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THE VERSATILE ROLE OF HOMOLOGOUS RECOMBINATION IN PLANT CELL: REPAIR OF DNA DAMAGE, STRESS-DIRECTED GENOME EVOLUTION AND FOREIGN DNA INTEGRATION

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A Thesis
Submitted to the Graduate Council
of the University of Lethbridge
in Partial Fulfillment of the
Requirements for the Degree

DOCTOR OF PHILOSOPHY

Department of Biological Sciences
University of Lethbridge
LETHBRIDGE, ALBERTA, CANADA

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Dedication

In loving memory of my grandparents,

Vasyl and Raisa Manenko

To my beloved parents,

Ludmyla and Oleksandr Boyko,

And to my Olena

For support and understanding

With gratitude to those I love and those who love me

Abstract

Homologous recombination represents a DNA repair pathway. Its role in a plant cell is not limited to double strand break repair. It also extends to genome evolution via rearranging of DNA sequences, and has an important application in foreign DNA integration in the plant genome. Our study demonstrated that effects exerted by stress on homologous recombination and genome stability are not restricted to the exposed generation. The progeny of plants exposed to stress exhibited elevated spontaneous homologous recombination, changes in DNA methylation and higher tolerance to stress. These heritable changes are mediated by an unknown stress-inducible epigenetic signal. Furthermore, we demonstrated that using factors that enhance homologous recombination can improve the efficiency of genetic transformation by *Agrobacterium*. We have developed and patented a plant growth medium enhancing homologous recombination and significantly increasing the transformation frequency. The role of several other chemicals for the improvement of transformation was also evaluated.

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List of Abbreviations

ADH1 – ALCOHOL DEHYDROGENASE1 gene

AP – apurinic/apyrimidinic sites

BTIs – VirB2-interacting proteins

CAF-1 – chromatin assembly factor 1

CAK2M – cyclin-dependent kinase-activating kinase

CaMV – cauliflower mosaic virus

cDNA – complementary DNA

ChIP – chromatin immunoprecipitation

chv – chromosomal virulence genes

CMT3 – CHROMOMETHYLASE3

COBRA – combined bisulphite restriction analysis

CpG – cytosine and guanine separated by a phosphate

CRE – callus regeneration efficiency

dATP – 2'-deoxyadenosine 5'-triphosphate

dCTP – 2'-deoxycytidine 5'-triphosphate

DDM1 – DECREASED DNA METHYLATION1

dGTP – 2'-deoxyguanosine 5'-triphosphate

DME – DEMETER DNA glycosylase

DML – DEMETER-LIKE protein

DNA-PKcs – DNA protein kinase catalytic subunit

dpg – day post germination

DPM – decays per minute

DREBs – dehydration-responsive-element-binding proteins

DRM – DOMAIN REARRANGED METHYLTRANSFERASE

DSB – double strand break

DSBR – double-strand break repair

dsT-DNA – double-stranded T-DNA

dTTP – 2'-deoxythymidine 5'-triphosphate

FLC – FLOWERING LOCUS C gene

FWA – LATE FLOWERING PHENOTYPE gene

GT – gene targeting

GUS – *β–glucuronidase* gene (*uidA*)

HDA6 – HISTONE DEACETYLASE6

HP1 – HETEROCHROMATIN PROTEIN1

HR – homologous recombination

HRF – homologous recombination frequency

IAA – indole-3-acetic acid

KYP – KRYPTONITE histone methyltransferase

LB medium – Luria-Bertani broth medium

LHP1 – HETEROCHROMATIN PROTEIN1

LTR – long terminal repeat

LUC – *luciferase* gene

MAPK – mitogen-activated protein kinase

MBDs – methyl-CpG-binding domain proteins

MET1 – METHYLTRANSFERASE1

miRNA – micro RNA

MMS – methyl methane sulfonate

MOM1 – MAINTENANCE OF METHYLATION

MOP1 – MEDIATOR OF PARAMUTATION1

MPK3 – mitogen-activated protein (MAP) kinase 3

MS medium – Murashige and Skoog medium

NAA – 1-naphthaleneacetic acid

nat-siRNA – natural antisense transcripts derived siRNAs

NCBI – National Center for Biotechnology Information

NHEJ – non-homologous end-joining

NPC – nucleus pore complex

P2ofC – the second progeny of control plants obtained from G1 plants grown under control conditions

P2ofS_25 – the second progeny of 25 mM NaCl treated plants obtained from G1 plants grown under control conditions

P2ofS_75 – the second progeny of 75 mM NaCl treated plants obtained from G1 plants grown under control conditions

PAMPs – pathogen-associated molecular patterns

PARP – Poly(ADP-ribose)polymerase

PCR – polymerase chain reaction

PDC1 – PYRUVATE DECARBOXYLASE1 gene

PofC – the first progeny of control plants

PofS_25 – the first progeny of 25 mM NaCl treated plants

PofS_75 – the first progeny of 75 mM NaCl treated plants

pol IVb – RNA Polymerase IVb

PPT – phosphinothricin

ptr-miRNAs – *Populus trichocarpa* micro RNAs

RAT5 – RESISTANT TO AGROBACTERIUM TRANSFORMATION5 gene

R-gene – resistance gene

ROPS – random oligonucleotide primed synthesis

ROS – reactive oxygen species

ROS1 – REPRESSOR OF SILENCING1

RR – recombination rate

SDSA – synthesis-dependent strand annealing

SE – somatic embryogenesis

siRNA – small interfering RNA

smRNA – small RNA

SMS – structure maintenance of chromosomes

SOS1-like – SALT OVERLY SENSITIVE1 like

SSA – single-strand annealing

ssDNA – single stranded DNA

ssDNA – single-stranded DNA

SSGR – single-strand-gap repair

ssT-DNA – single-stranded T-DNA

STF – stable transformation frequency

TBP – TATA-box Binding Protein

T-DNA – transferred DNA

TGI – Tobacco Genome Initiative

Ti – tumor-inducing plasmid

TIR1 – TRANSPORT INHIBITOR RESPONSE1

TMV – tobacco mosaic virus

UTR – untranslated region

UV – ultraviolet radiation

VIP1 – VirE2-interacting protein 1

vir – virulence genes

X-glu – 5-bromo-4-chloro-3-indolyl glucuronide

YEP medium – Yeast Extract Peptone medium

ZFN – zinc-finger nuclease

1. Introduction

Plant adaptation to changing environmental conditions represents a complex array of biochemical, molecular and metabolic responses, all of which are orchestrated by the cell's genetic material. High plasticity of the plant genome is one of the key determinants for quick acclimation to ever-changing growth conditions (Chinnusamy *et al.*, 2004; Turunen and Latola, 2005). Plants rely on a complex network of stress perception and stress signaling pathways, many of which may cross-talk at various steps (Zhu, 2001; Seki *et al.*, 2002, 2003; Chinnusamy *et al.*, 2004), leading to the activation of hundreds of genes, changes in cell transcription and metabolome profiles followed by adaptive changes to new growth conditions (Thomashow, 1999; Cook *et al.*, 2004; Kaplan *et al.*, 2004; Larkindale *et al.*, 2005; Kotak *et al.*, 2007; Zhu *et al.*, 2007a).

Successful adaptation to the new environment is not restricted to physiological changes but also requires effective mechanisms protecting the integrity of the plant genome under a negative influence of stress (Arnholdt-Schmitt, 2004; Madlung and Comai, 2004). Stress perception, signaling and defensive reactions frequently involve the overproduction of reactive oxygen species (ROS) in various cell compartments (Vranova *et al.*, 2002). Apart from their role as a secondary messenger and defense molecule (Mittler, 2002; Pastori and Foyer, 2002; Vranova *et al.*, 2002), ROS may cause an extensive damage to cellular macromolecules and organelles (Blokhina *et al.*, 2003) and even trigger cell apoptosis (Mittler, 2002). Thus, a tight control over the balance between ROS production and ROS scavenging must exist in a cell to prevent or minimize negative effects of oxidative damage under stress or normal physiological conditions (Desikin *et al.*, 2001, Knight and Knight, 2001; Mittler, 2002).

Oxidative damage to DNA resulting from the interaction with ROS represents a significant threat to genome integrity and carries a powerful mutagenic potential (Loeb and Preston 1986; Breimer, 1990; Evans *et al.*, 1993; Babiychuk, *et al.*, 1994). ROS-derived DNA damage challenges the DNA repair machinery. In fact, even a successful repair event may still result in altering an original DNA sequence due to either the imprecision of DNA repair pathways used or lower fidelity of repair polymerases, compared to replicative polymerases (Loeb and Preston, 1986; Strathern *et al.*, 1995; Gorbunova and Levy, 1999; Orel et. al., 2003; Kovalchuk *et al.*, 2004a; Puchta, 2005). One of the most critical forms of oxidative damage to DNA is represented by DNA double strand breaks (DSBs) (Breimer, 1990; Mittler, 2002: Blokhina *et al.*, 2003), which if left unrepaired can result in a loss of genetic information, chromosomal translocations, cell cycle arrest and apoptosis (Evans *et al.*, 1993; Critchlow and Jackson, 1998; Shrivastav *et al.*, 2008).

In fact, a number of natural stresses and various chemicals were shown to affect genome stability. Among those are light spectrum (Ries, *et al.*, 2000a) and day length (Boyko *et al.*, 2005), various types of ultraviolet (UV) (Puchta, *et al.,* 1995; Ries, *et al.*, 2000b; Molinier *et al.*, 2005; Boyko *et al.*, 2006b) and ionizing radiation (Kovalchuk, *et al.*, 1998), chemical mutagens (Brennan and Schiestl, 1998; Kovalchuk, *et al.*, 2001), toxic substances such as herbicides (Filkowski, *et al.*, 2003), temperature (Lebel, *et al.*, 1993; Jiang, *et al.*, 2003; Boyko *et al.*, 2005), salt (Puchta, *et al.*, 1995; Boyko *et al.*, 2006c, 2008; Boyko and Kovalchuk 2008b), water stress (Kalendar, *et al.*, 2000), and pathogen attack (Lucht, *et al.*, 2002; Kovalchuk, *et al.*, 2003a; Boyko *et al.*, 2007).

There are two major evolutionarily conserved DNA repair pathways available in a cell that can repair DSBs. These are homologous recombination (HR) and non-homologous end-joining (NHEJ). They both compete for the same substrate, the DSB substrate, and as their names suggest, they have different requirements with respect to a repair template (high homology vs. microhomology), thereby offering a different fidelity of DNA repair (Salomon and Puchta, 1998; Gorbunova and Levy, 1999; Orel *et. al.*, 2003; Dudas and Chovanec, 2004; Puchta, 2005; Bleuyard *et al.*, 2006; Shrivastav *et al.*, 2008). Moreover, a cell can discriminate in favor of using one of these two DSB repair pathways. In general, the balance between these two competitors is tightly controlled, and it depends on the availability of repair templates, cell cycle phase, proliferation rate, and functions of specific cell types (reviewed in Shrivastav *et al.*, 2008).

Recent literature reveals that DSB repair pathways and HR in particular are important in plant genome evolution and development of stress tolerance, with DSBs being raw material for increasing intragenome diversity and plasticity (Gorbunova and Levy, 1999; Kirik *et al.*, 2000; Puchta, 2005; Boyko *et al.*, 2007). In plant genomes that contain high amounts of repetitive DNA (Flavell, 1985), the elevated activity of HR may mediate rearrangements between repetitive sequences, and these rearrangements generate new alleles of existing genes. Importantly, many disease-resistance genes usually show high polymorphism and can be located in clusters, thereby being a good target for HR mediated rearrangements (Richter *et al.*, 1995, Stah *et al.*, 1999; Rocha *et al.*, 2002; Tornero *et al.*, 2002; Mauricio *et al.*, 2003; Meyers *et al.*, 2005).

A negative correlation between HR activity and the methylation level of repair templates (Engler *et al.*, 1993; Bender 1998; Bassing *et al.*, 2002) allows speculation that

DNA methylation can be used to protect important genes from undesirable rearrangements, or in contrast, DNA demethylation can encourage recombination when plants are exposed to certain stresses (Rizwana and Hahn, 1999; discussed in Boyko *et al.*, 2007). A correlation has been observed between tolerance to pathogen stress and the increased frequency of rearrangements in the *R-gene*-like loci in the progeny of virus-challenged plants (Boyko *et al.*, data not published). This notion supports a contribution of HR toward stress-accelerated evolution of sequences of a given gene.

Similarly, DNA methylation plays an important role not only in control of the activity of transposable elements and defence against foreign DNA but also in reversible modifications of gene expression profiles that can be inherited (Bender, 2004; Rassoulzadegan *et al.*, 2006). Importantly, various stresses were shown to trigger changes in DNA methylation (Steward *et al.*, 2000; Henderson and Dean, 2004). Some of these changes are essential for stress protection (Wada *et al.*, 2004; Sha *et al.* 2005; Dyachenko *et al.*, 2006). Information about stress-induced transcription profiles that might also confer tolerance to stress can be reversibly stored in DNA using epigenetic modifications (reviewed in Boyko and Kovalchuk, 2007, 2008a). The inheritance of stress-induced changes in gene expression is of great importance to plants, since equipping the progeny with a 'memory of stress' can enhance its tolerance and minimize damage delivered during the first exposure (reviewed in Boyko and Kovalchuk, 2007, 2008a). Increased tolerance to the same stress in the progeny of stress-treated plants was shown for cold (Blödner *et al.*, 2007), salt (Boyko *et al.*, 2008; Boyko and Kovalchuk 2008b), heavy metals (Boyko *et al.*, 2008), and pathogen stresses (Boyko *et al.*, data not published). Intriguingly, increased stress tolerance observed in the immediate progeny of stressed

plants correlated with elevated spontaneous frequency of HR (HRF), suggesting that HR may also contribute to a phenomenon of acquired transgenerational tolerance to stress (Boyko *et al.*, 2008; Boyko and Kovalchuk 2008b; Boyko *et al.*, data not published).

Epigenetic memory fixed in the form of DNA methylation can be further reinforced via numerous histone modification and chromatin remodelling processes that lead to the formation of heritable epialleles and paramutations (reviewed in Boyko and Kovalchuk, 2008a). Targeting chromatin structure followed by changes in gene expression and generation of new transcription profiles can be mediated via the activity of small RNA (smRNA) molecules (Bender 2004; Matzke *et al.*, 2004; Chan *et al.*, 2006; Matzke *et al.*, 2006; reviewed in Matzke and Birchler, 2005). In fact, a number of smRNAs reported to date were found to be regulated by a variety of stresses, including mechanical stress, dehydration, salinity, cold, pathogen attack, abscisic acid and nutrient deprivation (Sunkar and Zhu, 2004; Borsani *et al.*, 2005; Lu *et al.*, 2005; Katiyar-Agarwal *et al.*, 2006; reviewed in Sunkar *et al.*, 2007).

Similarly to epigenetic traits, an altered HRF can be inherited by the progeny of stressed plants, persisting in a population for one or several non-stressed generations (Kovalchuk *et al.*, 2003b; Molinier *et al.*, 2006; Boyko *et al.*, 2007, 2008; Boyko and Kovalchuk 2008b). Based on the fact that both meiotic and somatic HRF can be induced by a variety of biotic and abiotic stresses (Kovalchuk *et al.*, 2003a, 2003b, 2004a; Molinier *et al.*, 2005, 2006; Boyko *et al.*, 2005, 2006b, 2006c, 2007, 2008; Boyko and Kovalchuk 2008b), we suggest that HR can represent one of the mechanisms of stress adaptation.

Not only does HR play an important role as a DNA repair pathway and an evolutionary mechanism contributing to plant adaptation to stress, but it also has an essential application in biotechnology for foreign DNA integration in the host genome (Vergunst and Hooykaas, 1999; Puchta 2002; Hanin and Paszkowski, 2003; Reiss 2003; Lida and Terada, 2004). A homology-dependent mode of DNA repair possessed by the HR pathway makes it an invaluable tool for gene targeting (GT) technology in plants. Unfortunately, unlike NHEJ events that usually occur at high frequency in plant cells, the frequency of HR is quite low, thereby creating a major obstacle for GT in plants. In fact, there are only a very few reports of successful GT by HR in higher plants (Kempin *et al.*, 1997; Terada *et al.*, 2002).

Nowadays, one of the dominant technologies used for the delivery and integration of foreign DNA is represented by *Agrobacterium*-mediated genetic transformation. This approach relies on the ability of a natural plant soil pathogen, *Agrobacterium*, to transform its host with bacterial DNA. This leads to the expression of bacterial genes in a host cell. Current technology involves substitution of bacterial genes with sequences of interest that can be delivered to host cells instead (reviewed in Gelvin, 2003). A list of species that can be transformed using this technology includes various plants, fungi, yeast and even human cells, and it continues to grow (Tzfira and Citovsky, 2003; reviewed in Lacroix *et al.*, 2006). Increasing the efficiency of *Agrobacterium*-mediated genetic transformation is one of the critical problems in modern biotechnology.

Successful transgene integration into the recipient genome completely depends on the activity of host DNA repair proteins (van Attikum and Hooykaas, 2003; reviewed in Citovsky *et al.*, 2007). Therefore, the activity of related host factors has become a

bottleneck in the entire transformation procedure. In fact, transgene integration is preferentially targeted to DSB sites in the host genome (Chilton and Que, 2003; Tzfira *et al.*, 2003); hence, HR and NHEJ become key players during the last step of *Agrobacterium*-mediated transformation.

Different fidelity of DNA repair by HR and NHEJ is reflected in the precision and intactness of transgene integration via either of these two pathways. While the involvement of NHEJ usually results in multiple transgene insertions containing deletions and filling sequences (Salomon and Puchta, 1998; Chilton and Que, 2003), HR can mediate precise site-specific transgene integrations, which makes it a highly desired tool for GT (Vergunst and Hooykaas, 1999; Puchta 2002; Reiss 2003). Consequently, factors that could promote HR and/or inhibit NHEJ can considerably facilitate the improvement of *Agrobacterium*-mediated genetic transformation and GT in particular.

Our previous studies demonstrated that HRF can be greatly increased by a variety of abiotic and biotic stresses (Boyko *et al.*, 2005, 2006b, 2006c, 2007, 2008; Boyko and Kovalchuk 2008b). Based on our previous findings, we hypothesised that growing plants under mild salt stress, which is known to trigger ROS overproduction, will result in DNA damage. The high number of DNA DSBs will increase the activity of HR. Furthermore, continuous exposure to stress will result in the establishment of epigenetic 'stress memory' or other signal(s) that can be transmitted to the progeny of stressed plants. If our hypothesis is correct, increased tolerance to salt or other genotoxic stress accompanied by a modified DNA repair capacity and changes in DNA methylation will be seen in the progeny of stressed plants. Additionally, we hypothesised that using factors that increase HRF could influence the rate and quality of transgene integration via the *Agrobacterium*-

mediated plant transformation system. If our hypothesis is correct, we will see a higher number of stable transformants in plants which before transformation were grown in media containing factors which favor a higher HRF. Overall, we think that revealing the mechanisms that control HR could enhance our understanding of plant stress responses and facilitate the identification of factors that influence the efficiency of plant transformation by *Agrobacterium*.

The primary goal of our work is to identify cellular factors, abiotic and biotic stress elements which may control the level of chromosomal HR in plant somatic cells and elucidate the roles of the abovementioned factors in plant adaptation to stress. In order to reach the primary goal, two specific aims have been established:

– To analyze immediate and transgenerational changes in plant genome stability triggered by salt stress and investigate the role of HR in the development of adaptation to stress;
– To develop an efficient protocol that would allow the improvement of the rate and precision of transgene integration into the plant genome using a conventional *Agrobacterium*-mediated transformation system

In order to reach the goal, the following objectives were set:

Salt stress:

– to describe the influence of salt stress on genome stability of somatic cells;

- to describe changes in genome stability in the progeny of salt-exposed plants; and to elucidate the role of continuous stress exposure in the persistence of these changes;
- to describe the role of epigenetic changes in the maintenance of transgenerational genome stability and plant adaptation to stress

Improving plant transformation efficiency:

- to select the best medium composition that enhances the HRF and optimizes the rate of *Agrobacterium*-mediated plant transformation;
- to compare transformation rates between a conventional protocol and our novel protocol(s)
- to characterize transgene integrations via a segregation analysis and cloning of insertion sites

The major purpose of this study is to expand current knowledge on mechanisms underlying genome stability in plants, generation and inheritance of stress memory, and establishment of acclimation to stress. Our study attempted to develop an improved plant transformation protocol using factors and mechanisms that influence the rate of HR (RR) in a plant cell. Our approach was based on measuring the HRF as an indicator of genome stability via a sensitive detection system based on transgenic plants carrying a recombination substrate. This technique allowed a quick and accurate detection of factors that affect genome stability and HRF and made it possible to track these changes through several plant generations.

We showed that exposure of plants to salt results in a drastic change in genome stability and leads to dramatic changes in the response of their progeny to the same and different stresses. We were able to demonstrate that the progeny of stressed plants exhibited higher spontaneous HRF, and it was consistent with transcriptional activation of the *AtRad51* gene and a shift towards HR-mediated DSB repair. An increased HRF was inherited by the progeny of stressed plants as an epigenetic trait. The progeny of salt-treated plants displayed higher tolerance to salt and methyl methane sulfonate (MMS) stresses, which correlated with considerable changes in DNA methylation that were primarily observed within coding sequences. Transgenerational genome instability persisted only if plants were constantly exposed to stress. This suggests that the evolution of plant tolerance requires constant and prolonged exposure of several plant generations to stress.

We have managed to develop a fast screening system to search for various factors that influence the activity of HR and could be potentially applied in plant transformation. Using this system, we determined and evaluated several chemical factors that, if delivered to a growth medium, drastically improved the rate of *Agrobacterium*-mediated plant transformation. This allowed us to develop and successfully patent the growth medium composition that enhances HRF and increases the rate of plant transformation (Boyko and Kovalchuk, US Patent No. 11/466184). Overall, we strongly believe that our work will make a great contribution to the basic research and agricultural industry in Alberta.

2. Literature review

2.1. Plants and stress: major factors affecting genome stability

2.1.1. Plant environment and stress

During its life span, living matter is trying to respond and, if possible, to adapt to numerous environmental stimuli present in the environment. One of the most significant environmental factors is stress. Usually, stress has multiple negative effects on growth, development and reproduction of organisms (Arnholdt-Shmitt, 2004; Madlung and Comai, 2004). Stress often determines the distribution of species and more importantly exerts strong evolutionary pressure on a given population. The environmental stimuli that can be defined as stresses fall into several categories. Depending on their character, they can be subdivided into internal stresses, such as altered genome stability, polymerase errors and radical production upon cell metabolic reactions, and external stresses which can be further subdivided into two classes based on their abiotic or biotic nature (reviewed by Madlung and Comai, 2004).

Different strategies can be applied in order to minimize the influence of stress. These are tolerance, resistance and avoidance, or escape. The combination of these strategies may vary from species to species, but the most prominent difference can be observed when plants and animals are compared. Due to their sedentary life style, plants are usually restricted only to tolerance and resistance mechanisms. The persistence of stress on a given site for several plant generations would impose an additional challenge on plants, thus confirming the need to develop effective strategies for surviving.

2.1.2. Internal and external sources of oxidative damage and its effect on genome integrity

One of the most significant threats to plant cell integrity at a molecular level is the presence of ROS. Generation of ROS in different cell compartments under physiological conditions is an unavoidable consequence of aerobic metabolism (Foyer and Harbinson, 1994). The constant occurrence of two central aerobic processes, photosynthesis and respiration, in a plant cell results in a continuous production of various ROS in mitochondria, chloroplasts and peroxisomes. The reduction of molecular oxygen in these cellular compartments triggers spontaneous chain reactions, generating singlet oxygen, superoxide radicals, hydrogen peroxide and hydroxyl radicals. All of them are moderate or highly reactive molecules which may react with and damage cell proteins, unsaturated fatty acids in the plasma membrane, carbohydrates, and nucleic acids (Blokhina *et al.*, 2003). ROS may also trigger cell apoptosis, if they over-accumulate (reviewed in Mittler, 2002).

Oxidative damage to DNA resulting from the interaction with ROS is of special concern, as this form of damage possesses powerful mutagenic properties. Superoxide anion and hydrogen peroxide are normally not reactive towards DNA. However, in the presence of a ferrous or cuprous ion, they can be converted to the highly reactive hydroxyl radical via a Haber-Weiss and Fenton reaction. DNA exposure to the hydroxyl radical results in the release of free bases from DNA, and in base and sugar modifications. It generates DNA single and double strand breaks, DNA-protein crosslinks, and simple apurinic/apyrimidinic sites (AP) (Breimer, 1990).

DNA replication past AP sites by DNA polymerases results mainly in the insertion of dATP (review by Loeb and Preston 1986). In contrast, repair of abasic sites by base excision repair leads to the introduction of transient DNA strand breaks (Babiychuk, *et al.*, 1994). If left unrepaired, single and double strand breaks caused by ROS can block DNA replication, and therefore they can be lethal to a cell (Evans *et al.*, 1993). Moreover, repair of strand breaks frequently results in deletions, insertions, point mutations, and may lead to gene conversion, gene translocation, and duplication events (Loeb and Preston, 1986; Gorbunova and Levy, 1999; Orel et. al., 2003; Kovalchuk *et al.*, 2004a; Puchta, 2005). Importantly, no scavenger of hydroxyl radicals is produced in a plant cell (Apel and Hirt, 2004). Therefore, the avoidance of oxidative DNA damage and associated genotoxic effects is possible only by precise control over reactions that lead to the generation of this radical.

To avoid the negative influence of ROS, plants have developed complex and efficient enzymatic and non-enzymatic antioxidant defense systems that allow scavenging ROS and protecting cells from oxidative damage. These systems consist of a number of enzymes (superoxide dismutase, catalase, peroxidases, glutathione peroxidase, glutathione S- transferases, phospholipid-hydroperoxide glutathione peroxidase, ascorbate peroxidase, alternative oxidases) and low molecular mass antioxidants (ascorbate, glutathione, phenolic compounds, tocopherols) (Mittler, 2002; Blokhina *et al.*, 2003). The distinct subcellular localization of enzymatic components involved in the antioxidant defense and their differential inducibility allow them to achieve flexible control over ROS accumulation spatially and temporally (Vranova *et al.*, 2002). We can observe the equilibrium between ROS production and ROS scavenging under

physiological conditions. This delicate balance can be shifted toward a rapid production of ROS by a number of stress factors. A rapid increase in ROS accumulation is known as an oxidative burst, and it was shown to be a general response to various biotic and abiotic stresses (Vranova *et al.*, 2002).

A number of studies demonstrated the production of ROS to be triggered by various stress conditions, including drought and desiccation, high temperature, chilling, salt, heavy metals, UV, intensive light, air pollutants (ozone and SO_2), mechanical and nutrient deprivation, and pathogen attacks (Bowler, *et al.*, 1994; Mittler and Zilinskas, 1994; Allen, 1995; Noctor and Foyer, 1998; Desikin, *et al.*, 2001). In fact, ROS play a critical role of secondary messengers in stress-response signal transduction for the vast majority of stresses (Desikin *et al.*, 2001, Knight and Knight, 2001). Consequently, different stresses such as cold, drought or salinity result in the activation of similar signaling pathways (Seki *et al.*, 2002, 2003).

The latter notion is supported by a cDNA microarray analysis that used 7 000 independent *Arabidopsis* full-length cDNA sequences and revealed 299 drought-inducible genes and 213 high-salinity-stress-inducible genes (Seki *et al.*, 2002). More than half of high-salinity-stress-inducible genes were also induced by drought stress, indicating a significant crosstalk between these two responses (Seki *et al.*, 2002). Overall, a tightly regulated balance between different ROS-producing and ROS-scavenging mechanisms existing in a cell allows ROS to play a role as cellular stress indicators and secondary messengers associated with stress-response signal transduction pathways (Desikin *et al.*, 2001, Knight and Knight, 2001; Mittler, 2002).

2.1.3. Double strand break repair. Two competitors: non-homologous end-joining and homologous recombination

Repair of oxidative DNA damage caused by stress exposure is one of the keystones for a successful plant defense against stress. It helps preserve genome integrity and stability, and thereby reduces the risk of heritable DNA damage. Interactions of ROS derived from various exogenous and endogenous sources with DNA frequently result in the formation of DSBs that pose a major threat to genome integrity. If left unrepaired, DNA DSBs may lead to loss of genetic informationa, and chromosomal translocations. They may cause replication arrest and even trigger apoptosis, if a cell suffered extensive DNA damage (Evans *et al.*, 1993; Critchlow and Jackson, 1998; Shrivastav *et al.*, 2008) (Figure 2.1). In contrast, the presence of DSBs is absolutely required for initiating DNA strand exchange via HR and generating genetic diversity in gametes during meiosis (Richardson *et al.*, 2004). Similarly, DSBs are necessary during the development of T and B lymphocytes in vertebrates, where generation of antigen-receptor and immunoglobulin diversity is critical for proper immune system functions (Rooney *et al.*, 2004).

There are two major evolutionarily conserved DNA repair pathways available in a eukaryotic cell that have an impact on DSBs and insure the maintenance of genome stability: NHEJ and HR. NHEJ acts independently of significant homology between interacting DNA molecules, and HR requires extensive sequence homology for repair events to occur. NHEJ involves the direct rejoining of break ends. If it is not possible, NHEJ searches for microhomology, aligns one or few complementary bases to direct repeats, and removes the DNA in between (Bleuyard *et al.*, 2006; Shrivastav *et al.*, 2008).

Consequently, NHEJ is a relatively inaccurate DNA repair pathway which is frequently associated with small and large-scale deletions (from 1 bp up to > 1 kb), insertions (up to 1.2 kb long) or point mutations (Gorbunova and Levy, 1999). In contrast, HR can be considered to be a more precise DNA repair pathway with a 100% accuracy, if a perfectly homologous template like a sister chromatid, a homologous chromosome, or a repeated region are used to prime repair synthesis. Alternatively, if a template with imperfect homology is used for repair, HR may result in gene conversion events leading to loss of heterozygosity. It may lead to gene translocation and duplication events or generate large deletions, if intrachromosomal recombination occurs within the DNA region containing multiple repeats (Orel *et. al.*, 2003; Dudas and Chovanec, 2004; Puchta, 2005). Finally, lower fidelity of repair polymerases, if compared to replicative polymerases, leads to an increase in point mutation frequency at the sites of DSBs, even if a perfectly homologous template is used for repair (Strathern *et al.*, 1995).

Repair of DSBs via a HR pathway can be described by several different models (reviewed in Puchta, 2005). The first one, a synthesis-dependent strand annealing (SDSA) model, postulates that induction of DSB is followed by creation of a 3’ overhang in the acceptor molecule using exonucleases. The resulting 3’ end invades a double stranded donor forming a D-loop and initiates repair synthesis. Two scenarios are possible at this stage. If the 3’ end of the acceptor molecule is elongated up to the homology of the second 3’ end of the break, single strands anneal and a DNA molecule is repaired. Alternatively, if the 3’ elongated end of the acceptor molecule is not complementary to the 3’ end of the break, the break is closed via NHEJ, sometimes using microhomology of a few nucleotides (Puchta, 2005). In contrast to SDSA, the single-

strand annealing (SSA) model postulates the generation of 3' single-stranded overhangs by exonucleases at both ends of DSB. If overhangs carry complementary sequences, they can anneal forming a chimeric DNA molecule. The presence of non-complementary sequences on the 3' end of overhangs will lead to the formation of overhanging ends in a chimeric DNA that will be resected. Similarly, gaps in single-stranded regions can be filled by DNA repair synthesis (Puchta, 2005). Clearly, the most likely targets for repair via the SSA mechanism can be found within DNA regions containing repeated sequences, and SSA-mediated damage repair may lead to a deletion of DNA sequences flanked by complementary repeats.

While both SDSA and SSA are frequently observed in somatic tissues, HR in cells undergoing meiosis can occur via the classic DSB repair (DSBR) mechanism that involves the formation of a double Holiday junction and its resolution, leading to gene conversion and crossover events (Gorbunova and Levy, 1999). To reduce risks of crossover and translocation, the SDSA mechanism is predominantly used in somatic cells. Moreover, the prevalence of the SDSA mechanism over DSBR in somatic plant cells may be critical, since plants contain high amounts of repetitive DNA in their nuclei (Flavell, 1985) that can increase the risk of unpredictable deleterious genome rearrangements by crossovers. In contrast to DSBR, the SDSA mechanism represents a safer option, since it may result only in gene conversion events (Gorbunova and Levy, 1999; Puchta, 2005).

There exist a number of proteins that influences both HR and NHEJ DSB repaur pathways. This includes: the MRE11/RAD50/NBS1(XRS2) complex; BRCA1; histone H2AX; DNA-PKcs; PARP-1, RAD18 and ATM (Bleuyard *et al.*, 2006 and references

therein; Shrivastav *et al.*, 2008 and references therein). The MRE11/RAD50/NBS1(XRS2) complex processes the ends of a DSB prior to its repair. BRCA1 may negatively regulate end-processing by the MRE11-containing complex, thereby enhancing fidelity of NHEJ. BRCA1 also promotes HR via interactions with a RAD51 and BRCA2 protein. Phosphorylation of histone H2AX may result in activation of the DNA damage checkpoint and recruitment of a INO80 chromatin remodeling complex. Finally, DNA-PKcs facilitates the alignment of non-complementary ends and phosphorylates the key proteins involved in NHEJ and some of proteins involved in HR. In contrast, some proteins such as RAD51 and a KU heterodimer that consists of Ku70 and Ku80 (or Ku86) function exclusively in one of the two pathways. The RAD51 sequence is a highly conserved one among eukaryotes. It plays a critical role in HR via the formation of the Rad51/ssDNA nucleofilament from the 3' overhang of a DSB and promotes strand exchange reactions (Bleuyard *et al.*, 2006 and references therein). Consistently with its role in HR, an *Arabidopsis rad51* mutant displays an extremely severe sterility phenotype caused by the failure to synapse and repair meiotic DSBs during prophase I of meiosis which results in extensive chromosomal fragmentation (Li *et al.*, 2004a). The KU heterodimer mediates NHEJ and represents a part of DNA-PK. KU binds to various types of DNA ends including single-stranded gaps, helps protect them and forms a bridge between two ends of a break, thereby contributing to their juxtaposition (Bleuyard *et al.*, 2006 and references therein). Overall, the complexity of a system of protein factors involved in DNA repair indicates the existence of a multilevel regulatory network controlling DSB repair management in a cell.

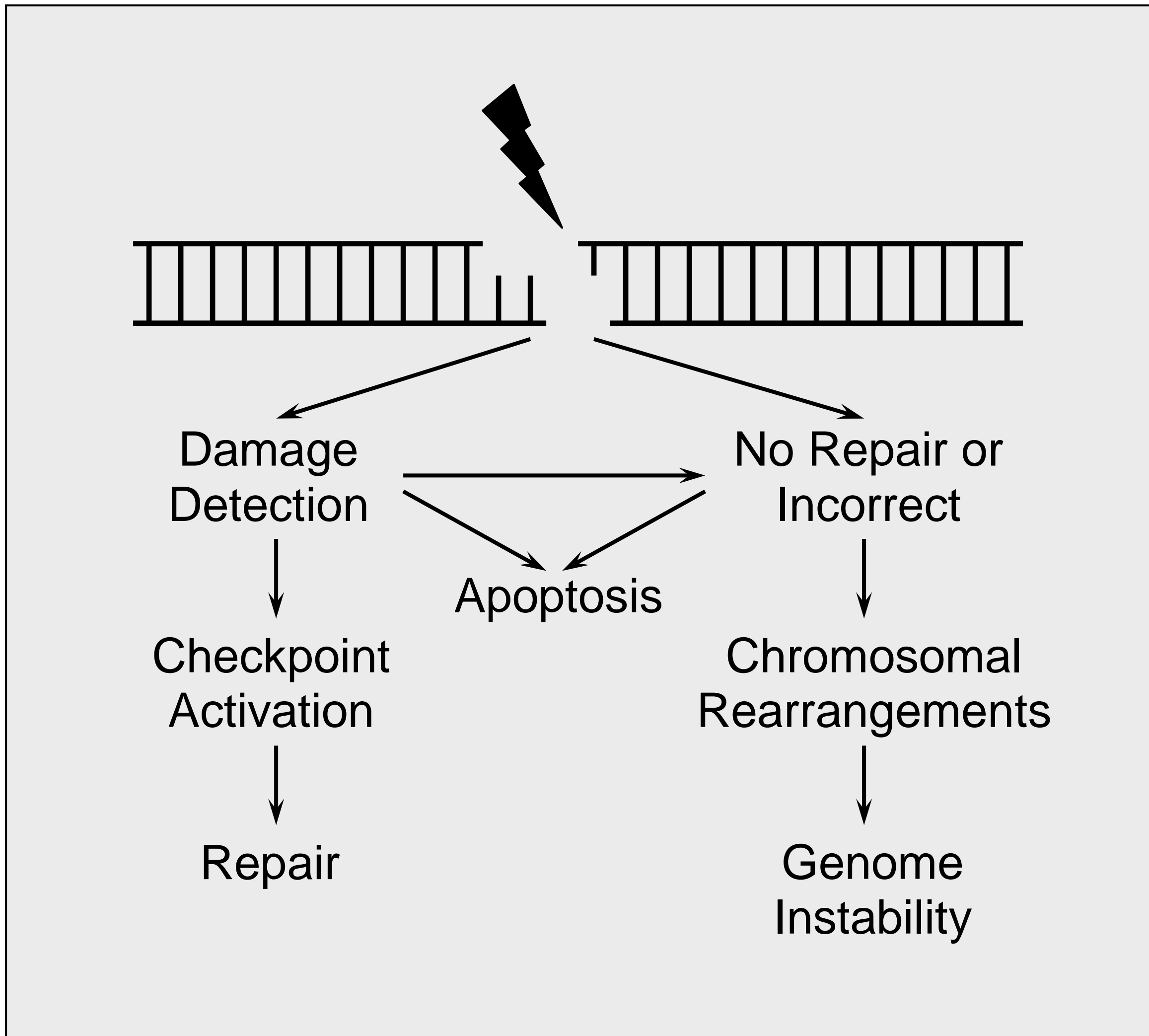

Figure 2.1. Effects of double strand breaks on plant cells

2.1.4. Assaying double strand break repair pathway choice

Competing for the same substrate, DSB repair pathways HR and NHEJ have different requirements with respect to a repair template, and thus offer different repair fidelity. Therefore, the balance between HR and NHEJ should be tightly controlled. Indeed, a cell can discriminate in favor of using one of these two DSB repair pathways, depending on the availability of repair templates, cell cycle phase, proliferation rate, and functions of specific cell types (reviewed in Shrivastav *et al.*, 2008). Prominent examples of such discrimination can be found when yeast is compared to higher eukaryotes. While HR is the dominant mode for DSB repair in yeast (Liang *et al.*, 1998; Paques *et al.*, 1999; Aylon and Kupiec, 2004), the vast majority of breaks in higher eukaryotes are processed by NHEJ (Salomon and Puchta, 1998; Kirik *et al.*, 2000). In contrast to the small yeast genome comprised mainly of coding sequences, higher eukaryotes have large genomes with a very high content of non-coding sequences (Beaton and Cavalier-Smith, 1999). HR as a dominant mode of repair allows yeast to prevent generation of random changes in coding DNA that could occur if the same damage were repaired using NHEJ. In contrast, small-scale deletions and insertions caused by NHEJ (Gorbunova and Levy, 1997, 1999) can be easily accommodated by higher eukaryotes due to their large genomes with a low content of coding sequence. Furthermore, NHEJ as a dominant mode of repair helps resolve the challenge of locating a homologous template in a large genome (Sonoda *et al.*, 2001; Sonoda *et al.*, 2006).

Similarly, when NHEJ is dominant during the G1 phase of a cell cycle, the upregulation of HR can be observed during S and G2 phases if sister chromatids are available (reviewed in Shrivastav *et al.*, 2008). Control over the shift from NHEJ toward

HR at the molecular level is mirrored by increasing expression of Rad52 and Rad51 during the S phase (Chen *et al.*, 1997). In fact, the balance between these two repair pathways is cell-cycle phase-dependent. Moreover, it changes during plant development and differs in various plant tissues (Boyko *et al.*, 2006a, 2006d). These changes are consistent with a different cell genome content and ploidy level in different plant tissues during different developmental stages as a result of somatic polyploidisation (Galbraith *et al.*, 1991; Joubes and Chevalier, 2000). Taking into consideration large amounts of repetitive DNA in the plant genome (Flavell, 1985), it becomes clear why high levels of HR may pose a threat to genome stability and lead to large-scale deletions and chromosomal translocations (Swoboda, *et al.*, 1994; Gorbunova and Levy, 1999; Puchta, 2005).

The existence of passive competition between HR and NHEJ can be supported by increased HR in cells deficient for various NHEJ proteins (Pierce *et al.*, 2001; reviewed in Shrivastav *et al.*, 2008). Recently, Shrivastav *et al.* (2008) suggested a model explaining an active regulation of DSB repair pathway choice via DNA-PKcs and ATM. The KU heterodimer binding to DSB ends recruits DNA-PKcs, which being bound to DNA becomes activated and phosphorylates itself, KU and other proteins. There are two phosphorylation clusters available at DNA-PKcs, T2609 and S2056. In contrast to the T2609 cluster that can be autophosphorylated either by the DNA-PKcs kinase domain or ATM, the S2056 cluster is a subject to autophosphorylation only. Phosphorylation of the T2609 cluster is sufficient to release DNA-PKcs from the break site, allowing an access of HR and NHEJ proteins. In contrast, phosphorylation of the S2056 cluster is required for initiation of the NHEJ repair pathway. Mutations in the S2056 cluster that block

phosphorylation lead to a significant increase of HR activity above a wild type level. However, the level of HR in the S2056 cluster mutants does not exceed the HR level observed in DNA-PKcs null cells (Shrivastav *et al.*, 2008).

To further investigate the role of DNA-PKcs, Shrivastav *et al.* (2008) generated a cell line that expressed a mutant form of DNA-PKcs in which inactivation of a kinase domain was achieved without introducing major changes in gross domain structure. This modification of a protein domain did not interfere with its ability to be phosphorylated by ATM. It also led to a 3-fold increase in the level of DSB-induced HR as compared to DNA-PKcs null cells (Shrivastav *et al.*, 2008). The foregoing indicates a combined positive effect of preventing phosphorylation at the S2056 cluster and the absence of competition with NHEJ. Importantly, ATM also promotes the HR pathway via phosphorylation of 12 other targets that play a role in HR; and the level of ATM can be stabilized by DNA-PKcs. Interestingly, six ATM targets can also be phosphorylated by DNA-PKcs (Shrivastav *et al.*, 2008).

Another possible mechanism of control over the balance between NHEJ and HR can be provided by Poly(ADP-ribose)polymerase (PARP) that covalently attaches ADP-ribose moieties to target proteins and mediates one of the earliest cell responses to DSBs (Ame *et al.*, 2004). Various biochemical studies demonstrated physical interactions between PARP and the Ku/DNA-PKcs complex (Sonoda *et al.*, 2006 and references therein). Wang *et al.* (2006) recently demonstrated that PARP competed with KU for DSB ends, thereby promoting HR and antagonizing NHEJ (Saberi *et al.*, 2007). Experimental evidence presented by Li *et al.* (2004b) suggested that PARP acted by decreasing the affinity of KU for DSB.

2.1.5. The role of double strand break repair pathways in plant genome evolution

To describe DSB repair pathways exclusively as factors maintaining genome stability would underestimate their role in a cell. Mistakes generated during DSB repair provide a raw material for evolution and also increase intragenome diversity and plasticity. A great deal of experimental evidence produced during the past several years indicates the involvement of DSB repair mechanisms in plant evolution and even in the development of tolerance to stress (Gorbunova and Levy, 1999; Kirik *et al.*, 2000; Puchta, 2005; Boyko *et al.*, 2007). An intriguing hypothesis is that stress can guide plant genome evolution using repair pathways, particularly HR, to trigger loci-specific genome rearrangements, thereby accelerating evolution of targeted sequences and development of tolerance to stress (reviewed in Boyko and Kovalchuk, 2007, 2008a).

2.2. Effects of stress on plant genomes: genetic and epigenetic regulations

2.2.1. Physiological aspects of salt stress

Soil salinity is one of the most significant stresses in agriculture. It represents a major constraint on food production in the world (Zhu, 2000). According to the United Nations Environment Program, approximately 20% of agricultural land and 50% of cropland suffer from salt stress (Yokoi *et al.*, 2002). Salinity conditions are usually caused by the presence of high NaCl concentrations in a plant growth environment. The osmotic component of salt stress makes effects of NaCl-mediated stress similar to those of drought and cold stresses (Seki *et al.*, 2003). In contrast, a unique component of NaCl-mediated stress, an ionic stress, is caused by an excessive amount of Na^{+} (Hasewaga *et al.*, 2000; Zhu, 2000, 2002). Nevertheless, both osmotic and ionic stresses are complemented by secondary oxidative stress (Niu *et al.*, 1995, Zhu *et al.*, 1997, Hasewaga *et al.*, 2000, Zhu, 2000) that represents a significant challenge to the DNA repair machinery. However, knowledge of possible salt effects on genome stability in plants remains fragmentary and requires further analysis.

2.2.2. Exposure to stress may mediate rapid establishment of stress tolerance to the same or different stresses

Continuous exposure to stress may not always have only a negative impact on plants and their metabolism. In many cases, exposure to mild stress is followed by acquired acclimation to more severe conditions. The role of stress exposure in plant adaptation was first described for a phenomenon known as cold acclimation in plants originating from temperate regions, where increased freezing tolerance was established via exposure to chilling temperatures (Levitt, 1980). Experimental evidence has been accumulating suggesting the existence of a similar phenomenon of fast stress-mediated acclimation to drought, cold and freezing, high temperature, high light and UV-B radiation stresses (reviewed in Chalker-Scott and Scott, 2004; Turunen and Latola, 2005; Caldwell *et al.*, 2007). It is apparent that the existence of a such mechanism is of primary importance for plants. A continuous presence of mild stress conditions, like chilling, may indicate an upcoming period of freezing temperatures, thereby allowing plants to activate their stress-defense systems and undergo hardening for more severe upcoming stress. In fact, Beck *et al.* (2004) demonstrated that such seasonal hardening in pine trees mediates the establishment of moderate freezing tolerance that can reach down to – 20 °C for the first hard frost. Moreover, the first subfreezing event triggers a second wave of hardening which together with seasonal hardening leads to acquired tolerance to temperatures as low as – 70 °C (Beck *et al.*, 2004). Strikingly, such extreme tolerance can be developed within a one-month period (Beck *et al.*, 2004).

A positive contribution of cold treatment to the development of tolerance to subsequent chilling or freezing stresses is not restricted to the exposed generation; it can

be observed in the immediate progeny of cold-exposed plants (Blödner *et al.*, 2007). Blödner *et al.* (2007) demonstrated that the progeny of plants grown in moderate cold conditions from bolting until the seed maturity stage showed a faster recovery of the photosynthetic yield under chilling and freezing conditions, compared to the progeny of plants produced at warm temperatures. The development of acquired tolerance by the progeny of plants whose ancestors were exposed to cold is consistent with recent reports on salt (Boyko *et al.*, 2008; Boyko and Kovalchuk 2008b) and pathogen (Boyko *et al.*, data not published) stresses demonstrating similar phenomena. Overall, it may represent an important stress tolerance mechanism in plants as sedentary organisms.

It is evident that the establishment of acquired tolerance to stress is achieved via numerous changes in gene expression and the molecular spectrum of metabolites in a plant cell (Thomashow, 1999; Larkindale *et al.*, 2005; Kotak *et al.*, 2007; reviewed in Zhu *et al.*, 2007a). Indeed, cold acclimation is mainly associated with transcriptional activation of hundreds of genes that code for transcription factors (Lee *et al.*, 2005). This indicates that a multitude of transcriptional cascades are activated during acclimation. Consistently, deacclimation is primarily mediated via transcriptional repression that targets many cold-inducible genes (Oono *et al.*, 2006).

Extensive changes in the cell transcriptome lead to a significant reconfiguration of the metabolome (Cook *et al.*, 2004; Kaplan *et al.*, 2004) which is reflected in synthesis and accumulation of various metabolites that perform protective functions (Chen and Murata, 2002; Stitt and Hurry, 2002; Kaplan and Guy, 2005). In fact, 75% of 400 poplar metabolites analyzed by Cook *et al.* (2004) for their response to cold stress showed an increase in concentration upon cold treatment. Similar changes in the metabolome can be

achieved under warm temperature conditions via ectopic expression of dehydration-responsive-element-binding proteins (DREBs) transcription factors that activate a set of cold inducible genes (Cook *et al.*, 2004). The foregoing underlines a major role of cold-inducible genes in the establishment of acquired tolerance to cold stress via modification of a cell's metabolite spectrum.

Plants rely on the perception of various abiotic stresses through a complex network of stress recognition and signaling pathways, some of which may be shared by different stresses, and which may cross-talk at various steps (Zhu, 2001; Seki *et al.*, 2003; Chinnusamy *et al.*, 2004). This implies that cold acclimation mentioned above represents a general strategy of response to abiotic stress rather than a specific response to cold stress. This is consistent with the stress cross-protection theory that was first suggested by Levitt (1980). It postulated that exposure to one stress can result in acquired tolerance to another stress, even if previously a plant had no experience of being exposed to it.

A number of studies conducted in the past ten years supported Levitt's theory (Levitt, 1980). They demonstrated that exposure to UV-B can increase tolerance to subsequent freezing (Dunning *et al.*, 1994; Richer and Hoddinott, 1997; Binder and L'-Hirondelle, 1999; Mendez *et al.*, 1999; Chalker-Scott and Scott, 2004), high temperatures (Teklemariam and Blake, 2003), drought and high light (Poulson *et al.*, 2002). It can also reduce levels of insect herbivory (Roberts and Paul, 2006) and fungal attack (Raviv and Antignus, 2004). Similarly, high light treatment can induce freezing tolerance in rye plants (Gray *et al.*, 1997); low temperature stimulates resistance to photoinhibition and snow moulds (Gaudet *et al.*, 2003). Plants that are more tolerant to cold temperatures demonstrate higher resistance to UV-B (Petropoulou *et al.*, 2001).

2.2.3. Epigenetic changes as a short-term strategy to minimize stress influence

Salinity stress represents a type of abiotic conditions that can persist at a given site for a prolonged period of time, thereby challenging plant defense systems continuously during many generations. As has already been mentioned, plants have a sedentary lifestyle and cannot use escape as a strategy to minimize the influence of stress. Hence, plants require the presence of efficient short-term defense strategies that are based on the manipulation of existing genetic information directed to the fast development of stress tolerance. These strategies may include an alteration of plant homeostasis during somatic growth (Shinozaki *et al.* 2003; Sung and Amasino, 2004) and heritable (also called transgenerational) modifications of gene expression (Whitelaw and Whitelaw, 2006).

These modifications can occur without changing the original DNA sequence, and are known as epigenetic modifications. They can be achieved on several interdependent levels, including reversible methylation of DNA sequences, numerous histone modifications and chromatin remodeling (Wagner, 2003; Vanyushin, 2006). All these modifications may be regulated by a number of physiological and developmental stimuli including stress (reviewed in Boyko and Kovalchuk, 2008a). The spectrum of external and internal influences experienced by an organism during its lifespan may lead to the generation of specific changes in gene expression that could be epigenetically (without changing the DNA sequence) fixed and passed to the progeny forming an epigenetic memory.

The maintenance of changes in gene expression in prokaryotic and eukaryotic organisms over several cell generations was well documented (Bender, 2004). In fact, the notion that transgenerational changes in DNA methylation are more frequently observed in plants than in animals (Takeda and Paszkowski, 2006) is consistent with a sedentary lifestyle of plants. In contrast to animals, plants establish the germ line late during development, thus allowing the transmission of epigenetic memory accumulated during their lives to the following generations.

2.2.4. DNA methylation is an epigenetic mark of primary importance

DNA methylation plays a major role in the regulation of gene expression, in the activity of transposable elements, in the defense against foreign DNA, and even in the inheritance of specific gene expression patterns (Rassoulzadegan *et al.*, 2006). The major differences in methylation patterns between plants and animals include a substantially higher percentage of modified cytosines and the absence of CpNpG and asymmetrical cytosine methylation in animals (Finnegan *et al.*, 1998b; Bender, 2004). Symmetrical CpG and CpNpG methylation is inherited during DNA replication in the form of hemimethylated sequences. Hence, it provides a methylation imprint memory on the parental DNA and also guides the activity of methyltransferases (Bender, 2004). On the contrary, asymmetrical cytosine methylation must be reestablished *de novo* after each replication cycle, since there is no complementary methylated sequence available to guide remethylation (Ramsahoye *et al.*, 2000; Gowher and Jeltsch, 2001). Experimental evidence suggests the existence of three distinct classes of enzymes responsible for cytosine methylation.

The first class is represented by a plant homologue of mammalian Dnmt1 methyltransferases, METHYLTRANSFERASE1 (MET1). Plants defective in MET1 show a lack of widespread CpG methylation (Lindroth *et al.*, 2001). The second class of methyltransferases, CHROMOMETHYLASE3, is unique to plants (Table 2.2.1). A loss-of-function *cmt3* mutant is characterized by a genome-wide loss of CpNpG methylation, especially at centromeric repeats and transposons (Lindroth *et al.*, 2001; Tompa *et al.*, 2002). Recent studies by Kato *et al.* (2003) on the activation of a normally silenced *CACTA* transposon in the *met1* and *cmt3* single and double mutants indicated redundancy

in the function of CMT3 with MET1 in CpG and CpNpG methylation. The last known class, DOMAIN REARRANGED METHYLTRANSFERASES, is composed of DRM1 and DRM2; it shows homology to the mammalian Dnmt3 methyltransferase (Cao *et al.*, 2000). DRM1 and DRM2 are mainly directed on *de novo* methylation of asymmetric sites (Cao and Jacobsen, 2002b), and they are capable of methylating CpNpG sites along with CMT3 (Cao *et al.*, 2003) (Table 2.2.1).

While the presence of DNA methylating enzymes is well proven, the existence of direct DNA demethylation mechanisms remains controversial. A passive loss of DNA methylation may occur due to the inhibition of *de novo* methylation or inability to maintain the parental imprint after DNA replication observed in *met1* mutants (Kankel *et al.*, 2003). Alternatively, active demethylation may occur via the glycosylase activity by removing methylcytosines from DNA (Zhu *et al.*, 2000; 2007b; Agius *et al.*, 2006; Morales-Ruiz *et al.*, 2006). It may play a critical role in preventing the formation of stable hypermethylated epialleles in the plant genome (Penterman *et al.*, 2007a). Indeed, the demethylation activity of *Arabidopsis* DNA glycosylase DEMETER (DME) regulates the gametophyte-specific activation of flowering time (*FWA)* gene expression (Kinoshita *et al.*, 2004). It also reverses imprinting of maternal copies of a *MEDEA* allele in the endosperm (Choi *et al.*, 2002). Gong *et al.* (2002) isolated a REPRESSOR OF SILENCING1 (ROS1), a DNA glycosylase/lyase, functioning on methylated and not demethylated DNA substrates (Table 2.2.1). To date, four members of the DEMETER DNA glycosylase family involved in DNA demethylation are known in *Arabidopsis*. They are DME and three DME-like proteins, DEMETER-LIKE2 (DML2), DML3, and ROS1 (Penterman *et al.*, 2007a, 2007b).

Table 2.2.1. Plant factors involved in epigenetic regulations

Name and function	Effects on chromatin	Effects of mutation and involvement in stress response	Modification/ Transcription	References
DNA methylation				
METHYLTRANSFERASE1 (MET1)/ Methyltransferase	Methylation of symmetrical CpG sites; post replicative *de novo* CpG methylation; not required for establishing new methylation imprints	Lack of CpG methylation; passive loss of DNA methylation throughout generations. MET1 is repressed in response to stress, leading to activation of repressed genes	Global/ Repression	Finnegan *et al.*, 1996; Steward, *et al.*, 2000; Kankel *et al.*, 2003; Wada *et al.*, 2004
CHROMOMETHYLASE3 (CMT3)/ Methyltransferase	CpNpG methylation; functionally redundant with MET1 and DRM in methylation of CpG and asymmetrical sites, respectively; targets centromeric repeats and transposons	Loss of CpNpG methylation	Global/ Repression	Bartee *et al.*, 2001; Lindroth *et al.*, 2001; Tompa *et al.*, 2002;
DOMAIN REARRANGED METHYLTRANSFERASES (DRM1, DRM2)/ Methyltransferase	*De novo* methylation of asymmetric sites; functionally redundant with CMT3 in CpNpG methylation; possibly reinforces preexisting methylation	Loss of *de novo* asymmetric methylation at non-CpG sites	Global/ Repression	Cao *et al.*, 2000; 2003; Cao and Jacobsen, 2002a; 2002b
DEMETER (DME)/ DNA glycosylase	Demethylation of previously silenced sequences, possibly in a tissue-specific manner	Inability to activate imprinted genes; inheritance of a mutant maternal allele results in seeds abortion	Local, promoters/ Activation	Kinoshita *et al.*, 2004; Morales-Ruiz *et al.*, 2006; Penterman *et al.*, 2007a
REPRESSOR OF SILENCING1 (ROS1)/ DNA glycosylase/lyase	Demethylation activity on methylated and not on demethylated DNA substrates	Hypermethylation and transcriptional silencing of specific genes; enhanced sensitivity to genotoxic agents	Local, promoters/ Activation	Gong *et al.*, 2002; Kapoor *et al.*, 2005; Agius *et al.*, 2006; Penterman *et al.*, 2007a; Zhu *et al.*, 2007b

Table 2.2.1. Plant factors involved in epigenetic regulations (*continued*)

Name and function	Effects on chromatin	Effects of mutation and involvement in stress response	Modification/ Transcription	References
DNA methylation				
DEMETER-LIKE (DML) proteins: DML2 and DML3/ DNA glycosylase/lyase	Demethylation activity is primarily localized at the 5′ and 3′ ends of genes, preventing the accumulation of methylation at or near genes. DML proteins remove aberrant 5′ and 3′ methylation from genes and prevent the formation of highly methylated stable epialleles.	Hypermethylation of gene sequences at either the 5′ or 3′ end; *dml* mutant hypermethylation has a negligible effect on gene expression	Local, 5′ and 3′ ends of genes/ Mostly unaffected	Penterman *et al.*, 2007a, 2007b
Histone modifications				
SUVH1/ Histone methyltransferase	Methylation of histone H3K9; has a minor impact on heterochromatin reinforcement.	Loss of H3K9 methylation	Global/ Repression	Naumann *et al.*, 2005
SUVH2/ Histone methyltransferase	Methylation of histones H3K9, H3K27, H4K20; heterochromatin reinforcement; SUVH2 mediated gene silencing depends on MET1 and DDM1	Loss of H3K9; H3K27 and H4K20 methylation; reduction of DNA methylation in heterochromatin	Global/ Repression	Naumann *et al.*, 2005
SUVH4 (KRYPTONITE) (SUVH4/KYP)/ Histone methyltransferase	Methylation of histone H3K9; activity is dependent on CpG DNA methylation in the given loci; has a minor impact on heterochromatin reinforcement	Loss of H3K9 methylation; a negative effect on CpNpG methylation	Global/ Repression	Jackson *et al.*, 2002; Johnson *et al.*, 2002; Jasencakova *et al.*, 2003; Naumann *et al.*, 2005
HISTONE DEACETYLASE6 (HDA6)/ Histone deacetylase	Reinforcing CpNpG methylation induced by RNA-directed transcriptional silencing	Reactivation of previously silenced transgenes	Local/ Repression	Aufsatz *et al.*, 2002
Chromatin remodeling				
METHYL-CpG-BINDING DOMAIN PROTEINS (MBD1 – MBD13)/ 5-methylcytosine binding proteins	Bind methylated CpG and change local chromatin structure through recruiting enzymes involved in modification of core histone proteins; promote heterochromatin formation and repeat silencing	Late flowering and reduced fertility (*mbd11*); shoot branching and early flowering due to transcriptional repression of *FLC* (*mbd9*)	Local/ Repression, activation	Ben-Porath and Cedar, 2001; Zemach and Grafi, 2003, 2007 and references therein

Table 2.2.1. Plant factors involved in epigenetic regulations (*continued*)

Name and function	Effects on chromatin	Effects of mutation and involvement in stress response	Modification/ Transcription	References
Chromatin remodeling				
LIKE HETEROCHROMATIN PROTEIN1 (LHP1) Chromodomain protein	Binds to histone H3K9; chromatin condensation and coating	Inability to repress expression of euchromatic genes associated with a specific developmental stage	Global/ Repression	Gaudin *et al.*, 2001; Mylne *et al.*, 2006
DECREASED DNA METHYLATION1 (DDM1)/ SWI2/SNF2 DNA helicase	Controls DNA methylation, possibly binds methyl-CpG binding domain proteins, and affects their subnuclear localization	Decondensation of centromeric heterochromatin, redistribution of remaining DNA methylation, changes in a pattern of histone methylation Silencing of *R-genes* and retrotransposons; involved in DNA damage response	Global/ Repression	Singer *et al.*, 2001; Johnson *et al.*, 2002; Soppe *et al.*, 2002; Stokes *et al.*, 2002; Zemach *et al.*, 2005; Shaked, *et al.*, 2006
DEFECTIVE FOR RNA-DIRECTED DNA METHYLATION1 (DRD1)/ SWI/SNF-like protein	Directing non-CpG DNA methylation in response to a RNA signal; mediates full erasure of methylation when the signal is removed; together with pol IVb acts downstream of smRNA biogenesis pathway, possibly interacting with DNA methyltransferases and DNA glycosylases; preferentially targets promoter and LTRs in euchromatin	Mutants do not show significant defects in CpG methylation but exhibit loss of non-CpG methylation at previously silenced euchromatic promoters and transposons; down regulation of *ROS1* and *DME*	Local, euchomatic promoters/ Repression, activation	Kanno *et al.*, 2004; 2005a; Matzke *et al.*, 2006 and references therein; Pikaard, 2006
RNA POLYMERASE IVb (pol IVb) (subunits NRPD2a and NRPD1b)/ Nuclear RNA polymerase	Guides cytosine methylation of homologous DNA regions by an unknown mechanism using smRNA signals; NRPD1b possibly recruits DNA methyltransferases involved in *de novo* cytosine methylation at asymmetric sites; together with DRD1 reversibly controls promoters and LTRs in euchromatin	Mutants do not show significant defects in CpG methylation but exhibit loss of non-CpG methylation at on previously silenced euchromatic promoters and transposons	Local, euchomatic promoters/ Repression, possibly activation	Kanno *et al.*, 2005b; Matzke *et al.*, 2006 and references therein; Pikaard, 2006
MAINTENANCE OF METHYLATION1 (MOM1)/ Similar to SWI2/SNF2	Regulation of silent heterochromatic regions transcription; transgene silencing; preventing transcription of 180 bp satellite repeats and not of transposons	Release of transcriptional gene silencing and 5S repeat silencing; no effect on heterochromatin organization and DNA methylation.	Global/ Repression	Amedeo *et al.* 2000; Vaillant *et al.*, 2006

2.2.5. Histone modifications: reinforcing DNA-methylation imprints

Since gene transcription occurs within a nucleosome consisting of DNA wrapped around an octamer histone core, modifications of histone proteins via (de)methylation and (de)acetylation regulate gene expression. It was demonstrated that the euchromatin state is dependent on hyperacetylation of histones H3 and H4 along with methylation of H3 at the lysine K4 position (Bender, 2004). In contrast, the formation of heterochromatin structure requires underacetylation of H3 and H4, methylation of K9, and demethylation of K4 residues of H3 (Bender, 2004). It was suggested, however, that different methylation states of histone H3 might also result from the deposition of two independent H3 variants that differ in sequence and posttranslational modifications, particularly in an enrichment of methylated K9 and K27 (Zilberman and Henikoff, 2005).

There are several lines of experimental evidence suggesting the interdependence of DNA and histone methylation. It was shown that CpG methylation loss in the *met1* mutant results in H3K9 methylation loss (Soppe *et al.*, 2002; Tariq *et al.*, 2003). In contrast, H3K9 methylation loss in the KRYPTONITE (KYP) histone methyltransferase *kyp* mutant does not affect CpG methylation (Jasencakova *et al.*, 2003). This suggests that H3K9 methylation acts downstream of CpG methylation and reinforces heterochromatin. On the contrary, DNA methylation at CpNpG sites appears to be partially dependent on the activity of KYP (Jackson *et al.*, 2002).

Histone methylation can recruit other proteins such as HETEROCHROMATIN PROTEIN1 (HP1) that binds to methylated H3K9 (Lachner *et al.*, 2001) and helps propagate heterochromatin to adjacent regions on the chromosome (Grewal and Moazed, 2003). Also, an *Arabidopsis* homologue of HP1, HETEROCHROMATIN PROTEIN1

(LHP1), is involved in regulating flowering time in response to environmental stimuli (Gaudin *et al.*, 2001; Mylne *et al.*, 2006).

Methylated DNA serves as a substrate for binding nuclear proteins named methyl-CpG-binding domain proteins or MBDs. These proteins bind to 5-methylcytosines, recruit enzymes that modify core histone proteins, and change local chromatin structure (Ben-Porath and Cedar, 2001) (Table 2.2.1). However, it must be noted that not all MBDs are able to bind methylated CpG *in vitro*. These MDBs can be possibly involved in control of chromatin structure through other mechanisms (reviewed in Zemach and Grafi, 2007).

2.2.6. Chromatin remodeling proteins: shaping and maintaining chromatin structure

Control of gene expression through DNA methylation and histone modifications is complemented by the activity of chromatin remodeling proteins. Among them, there are members of the SWI2/SNF2 DNA helicase family that are of crucial importance in DNA repair, recombination, gene expression and replication (Havas *et al.*, 2001). The SWI2/SNF2 family proteins alter chromatin structure through the disruption of DNA-histone interactions (Geiman and Robertson, 2002).

The DECREASED DNA METHYLATION1 (DDM1) protein was the first member of this family described. It controls methylation directly and indirectly by changing histone methylation (Johnson *et al.*, 2002) (Table 2.2.1). Recently, Zemach *et al.* (2005) demonstrated that MBDs bind DDM1. They also reported a disrupted localization of MBDs at chromocenters in the *ddm1* mutant. This suggests that DDM1 may facilitate the localization of MBDs at specific nuclear domains. The *ddm1* mutant shows a 70% reduction in global genome methylation (Jeddeloh *et al.*, 1999), activation of transposable elements (Miura *et al.*, 2001; Singer *et al.*, 2001), and phenotypical instability (Kakutani *et al.*, 1996). *ddm1*-induced hypomethylation also results in transcriptional activation of a previously silent disease-resistance gene array (Stokes *et al.*, 2002), and activates a number of retrotransposons (Kato *et al.*, 2004).

Another control mechanism of gene expression is represented by the nuclear MAINTENANCE OF METHYLATION1 (MOM1) protein with limited homology to DDM1 (Amedeo *et al.* 2000). MOM1 is involved in DNA-methylation-independent silencing of repetitive sequences in *Arabidopsis* (Vaillant *et al.*, 2006). MOM1 prevents

transcription of 180 bp satellite repeats of transposons (Vaillant *et al.*, 2006). In *mom1* mutants, a release of transgene silencing (Amedeo *et al.* 2000) and 5S repeat repression (Vaillant *et al.*, 2006) occurs without reducing or altering their DNA and histone methylation patterns (Table 2.2.1). This suggests the existence of two distinct epigenetic silencing pathways, DNA-methylation-dependent and DNA-methylation-independent.

2.2.7. Epigenetic modification – a stress response mechanism controlling gene regulation

Until recently, the link between stress exposure and sequence-specific changes in DNA methylation was hypothetical. It was demonstrated that prolonged exposure to cold triggers stable transcriptional silencing of *FLC* that leads to flowering inhibition (Henderson and Dean, 2004). Moreover, flowering time directly correlates with the level of DNA methylation in *MET1* gene antisense knockouts (Finnegan *et al.*, 1998a). Therefore, *met1* mutants do not require cold treatment to initiate flowering. This proves that a developmental switch was epigenetically controlled.

Cold exposure of root tissues of maize seedlings resulted in DNA demethylation at nucleosome core regions (Steward *et al.*, 2000). In fact, DNA replication was strongly reduced in chilled tissues, leading to speculation that genome hypomethylation was the result of active rather than passive demethylation. Cold-induced demethylation of a nucleosome core and relaxation of chromatin structure could serve as a stress-induced transcriptional switch for many stress-regulated genes (Steward *et al.*, 2002).

Several other papers suggest that changes in DNA methylation are required for stress protection. Dyachenko *et al.* (2006) demonstrated a two-fold increase in the level of CpNpG methylation in the nuclear genome of *M. crystallinum* plants exposed to high salinity. An increase in methylation was associated with switching from C3- to C4-type photosynthesis. Similarly, Sha *et al.* (2005) reported that an age-dependent increase in methylation confers resistance to the blight pathogen *X. oryzae* in rice.

Methylation contributes greatly to the plant's ability to respond to stress. Hypomethylation found in *met1* results in specific expression of 31 genes, most of which

are related to stress response (Wada *et al.*, 2004). Demethylation of the *NtAlix1*gene occurs also as a result of viral infection, thus confirming that induction of this gene under natural stress conditions requires sequence demethylation. Steward *et al.* (2000) also showed that transcriptional activation of a *ZmMI1* gene in maize seedlings was dependent on cold-induced sequence demethylation. The *ZmMI1* gene contains a retrotransposon-like sequence, and its activation mirrored cold-induced root-specific demethylation in *Ac/Ds* transposon regions followed by their activation (Steward *et al.*, 2000).

Activation of transposons in response to stress is a common phenomenon. Low temperature treatment decreases methylation and increases the rate of transposon *Tam3* excision by binding its transposase to GCHCG (H=not G) sites immediately after DNA replication, and thus preventing *de novo* sequence methylation (Hashida *et al.*, 2003; 2006). Stress induces the activity of *Tos17* (rice) (Hirochika *et al.*, 1996), *Tto1* (tobacco) (Takeda *et al.*, 1999), *Tnt1* (tobacco) (Beguiristain *et al.*, 2001) and *BARE-1* (barley) (Kalendar *et al.*, 2000) retrotransposons. An intriguing hypothesis that stress-activated transposons could positively contribute to genome adaptation to growth in colder climates was supported by the detection of *mPing* transposition into a rice homologue of the flowering time gene *CONSTANS* in stressed cultivars (Jiang *et al.*, 2003). Indeed, Song *et al.* (1997) suggested that a number of transposable elements and their derivatives present in resistance gene (*R-gene*) loci played a significant role in a rapid diversification of this gene family. These publications support the long-standing hypothesis proposed by Barbara McClintock. She suggested that all kinds of stresses could potentially reshape plant genomes via transposon activation (McClintock, 1984).

Similarly, histone modifications represent another stress response mechanism acting by epigenetic control over gene expression. Chua *et al.* (2003) established a link between light-dependent transcriptional induction of a pea plastocyanin gene and histone acetylation. It was suggested that binding an enhancer to a nuclear matrix activates transcription through alteration of local chromatin structure, thus increasing acetylation of the promoter and 5' coding region (Chua *et al.*, 2003). Tsuji *et al.* (2006) demonstrated that transcriptional activation of submergence-inducible genes *ADH1* and *PDC1* in rice was reversibly mediated through histone H3K4 methylation and H3 acetylation.

Consistently with the previously mentioned types of epigenetic modifications, the involvement of chromatin remodeling factors in stress response was supported by extensive studies of plants impaired in their functions. DDM1 deficient plants were shown to be more sensitive to UV-C and γ-radiation than wild type and *met1* mutant plants (Shaked *et al.*, 2006). This indicates that increased radiation sensitivity can be mediated by disrupting chromatin remodeling functions rather than cytosine methylation. It should be noted, however, that Shaked *et al.* (2006) did not analyze the double *met1cmt3* mutant. If they considered any possible functional redundancy of MET1 and CMT3 (Kato *et al.*, 2003), they could have made different conclusions.

Other reports also supported the link between chromatin maintenance and stress response. Mutants of a nuclear protein BRU1 involved in the maintenance of chromatin structure were highly sensitive to genotoxic stress and were characterized by increased intrachromosomal homologous recombination (Takeda *et al.*, 2004). Similarly, expression of the *MIM1* gene involved in the maintenance of chromosome structure and

required for efficient homologous recombination was significantly increased by DNA-damaging agents (Hanin *et al.*, 2000).

Another SWI/SNF like protein, DEFECTIVE FOR RNA-DIRECTED DNA METHYLATION1 (DRD1), represents a novel plant-specific chromatin remodelling protein that is required for RNA-directed *de novo* methylation of target promoters (Kanno *et al.*, 2004). It is also necessary for full loss of induced *de novo* DNA methylation after a silencing RNA trigger is withdrawn (Kanno *et al.*, 2005a). DRD1 interacts with two other factors, NRPD1b and NRPD2a, that represent subunits of a novel plant-specific RNA polymerase, pol IVb (Kanno *et al.*, 2005b). Together, DRD1 and a pol IVb complex act downstream of a small RNA (smRNA) biogenesis pathway. Thus, they direct reversible silencing of euchromatic promoters in response to RNA signals possibly through recruitment of DNA methyltransferases to methylate homologous DNA sequences (Matzke *et al.*, 2006) (Table 2.2.1). It is noteworthy that among putative DRD1 targets are DNA glycosylases ROS1 and DME that both are involved in active DNA demethylation (Morales-Ruiz *et al.*, 2006; Penterman *et al.*, 2007a). Down-regulation of ROS1 in *drd1* and *pol IVb* mutants confirms the importance of a DRD1/pol IVb pathway for active loss of induced *de novo* DNA methylation (Kanno *et al.*, 2005a).

2.2.8. smRNAs as a sensitive and selective trigger that directs epigenetic modification

A significant advantage of epigenetic regulation is a fast stimulus-directed generation of new transcriptional states that are heritable and reversible. One of the key mechanisms involved in targeting chromatin structure and modifying a gene expression pattern in response to environmental stimuli is based on the activity of smRNAs. They were shown to guide transcription repression through modifications of DNA and histones (Bender 2004; Matzke *et al.*, 2004; Chan *et al.*, 2006).

There are two current models that explain how smRNAs interact with a target locus in DNA. They suggest a homology-based pairing of smRNAs with either genomic DNA sequences (a DNA-recognition model) or nascent RNA transcribed from the target locus (an RNA-recognition model) (reviewed in Matzke and Birchler, 2005). In accordance with the DNA-recognition model, the interaction of smRNAs with genomic DNA sequences could provide an attractive substrate for cytosine methyltransferases. The ability of a DRD1/pol IVb complex to interact with DNA methyltransferases and DNA glycosylases suggests the involvement of this complex in the maintenance of reversible epigenetic states of euchromatic promoters in response to RNA signals (Matzke *et al.*, 2006).

An initial DNA methylation imprint in response to a stress-induced RNA signal can be created by DRMs at asymmetric sites and then perpetuated at symmetric CpG and CpNpG sites by MET1 and CMT3, respectively (Cao and Jacobsen, 2002a; 2002b). In contrast to non-CpG methylation that is substantially reduced when a signal is removed, CpG methylation can be maintained through several generations (Bender, 2004). These

findings are intriguing, since non-CpG methylation is very unique to plants. It may represent an additional degree of complexity added to a gene expression control system to insure its fast and reversible response to environmental stimuli.

Small RNAs reported up-to-date are involved in the regulation of plant development (Chen, 2004; Juarez *et al.*, 2004; Kidner and Martienssen, 2004). They are tissue and organ specific (Sunkar and Zhu, 2004; Lu *et al.*, 2005) and are regulated by a number of abiotic stresses including mechanical stress, dehydration, salinity, cold, abscisic acid, and nutrient deprivation (Sunkar and Zhu, 2004; Borsani *et al.*, 2005; Lu *et al.*, 2005; reviewed in Sunkar *et al.*, 2007). A large number of non-conservative micro RNAs (miRNAs), available in some species and absent in the others, might support a hypothesis that the development of a specialized miRNA network was driven by physiological and stress conditions specific for each species (reviewed in Lu *et al.*, 2005).

Identification of 22 miRNAs from developing secondary xylem of *P. trichocarpa* stems (Lu *et al.*, 2005) further confirmed that species-specific miRNAs contribute to regulation of gene expression associated with specific growth/stress conditions. The expression of many ptr-miRNAs was induced in the developing xylem of stems in the presence of gravitropism-mediated mechanical stress (Lu *et al.*, 2005). This stress triggers upregulation of ptr-miR408 expression and regulates the plastocyanin-like protein mediating lignin polymerization. In addition, mechanical stress downregulates the expression of ptr-miR164 and ptr-miR171. These miRNAs target genes are involved in cell division and elongation in response to gravitropism.

Recent data by Sunkar and Zhu (2004) demonstrated the existence of stress-inducible changes in the *Arabidopsis* miRNA pool. The most interesting examples are

miRNA402 and miRNA407 regulated by dehydration, salinity, cold, and abscisic acid. Whereas miRNA402 targets ROS-like DNA glycosylase, miR407 targets a SET domain protein functioning in histone *Lys* methylation (Sunkar and Zhu, 2004). Some of miRNAs were shown to have multiple target sites within the same gene, which implies that different levels of gene repression might be achieved by binding different numbers of miRNAs to the target (Doench *et al.*, 2003).

Stress-induced miRNAs have a tissue-specific expression pattern. It reflects organ-specific functional and metabolic differences in response to stress. Indeed, miR393 downregulates TIR1, a positive regulator of auxin signalling, and has its strongest expression in the inflorescence under physiological conditions. Hence, strong miR393 induction by stress is consistent with inhibition of plant growth under stress conditions (Sunkar and Zhu, 2004). Consistently with the role of miRNAs in the establishment of a stress-induced gene expression pattern, *Arabidopsis* mutants *hen1-1* and *dcl1-9* partially impaired in the production of miRNAs were shown to be hypersensitive to abiotic stresses (Sunkar and Zhu, 2004).

Recently, nat-siRNAs (a new class of small interfering RNAs (siRNAs) that derives from natural antisense transcripts) have been reported (Borsani *et al.*, 2005). They are involved in stress-mediated regulation of genes located in antisense overlapping pairs that results in generation of complimentary transcripts. The authors showed that induction of one of these genes in an antisense pair by stress results in the production of nat-siRNA, which guides the cleavage of other gene transcripts followed by downregulation of the gene activity. Similarly, studies of Katiyar-Agarwal *et al.* (2006) demonstrated induction of another specific nat-siRNA during *Pseudomonas syringae* infection that conferred

resistance to this pathogen. This mechanism may play an important role, since there are thousands of genes that are grouped in antisense overlapping pairs (Borsani *et al.*, 2005).

2.2.9. Inducible epigenetic changes may guide genome evolution and shape the plant genome

Epigenetic modification of DNA via selective cytosine methylation plays a crucial role in establishing a stable epigenetic mark in plants. Despite their reversibility, changes in DNA methylation are quite stable modifications that are not easily reset; and they are frequently transmitted for several generations. Indeed, backcrosses of a *ddm1* mutant to wild type plants do not revert the mutant phenotype. This demonstrates that hypomethylation can be stably transmitted during meiosis, gametogenesis, and mitosis, regardless of the presence of a functional *DDM1* gene (Kakutani *et al.*, 1999). The progeny of *MET1* antisense plants exhibit DNA hypomethylation independent of the presence of the transgene locus that previously triggered inhibition of *MET1* (Finnegan *et al.*, 1996).

Perhaps, the best examples of methylation-mediated heritable changes are epialleles representing different forms of the same gene regulated epigenetically. Epialleles can be formed in response to a number of stimuli, and they may play an important role in acclimation. Good examples of such epialleles are methylated and demethylated forms of the *FWA* gene. Both of them are equally stable and can be inherited as a true Mendelian trait based on methylation rather than on the sequence difference (Zilberman and Henikoff, 2005). Targeting DNA methylation to the *FWA* gene was triggered by positioning its promoter and transcription start site within two pairs of direct repeats (Soppe *et al.*, 2000). Moreover, two *Arabidopsis* ecotypes, L*er* and Da (1)-12, carry transposon insertions in the first intron of the *FLC* gene. This represents independent adaptive events that lead to the establishment of cold-independent flowering

initiation by preventing high expression of the *FLC* gene (Michaels *et al.*, 2003; Liu *et al.*, 2004a).

Paramutations represent another class of heritable epigenetic traits. They are caused by interactions between two alleles of a single locus. Paramutation is essentially a heritable change of one allele (a target) that is induced by the other allele (a trigger). The silenced state of a target allele induced by a trigger remains stable, even if a trigger allele segregates out in the next generation (Chandler *et al.*, 2000). Recent studies by Alleman *et al.* (2006) confirmed the critical role siRNAs play in production and maintenance of chromatin states in maize. They demonstrated that *MEDIATOR OF PARAMUTATION1 (MOP1)* gene that encodes the RNA-dependent RNA polymerase is required for production of paramutations (Alleman *et al.*, 2006). It can be hypothesized that a mi-/si-RNA-induced transgenerational response to stress indeed exists in plants.

The adaptive advantage of heritable stress memories was recently supported by observations that plant exposure to biotic (pathogen) or abiotic (salt) stresses leads to global and loci-specific changes in genome stability and methylation. It also results in elevated tolerance of the immediate progeny to previously applied stresses (Boyko *et al.*, 2008; Boyko and Kovalchuk, 2008b; Boyko *et al.*, data not published).

Genome rearrangements represent another epigenetic-sensitive mechanism affecting genome stability. The dual role of HR, as a DNA repair pathway and a putative evolutionary tool, was intensively discussed over the past several years (Puchta, 2005; Schuermann *et al.*, 2005 and references therein). It has been suggested that HR can be involved in genome evolution through rearrangements of existing sequences, frequently resulting in gene duplication or deletion events. Indeed, the degree of HR-mediated

V(D)J rearrangements depends on DNA methylation (Bassing *et al.*, 2002). It can be suggested that stress-directed changes in DNA methylation can stimulate or prevent rearrangements in different genomic loci (Rizwana and Hahn, 1999). Highly conserved gene families located in clusters could possibly increase their diversity using HR. Indeed, it has been suggested that the evolution of plant *R-genes* involved gene duplication and recombination events (Meyers *et al.*, 2005). The fact that meiotic and somatic HRF can be altered by a variety of biotic and abiotic stresses could suggest that changes in the HRF represent one of the mechanisms of stress adaptation (Kovalchuk *et al.*, 2003a, 2003b, 2004a; Molinier *et al.*, 2005, 2006; Boyko *et al.*, 2005, 2006b, 2006c, 2007, 2008; Boyko and Kovalchuk 2008b). Indeed, in several studies, the increased HRF was inherited by the progeny of stressed plants as an epigenetic trait persisting in a population for one or several non-stressed generations (Kovalchuk *et al.*, 2003b; Molinier *et al.*, 2006; Boyko *et al.*, 2007, 2008; Boyko and Kovalchuk 2008b).

Recent studies by Boyko *et al.* (2007) established a defined correlation between stress exposure, loci-specific epigenetic changes, and genome stability of exposed plants and their progeny using a well-studied model of TMV infection. We have demonstrated that the progeny of tobacco plants treated with TMV inherited elevated rates of HR that correlated with an increased frequency of rearrangements in *R-gene*-like loci. Importantly, the progeny of stressed plants displayed increased levels of global genome methylation and exhibited loci-specific hypomethylation. Namely, the *R-gene*-like loci that carry homology to the *N-gene* conferring resistance to TMV were found to be hypomethylated and, as a consequence, rearranged more frequently (Boyko *et al.*, 2007). Since plants used for infection (the SR1 cultivar) did not have the *N-gene*, it would be

correct to assume that locus-specific changes in methylation and rearrangements could be a plant strategy for creating an active *R-gene*. It is possible that such transgenerational changes in recombination and DNA methylation represent a general epigenetically controlled mechanism directed to selective relaxation of DNA sequences, thereby allowing faster evolution under the influence of various environmental stimuli. The foregoing might somewhat resemble a phenomenon reported in flax, where a number of heritable changes could be triggered by environmental changes relatively fast (Chen *et al.*, 2005; Cullis, 2005).

2.3. *Agrobacterium*-mediated genetic transformation: basic mechanisms and improvement strategies

2.3.1. Homologous recombination as a biotechnological tool

The importance of HR is not restricted to its critical role in meiosis and DSB repair. HR has increasing significance in the field of biotechnology and genetic engineering (Vergunst and Hooykaas, 1999; Puchta 2002; Reiss 2003; Hanin and Paszkowski, 2003; Lida and Terada, 2004). Transgene integration into a host genome using an HR repair pathway offers unique opportunities for site-specific transgene integration into a predetermined location. It has great prospects for developing GT technology in plants. The contribution of HR to genetic plant transformation and the ways it could be improved are the focus of our study and will be discussed below.

2.3.2. *Agrobacterium*: from a plant pathogen to a favoured genetic transformation tool

During the past decade, *Agrobacterium*-mediated genetic transformation became one of the dominant technologies used to produce a variety of genetically modified transgenic plants. The successful introduction of this technology can be partially explained by the fact that *Agrobacterium tumefaciens* used for donor DNA delivery is a typical plant pathogen present in soil. However, in contrast to many other plant pathogens, it has a unique ability of trans-kingdom DNA transfer (reviewed in Gelvin 2003). The natural ability to transform a host cell using bacterial DNA observed in *Agrobacterium* triggered an extensive search for other plant pathogens with similar properties. To date, three non-*Agrobacterium* species, all sharing the ability of plant genetic transformation, have been identified. This short list includes *Rhizobium sp.* NGR234, *Sinorhizobium meliloti* and *Masorhizobium loti* (Broothaerts *et al.*, 2005; reviewed in Chung *et al.*, 2006). However, *Agrobacterium* remains a favoured tool used for transgenesis. The foregoing is confirmed by a growing number of *Agrobacterium*-related patents being claimed every year (Roa-Rodriguez and Nottenburg, 2003), and by a continuously increasing range of host species including various plant species, yeast, mushrooms, filamentous and phytopathogenic fungi, and even a human cell (Tzfira and Citovsky, 2003; reviewed in Lacroix *et al.*, 2006). Importantly, the ability to transform human cells opens up great possibilities for using *Agrobacterium* not only in plant biotechnology but also in human and animal gene therapy.

Agrobacterium performs genetic transformation of recipient species by transferring and integrating the bacterium tumor-inducing plasmid (Ti) region

(approximately 10 to 30 kbp in size) into the host genome. The transferred DNA region is known as the T-DNA, and it usually represents less than 10% of the Ti plasmid. Borders of the T-DNA region are well-defined by the so-called border sequences that are represented by 25 bp direct long repeats, flanking the T-DNA right and left borders. Generally, there is only one T-DNA region present in the Ti plasmid, however Ti plasmids containing multiple T-DNA sequences are also known. T-region sizes of some naturally occurring Ti plasmids can reach approximately 23 kpb, allowing the introduction of many genes into the T-region (reviewed in Gelvin 2003, and Tzfira *et al.*, 2004a).

In the wild-type *Agrobacterium* strains, the T-DNA encodes a set of oncogenes and opine-catabolism genes, and its expression in a plant cell results in neoplastic growth and production of opines. In contrast, biotechnology relies on using the so-called recombinant or disarmed *Agrobacterium* strains that have an entire sequence of the native T-DNA replaced with any genes of interest, and therefore they are capable of plant transformation without causing tumour development (Tzfira and Citovsky, 2006 and references therein). Interestingly, recombinant *Agrobacterium* was shown to transfer large T-DNAs (up to 150 kbp) (Hamilton *et al.*, 1996) and 200 kbp (Miranda *et al.*, 1992).

The Ti plasmid also contains a set of *virulence* (*vir*) genes. These genes together with *chromosomal virulence* (*chv*) genes mediate processing of T-DNA from the Ti plasmid and its subsequent exporting from the bacterium to the host cell. Briefly, VirD1 and VirD2 act as site-specific endonucleases that recognize the T-DNA border sequences and release a single stranded (ss)T-DNA molecule from the Ti plasmid. The resulting

ssT-DNA with one molecule of VirD2 attached to the 5' end represents an immature T-complex that is exported by the VirB/D4 type IV secretion system into the plant cell along with several other Vir proteins, including VirE2, VirE3 and VirF. Once inside the host cell cytoplasm, the T-DNA covered with numerous VirE2 molecules can form a "telephone cord"-like structure of a mature T-complex, which is then actively transported to the nucleus using a cell dynein motor. A mature T-complex enters the nucleus through a nucleus pore complex (NPC) and is believed to be directed to DSB sites, where T-DNA is stripped of accompanying proteins and integrated into the host genome (reviewed in Tzfira *et al.*, 2004b; Tzfira and Citovsky, 2006). It is noteworthy that at various stages of T-DNA delivery and integration (including the immature T-complex delivery to the host cell cytoplasm, transport through the cytoplasm and nuclear import, intracellular transport, T-DNA uncoating and integration), the activity and factors of various host cellular mechanisms are actively utilized by *Agrobacterium* to complete transformation (reviewed in Citovsky *et al.*, 2007). The fact that *Agrobacterium* depends greatly on host factors for the successful T-DNA integration suggests a variety of promising strategies for improving plant transformation efficiency. These strategies are based on various manipulations with host factors participating in T-DNA integration.

2.3.3. Host systems involved in *Agrobacterium* infection

Among host factors used by *Agrobacterium* on its way to the nucleus are plant cell surface receptors. The attachment of *Agrobacterium* to host plant cells is an absolute requirement for transformation (McCullen and Binns, 2006). Recent studies made it possible to isolate several putative receptors and cell-attachment factors localized on the host cell surface and utilized by *Agrobacterium*. Among them are a vitronectin-like protein, a rhicadhesin-binding protein, a cellulose synthase-like gene and several VirB2-interacting proteins (BTIs) (reviewed in Citovsky *et al.*, 2007).

Once inside the host cytoplasm, the mature T-complex has to be delivered to the nucleus. The large size of the complex (with an outer diameter ~ 15.7 nm (Abu-Arish *et al.*, 2004) and the dense structure of cytoplasm impedes passive transport of the complex. In contrast, a model that suggested an active mechanism mediating transport of this complex to the nucleus was developed by Tzfira (2006) based on the experimental evidence available (Salman *et al.*, 2005; Tzfira, 2006). Tzfira (2006) suggested that active transport is performed by a dynein-like *Arabidopsis* protein DLC3, which interacts with another host factor, a VirE2-interacting protein 1 (VIP1), mediating the recognition of VirE2 by the nuclear import machinery (Citovsky *et al.*, 2007).

The large size of the T-complex no matching closing parameters compared to the NPC (~ 9 nm) implies that there should be an active mechanism in a plant cell that could deliver the T-complex to the nucleus. The host nuclear-import machinery itself is the best tool for doing this. Consistently with this hypothesis, both proteins interacting with T-DNA in the T-complex, VirD2 and VirE2 proteins, can also interact with host importin/karyopherin α (Ballas and Citovsky, 1997) and VIP1 (Tzfira *et al.*, 2001)

proteins, respectively. While VirD2 initiates nuclear import and is directly recognized by the nuclear-import machinery, VIP1 along with another bacterial factor, a VirE3 protein, plays a role of a molecular adaptor between VirE2 and host karyopherin α (reviewed in Tzfira and Citovsky, 2006). Consistent with a pivotal role for the importin α family in the T-complex import, *Arabidopsis* plants deficient in one of importin α genes are resistant to *Agrobacterium* infection (Zhu *et al.*, 2003a). Interestingly, a VirE3 protein may play a role of a bacterial host range factor (Hirooka and Kada, 1986) that compensates the absence or low levels of VIP1 in some plants and non-plant species (discussed in Citovsky *et al.*, 2007). Indeed, VirE3 promotes nuclear accumulation of VirE2 in mammal cells that lack VIP1 and in plants with suppressed expression of VIP1 (Lacroix *et al.*, 2005). Deng *et al.* (1998) reported interactions between VirD2 and host cyclophilins that serve as molecular chaperones and maintain the proper conformation of VirD2 during its transport to the nucleus.

Another group of host factors that influence nuclear import of the T-complex and that has been identified in *Arabidopsis* and alfalfa is represented by a nuclear cyclin-dependent kinase-activating kinase (CAK2M) (Bakó *et al.*, 2003) and by a type 2C serine/threonine phosphatase, DIG3 (Tao *et al.*, 2004). While the former factor phosphorylates VirD2 and promotes its nuclear import, the latter reverses phosphorylation and negatively regulates nuclear import of the T-complex. The recent report by Djamei *et al.* (2007) suggested that *Agrobacterium* is also able to use a part of the mitogen-activated protein kinase (MAPK) defence signalling pathway, MPK3 kinase, for phosphorylation of VIP1 that leads to its nuclear localization (reviewed in Dafny-Yelin *et al.*, 2008).

Delivery of the T-complex to its integration site in chromatin also requires the participation of host factors, particularly CAK2M, a TATA-box binding protein (TBP), VIP1, and core histones (reviewed in Citovsky *et al.*, 2007). The first two factors, CAK2M and TBP, bind to VirD2 (Bakó *et al.*, 2003). Moreover, CAK2M interacts with the largest subunit of RNA polymerase II, thus permitting recruitment of TBP that allows controlling transcription and transcription-coupled repair. Overall, interactions between CAK2M, TBP, and VirD2 target the T-complex to the host chromatin (Bakó *et al.*, 2003). Considering the involvement of VIP1 in chromatin decondensation, it is safe to assume that its interactions with VirE2 may play a role similar to that of the T-complex targeting to chromatin (Tzfira *et al.*, 2001). Consistently with that model, VIP1 was shown to interact *in planta* with the *Arabidopsis* core histone H2A (Li *et al.*, 2005a; Loyter *et al.*, 2005). An *Arabidopsis* histone H2A mutant was found to be deficient in T-DNA integration (Mysore *et al.*, 2000a). Finally, the involvement of the host transcription machinery in delivery of T-complexes to insertion sites can be further supported by the finding of Alonso *et al.* (2003) that T-DNA insertions into regulatory regions of plant genes occur at high frequencies.

During the integration stage, the interaction of the host DNA repair machinery with T-DNA requires uncoating T-DNA of its cognate proteins. Uncoating is completed via the plant-targeted proteolysis machinery. VirF, a bacterial F-box protein that functions as a subunit of E3 ubiquitin ligase, forms a ternary complex with VIP1 and VirE2. This results in their destabilization by targeted proteasomal degradation (Tzfira *et al.*, 2004b). The foregoing is consistent with a nuclear localisation of VirF and a negative effect of proteasomal inhibitors on early T-DNA expression (Tzfira *et al.*, 2004b). It is

possible, however, that unidentified plant F-box proteins are also involved in this process, since VirF is known to be dispensable for transformation of some plant species (Regensburg-Tuink and Hooykaas, 1993).

The final step of transformation, the actual integration of T-DNA into plant genomes, is almost completely host-dependent. It mainly determines the precision of transgene integration (Tzfira *et al.*, 2004a). Unfortunately, there are still some blank spots and data controversy regarding mechanisms and factors employed during this stage. Two mutually exclusive models explaining T-DNA integration were originally proposed (reviewed in Tzfira *et al.*, 2004a). The first, the DSBR model, postulates that T-DNA preferentially integrates into DSB sites. Integration via the DSBR model requires conversion of ssT-DNA molecules into dsT-DNA molecules before integration. In contrast, the single-strand-gap repair (SSGR) model suggests that ssT-DNA integrates into a nicked DNA and then provides a template for complimentary DNA strand synthesis that substitutes an original host DNA sequence at the integration site. Both models were later modified to incorporate new experimental evidence (reviewed in Tzfira *et al.*, 2004a).

Experimental evidence suggests targeted integration of T-DNA into DSB sites via double stranded intermediates (Chilton and Que, 2003). A number of studies reported that the induction of DSBs via X-ray irradiation (Kohler *et al.*, 1989) and using rare cutting endonucleases (Salomon and Puchta, 1998; Tzfira *et al.*, 2003) resulted in enhanced transgene integration. In fact, Tzfira *et al.* (2003) analysed sequences at insertion sites obtained from transformation of transgenic tobacco carrying an I-*Sce*I endonuclease recognition site with two *Agrobacterium* strains. One of the strains had the T-DNA

carrying an I-*Sce*I recognition site, and the other one allowed transient expression of I-*Sce*I to induce DSBs. Importantly, seven out of a total of 16 plants, which contained the T-DNA integrated into the I-*Sce*I site, contained the T-DNA digested with I-*Sce*I. Since the I-*Sce*I endonuclease cuts only double-stranded DNA, it was concluded that conversion of ssT-DNA into double stranded DNA occurred before an integration event (Tzfira *et al.*, 2003). Overall, it seems that ssT-DNA that enters the nucleus is being converted into a double stranded intermediate that later is directed to naturally occurring DSBs, and then it is integrated in the host genome using the host DNA repair machinery. This implies that host factors are required for conversion of a single stranded T-DNA to a double stranded T-DNA, for production of DSBs in the host genome, and finally for incorporation (ligation) of T-DNA molecule into these breaks.

As it has been mentioned, DSBs can be repaired using one of two alternative DNA repair pathways, NHEJ or HR. Both pathways differ in their fidelity of DNA repair (Gorbunova and Levy, 1999; Puchta, 2005) which may affect the intactness of the T-DNA sequence during integration. Moreover, using homologous templates for repair makes HR a likely candidate for generation of site-specific insertions and GT (Vergunst and Hooykaas, 1999; Puchta 2002; Reiss 2003). Unfortunately, the majority of DSBs in plant cells are repaired via NHEJ, and the contribution of HR is very low (Gorbunova and Levy, 1999; Puchta, 2005). This is a major drawback in the development of technology for a site-specific transgene integration in plant cells. A number of studies conducted in the past several years were focused on: identification of host DNA repair factors involved in T-DNA integration, detection of mechanisms that control a DSB repair pathway choice in a cell, and manipulation of these mechanisms during T-DNA integration in the host

genome (reviewed in Tzfira *et al.*, 2004a; Tzfira and Citovsky, 2006; and Shrivastav *et al.*, 2008).

2.3.4. Host DNA repair factors involved in T-DNA integration

Most of the experimental evidence supporting the role of host DNA repair factors in T-DNA integration was obtained using yeast as a model system. Using this system, it was first demonstrated that simultaneous inactivation of HR and NHEJ by mutations in *RAD52* and *KU70* genes inhibits T-DNA integration completely (van Attikum and Hooykaas, 2003), proving that the DSB repair machinery of a cell is absolutely required for successful transformation. Consistently, mutations inactivating either *RAD52* or *KU70* genes lead to preferential integration of T-DNA via the NHEJ pathway (van Attikum and Hooykaas, 2003) or the HR pathway (van Attikum *et al.*, 2001), respectively. This suggests that Ku70 and Rad52 enzymes play a key role in the determination of a T-DNA integration mode in yeast.

Using different yeast mutants deficient in DNA repair genes, van Attikum and Hooykaas (2003) demonstrated that integration of T-DNA via the NHEJ pathway requires the activity of Ku70, Rad50, Mre11, Xrs2, Lig4, and Sir4. In contrast, integration via the HR pathway is independent of Rad50, Mre11, Xrs2, Lig4, and Ku70 and requires the activity of Rad52 and Rad51 (van Attikum *et al.*, 2001) (Table 2.3.1).

Unfortunately, the results on T-DNA integration using the host's DNA repair pathways obtained in the yeast system can not be directly applied to plants. In yeast, HR is the predominant mode of DNA repair. In contrast, plants like other higher eukaryotes predominantly use the NHEJ pathway. The difference is also reflected at the molecular level: vertebrates and plants have three critical NHEJ-related factors that are missing in yeast, including DNA-PKcs, BRCA1, and Artemis (Bleuyard *et al.*, 2006 and references therein; Reidt *et al.*, 2006; reviewed in Shrivastav *et al.*, 2008). The presence of DNA-

PKcs in higher eukaryotes may play a critical role in the regulation of NHEJ and could account for its domination over HR (reviewed in Shrivastav *et al.*, 2008). Since a low HR frequency in vertebrate cells coexists with the presence of a yeast Rad52 homologue, the absence of Rad52 in plants (Iyer *et al.*, 2002) can not be the main cause of a low HR activity.

One of the key factors involved in T-DNA integration in plants is a Ku80 protein that in complex with Ku70 and DNA-PKcs proteins represents the NHEJ pathway. The role of Ku80 was supported by genetic studies using *Arabidopsis* insertional mutants in the *KU80* gene that were found to be deficient in T-DNA integration in somatic cells (Li *et al.*, 2005b). Immunoprecipitation of Ku80-dsT-DNA complexes from *Agrobacterium*-infected plants supported a physical interaction between Ku80 and T-DNA in the nucleus (Li *et al.*, 2005b). Consistently, overexpression of Ku80 in plant somatic cells resulted in an increased susceptibility to *Agrobacterium* infection (Li *et al.*, 2005b). In contrast to somatic cells, data on the involvement of Ku80 in T-DNA integration in germ-line cells remain controversial. While Friesner and Britt (2003) reported Ku80 to be essential for transformations, the study of Gallego *et al.* (2003) demonstrated its dispensability. Similarly, another NHEJ factor, *Arabidopsis* LIG4 ligase, was dispensable for transformation in somatic cells (van Attikum *et al.*, 2003) and required in germ-line cells (Friesner and Britt, 2003) (Table 2.3.1). Interestingly, some *Arabidopsis* mutants, like *rat1,* that show resistance to *Agrobacterium* somatic transformation (Zhu *et al.*, 2003b) also display susceptibility to germ line transformation (Mysore *et al.*, 2000b). This phenomenon may indicate the difference in a spectrum of host factors required for transformation in somatic (Zhu *et al.*, 2003b) and germ-line cells (Ye *et al.*, 1999).

Experimental evidence provided by studies of Mysore *et al.* (2000a) demonstrated that core histones represent another important factor influencing the last step of plant transformation. Histone H2A expression in various plant tissues was positively correlated with their susceptibility to *Agrobacterium* infection (Yi *et al.*, 2002). Moreover, inactivation of the *AtH2A* gene in the *Arabidopsis rat5* mutant leads to deficiency in T-DNA integration (Mysore *et al.*, 2000a). Overall, histone H2A may promote T-DNA integration via relaxation of a host chromatin structure (Mysore *et al.*, 2000a). Consistently, the importance of chromatin structure in T-DNA integration was further supported using *fas1* and *fas2* mutant plants (Endo *et al.*, 2006). FAS1 and FAS2 represent two of three subunits of Chromatin Assembly Factor 1 (CAF-1) (Kaya *et al.*, 2001; Henning *et al.*, 2003) that mediates deposition of H3/H4 histones onto replicating DNA, thereby promoting chromatin condensation (Tagami *et al.*, 2004). Reduced heterochromatin in *fas* mutants (Schonrock *et al.*, 2006) was consistent with an enhanced frequency of T-DNA integration into the plant genome (Endo *et al.*, 2006).

The significance of host chromatin structure for T-DNA integration is consistent with a genome-wide T-DNA insertional analysis that included more than 80 000 independent insertions and demonstrated preferential integration of T-DNA in intergenic regions, including promoters, 5' and 3' UTRs, in which active transcription usually occurs (Alonso *et al.*, 2003). However, it should be taken into consideration that the authors who reported preferential integration of T-DNA into transcriptionally active regions of the host genome (Alonso *et al.*, 2003; Tzfira *et al.*, 2003) used an active selection during the callus regeneration step. This could result in a preferential selection of actively expressed T-DNA insertions located in transcriptionally active regions,

thereby discriminating the selection of transgenic plants containing T-DNA insertions within heterochromatin or within regions with a low transcriptional activity. In fact, recent studies that used non-selective conditions supported the idea that T-DNA integration occurs more randomly in a genome, and integration events can be found also in heterochomatic regions at relatively high frequencies (Francis and Spiker, 2005; Kim *et al.*, 2007).

Table 2.3.1. Summary of yeast and *Arabidopsis* DNA repair proteins involved in T-DNA integration and their functions

Host gene		Role in DNA repair	Yeast		*Arabidopsis*	References
Yeast	*Arabidopsis*		HR	NHEJ		
KU70	*AtKu70*	Binding and stabilization of various types of DNA ends; DSB end juxtaposition	Not required	Required	Not determined	van Attikum *et al.*, 2001; Tamura *et al.*, 2002; van Attikum and Hooykaas, 2003
KU80	*AtKu80*	Binding and stabilization of various types of DNA ends; DSB end juxtaposition	Not determined	Not determined	Required for T-DNA integration in somatic cells; reported both essential and dispensable for integration in germ-line cells; overexpression in somatic cells increases transformation frequency	Tamura *et al.*, 2002; West *et al.*, 2002; Friesner and Britt, 2003; Gallego *et al.*, 2003; Li *et al.*, 2005b
LIG4	*AtLig4*	ATP-dependent ligase; joining DSB ends	Not required	Required	Dispensable for transformation in somatic cells; required for transformation in germ-line cells	Friesner and Britt, 2003; van Attikum *et al.*, 2001; van Attikum and Hooykaas, 2003; van Attikum *et al.*, 2003; Calsou *et al.*, 2003; Zhu *et al.*, 2003b
SIR4	Unknown homolog	Interacts with Ku70; forms a complex with Sir3-Sir2; may be involved in histone acetylation	Not determined	Required	–	van Attikum *et al.*, 2001; van Attikum and Hooykaas, 2003
RAD50	*AtRad50*	DSB end juxtaposition and end processing; together with Mre11 and Xrs2 forms a complex that has the exonuclease activity	Not required	Required	Not determined	Gherbi *et al.*, 2001; Gallego *et al.*, 2001; van Attikum *et al.*, 2001; van Attikum and Hooykaas, 2003; Bleuyard *et al.*, 2004
MRE11	*AtMre11*	DSB end juxtaposition and end processing; together with Rad50 and Xrs2 forms a complex that has the exonuclease activity	Not required	Required	Not determined	Bundock and Hooykaas, 2002; van Attikum *et al.*, 2001; van Attikum and Hooykaas, 2003; Puizina *et al.*, 2004

Table 2.3.1. Summary of yeast and *Arabidopsis* DNA repair proteins involved in T-DNA integration and their functions (*continued*)

Host gene		Role in DNA repair	Yeast		*Arabidopsis*	References
Yeast	***Arabidopsis***		**HR**	**NHEJ**		
XRS2	Unknown homolog	DSB end juxtaposition and end processing; together with Rad50 and Mre11 forms a complex that has the exonuclease activity	Not required	Required	–	van Attikum *et al.*, 2001; van Attikum and Hooykaas, 2003
RAD51	*AtRad51*	Formation of Rad51/ssDNA nucleofilament and strand invasion	Required	Not required	Not determined	Doutriaux *et al.*, 1998; van Attikum *et al.*, 2001; van Attikum and Hooykaas, 2003; Li *et al.*, 2004a
	AtRad5	Close homolog of yeast *RAD51*; ATP-dependent homologous function in DNA pairing	–	–	*rad5* mutant is deficient in T-DNA integration in somatic cells	Sonti *et al.*, 1995
RAD52	Unknown homolog	Binds ssDNA and supports Rad51/ssDNA nucleofilament assembly prior to Rad51-mediated strand invasion	Required	Not required	–	van Attikum *et al.*, 2001; van Attikum and Hooykaas, 2003
RAD54	*AtRad54*	Supports the extension of the heteroduplex region after Rad51-mediated strand invasion	Not determined	Not determined	Overexpression of yeast RAD54 in *Arabidopsis* drastically increases the frequency of site-specific transgene integration	Shaked *et al.*, 2005, 2006; Osakabe *et al.*, 2006
Unknown homolog	*AtH2A*	Core histone; DNA packaging	–	–	Dispensable for integration in germ-line cells and required in somatic cells; overexpression in somatic cells increases transformation frequency	Mysore *et al.*, 2000a
Unknown homolog	*FAS1* and *FAS2*	Subunits of a chromatin assembly factor 1 (CAF-1) that mediates the deposition of H3/H4 histones onto DNA following replication and nucleotide excision repair	–	–	*fas* mutant reduced heterochromatin, drastically upregulated HRF, increased the activity of *AtRAD51* and *AtRAD54* genes, and enhanced T-DNA integration frequency	Tagami *et al.*, 2004; Endo *et al.*, 2006; Schonrock *et al.*, 2006

2.3.5. Genetic manipulation of the host for improving transformation efficiency

The existence of multiple host factors required for *Agrobacterium*-mediated transformation suggests a promising possibility for improving transformation efficiency via manipulations of a host rather than of bacterium factors. Moreover, using this approach should help us to gain the maximum control over the last step of transformation, T-DNA integration, which greatly relies on the host DNA repair machinery. This machinery may help direct T-DNA integration preferentially via the HR pathway which increases the precision and site-specificity of transgene integration.

Different strategies can be suggested for improving plant transformation via manipulation of host factors. The first strategy, which is probably the most used nowadays, is overexpression of host factors that have a positive effect on plant transformation. Indeed, overexpression of histone H2A in wild-type *Arabidopsis* plants greatly increased their susceptibility to *Agrobacterium* infection (Mysore *et al.*, 2000a). Similarly, overexpression of BTI in *Arabidopsis* (Hwang and Gelvin, 2004) and VIP1 in tobacco (Tzfira *et al.*, 2002) increased their susceptibility to genetic transformation. Overexpression of DNA repair proteins derived from different organisms in plant cells may increase the frequency of site-specific integration events. In fact, expression of the *E. coli RecA* gene in transgenic tobacco plants increased HR frequency between sister chromatids by 2.4-fold as compared with wild type plants (Reiss *et al.*, 2000). In a similar way, expression of the bacterial resolvase RuvC resulted in more than a 10-fold increase of somatic crossover and intrachromosomal recombination in transgenic tobacco plants

(Shalev *et al.*, 1999). Nevertheless, despite its positive effect on HR, overexpression of RecA and RuvC in plant cells had no significant effect on the GT frequency.

In contrast, expression of the yeast *RAD54* gene, a member of the SWI2/SNF2 superfamily of chromatin remodelling genes in *Arabidopsis,* increased the GT frequency by one to two orders of magnitude (Shaked *et al.*, 2005). This specific induction is consistent with reduced rates of GT previously reported in yeast, mouse stem cells, and chicken DT40 cells with the inactivated *RAD54* gene or its homologs (Bezzubova *et al.*, 1997; Essers *et al.*, 1997). An important role of chromatin remodelling factors in controlling the HRF was also supported by early studies of Hanin *et al.* (2000), who identified the *Arabidopsis MIM* gene encoding for a protein belonging to the SMS (structure maintenance of chromosomes) family. Overexpression of a MIM protein in plant cells resulted in a 2-fold increase in the intrachromosomal HR frequency as compared with the wild type (Hanin *et al.*, 2000). Mysore *et al.* (2000a) demonstrated that overexpression of the *RAT5* histone H2A gene in wild-type *Arabidopsis* plants increases transformation efficiency.

Inactivation of host genes that negatively influence T-DNA transformation and the HR pathway may represent an alternative to the strategy of overexpression. In fact, mutation in the *Arabidopsis AtRad50* gene that encodes a protein involved in the NHEJ pathway stimulated HR and caused a hyper-recombinant phenotype (Gherbi *et al.*, 2001). Similarly, other host factors that negatively control T-DNA integration, like the protein phosphotase DIG3 that inhibits a nuclear import of VirD2 (Tao *et al.*, 2004), can be considered to be possible targets for downregulation during genetic transformation. In fact, the studies of Ninomiya *et al.* (2004) using *Neurospora* strains deficient in human

KU70 and *KU80* homologs demonstrated that the rate of HR and GT can be greatly increased by suppression or blocking of the NHEJ repair pathway. Similarly, *Arabidopsis fas1* and *fas2* mutant plants deficient in the activity of a CAF-1 protein involved in nucleosome assembly and chromatin condensation were hypersusceptible to *Agrobacterium* transformation (Endo *et al.*, 2006). Moreover, *fas* mutants displayed a hyper-recombinant phenotype which was consistent with enhanced transcription of *AtRad51* and *AtRad54* genes (Endo *et al.*, 2006).

In plant species recalcitrant to *Agrobacterium,* transient overexpression of specific host factors during co-transformation can be problematic. Hence, it can be suggested to use an *Agrobacterium*-independent transformation technique (like biolistics) for achieving transient overexpression of specific host factors during inoculation with *Agrobacterium* (Tzfira and Citovsky, 2006). Alternatively, it would be interesting to express specific host factors fused to a bacterial export signal in *Agrobacterium* itself, so they could be delivered to host cells in a manner similar to that used for other Vir proteins (Tzfira and Citovsky, 2006). Successful expression in *Agrobacterium* and the following export of a chimeric Cre recombinase fused to a VirF protein export signal to a plant cell during transformation (Vergunst *et al.*, 2005) hold considerable promise for the application of this strategy for enhancing transformation efficiency on a routine basis.

The choice of plant tissues for subsequent transformation can also greatly influence transformation efficiency. Early reports by Grevelding *et al.* (1993) demonstrated that transformation of *Arabidopsis* root tissues yields plants with fewer T-DNA insertions than transformation of leaf disks. Similarly, tissue-specific variations in histone H2A expression in *Arabidopsis* (Yi *et al.*, 2002) underline the importance of the

tissue type selected for transformation. The age of plant explants chosen for transformation was also reported to be an important factor that influenced transformation frequency in cauliflower (Chakrabarty *et al.*, 2002) and wheat (Pastori *et al.*, 2001). In both cases, explants taken from young donors performed better compared to those obtained from older plants (Pastori *et al.*, 2001; Chakrabarty *et al.*, 2002). Overall, the creation of complex profiles describing tissue- and stage-specific expression of various host factors that contribute to transformation could greatly facilitate a search for optimal conditions for genetic modifications.

Proliferation rates in tissues used for transformation, cell cycle phases and cell types used can represent another important component for improving transformation efficiency. In fact, early data from the synchronized petunia cell population indicated that entry in the S-phase of the cell cycle was essential for stable genetic transformation (Villemont *et al.*, 1997). A very low frequency of HR in vertebrate somatic cells that represents a serious obstacle for efficient GT was overcome by choosing cell types that initially displayed high HRFs (Yamazoe *et al.*, 2004) and actively suppressed NHEJ (Orii *et al.*, 2006). This allowed the successful application of chicken B lymphocyte cells (D-40 system) and mouse embryonic stem cells for genetic transformation and GT (Yamazoe *et al.*, 2004).

Another important consideration on the way of improving plant transformation is that even disarmed *Agrobacterium tumefaciens* used for transformation can activate host defence mechanisms (Djamei *et al.*, 2007; reviewed in Dafny-Yelin *et al.*, 2008). A number of economically important plants like maize and soybean are susceptible to transient transformation, but stable transformants are extremely hard to obtain (Gelvin

2003 and references therein). This can be explained by activation of the host defence system triggered by bacterium infection, and it may lead to apoptotic responses and multiple necroses, thereby drastically decreasing the yield of stable transformants of agronomically important plants like grape (Pu and Goodman, 1992; Deng *et al.*, 1995) and maize (Hansen, 2000). This obstacle was successfully overcome by using antioxidants that made it possible to significantly increase the number of regenerated stable transformants of grape, rice, maize, and soybean (Gelvin, 2003 and references therein). Similarly, overexpression of the baculovirus apoptotic suppressor genes *p35* and *iap* greatly reduced necrotic responses and endogenous DNA cleavage in cells transformed by *Agrobacterium* (Gelvin, 2003 and references therein).

Among other types of host defence mechanisms activated during *Agrobacterium* infection is a perception of bacterial pathogen-associated molecular patterns (PAMPs) that leads to the enhanced production of ROS and increased callose deposition that reinforces cell walls (reviewed in Nürnberger *et al.*, 2004). A negative effect of PAMP perception on genetic transformation can be removed via mutation of the *EFR* plant gene that encodes a receptor kinase for perception of the bacterial EF-Tu PAMP. In fact, inactivation of the *EFR* receptor kinase gene results in super-susceptibility of the host to transformation (Zipfel *et al.*, 2006).

Taking into consideration that the number of DSBs available in the host genome represents a limiting factor for T-DNA integration (Salomon and Puchta, 1998), transient induction of DSBs by X-rays or chemical agents may be considered as a possible strategy to increase transformation efficiency. The main disadvantage of this approach is a negative effect DSBs have on genome stability and cell viability, which affects the final

yield and quality of transgenic plants. A good alternative would be a directed site-specific generation of DSBs in genetic loci in which T-DNA integration is desirable. There are several strategies that use site-specific DSBs for improving precision of transformation.

The first system is based on using rare cutting endonucleases (I-*Sce*I, I-*Ceu*I), which can be transiently expressed in cells during co-transformation with two *Agrobacterium* strains that carry T-DNA containing the genes of interest and T-DNA containing the endonuclease gene (Tzfira *et al.*, 2003). Two major drawbacks of this approach are: a rare and random dispersion of endonuclease recognition sites through the plant genome (Puchta, *et al.*, 1993; Li *et al.*, 2007) that could drastically decrease precision of integration, and a need for the previous generation of transgenic plants that have a recognition site in a desired location.

The second approach that results in effective targeting of T-DNA into the desired loci is represented by the Cre-*lox* system (reviewed in Gilbertson, 2005). This bacteriophage derived system consists of the Cre recombinase and the 34 bp *lox* site. Cre binds to 13 bp long inverted repeats in the *lox* sites and catalyzes a crossover in the 8 bp spacer region of two *lox* sites. This system can be effectively used for both site-specific transgene integration and selectable marker gene removal (reviewed in Gilbertson, 2005). However, this system also has several drawbacks. Despite the absence of wild type *lox* sequences in plant genomes, they still contain pseudo-*lox* sites, which display more than 50% similarity with *lox* sites and can still be involved in recombination (Schmidt *et al.*, 2000; Silver and Livingston, 2001). Moreover, it was demonstrated that high expression of *cre* results in chromosomal rearrangements in mammalian cells (Schmidt *et al.*, 2000; Silver and Livingston, 2001) and produces aberrant phenotypes in plants including

chlorosis, leaf deformation, stunted growth, and delayed flowering (Que *et al.*, 1998; Coppoolse *et al.*, 2003). High levels of *cre* expression trigger recombination in chloroplasts between the *lox* site and AT-reach sequences located about 500 bp away from it (Hajdukiewicz *et al.*, 2001). Finally, Cre-*lox* as well as the I-*Sce*I system require preceding integration of *lox* sites and *Sce*I recognition sites in a location selected for site-specific transgene integration.

The third approach uses zinc-finger nucleases (ZFNs) that are specifically designed to recognise and introduce DSBs into predicted locations within the host genome (Lloyd *et al.*, 2005; Wright, *et al.*, 2005). This ZFN is a dimmer of two ZFNs, each consisting of a set of zinc-finger motifs allowing specificity in recognition of target DNA sequences and a non-specific endonuclease (reviewed in Wright, *et al.*, 2005). Each pair of three-finger ZFNs has a recognition sequence of 18 pb, which should occur in a genome every 4^{18} bp. It means that three-finger ZFNs could provide enough specificity in genomes of a size of about 10^{10} bp (Lloyd *et al.*, 2005). Moreover, recognition specificity can be further increased by using four-finger ZFEs, which have a recognition sequence of 24 bp. Overall, this strategy has great potential for plant transgenesis and GT technology. Its routine application in biotechnology has currently been the subject of many ongoing studies (reviewed in Wu *et al.*, 2007).

2.3.6. Alternative strategies for improving transformation efficiency via manipulation of the host

In contrast to direct overexpression, inactivation and co-delivery of host factors during transformation, indirect stimulation and preparation of the host for future transformation can be viewed as possible approaches for improving transformation efficiency. Reports in the early nineties suggested some promising ideas for transformation enhancement. It was demonstrated that supplementation of growth media of a future host with various chemicals can influence DNA repair. Waldman and Waldman (1991) grew mammalian cells in the continuous presence of 2 mM 3-methoxybenzamide, a competitive inhibitor of PARP (Waldman and Waldman, 1990), and they were able to demonstrate a 3–4-fold increase of intrachromosomal HR (Waldman and Waldman, 1991). Similarly, the use of another PARP inhibitor, niacinamide, for biolistic-mediated wheat transformation increased the number of low-copy transgene integration events (De Block *et al.*, 1997). Moreover, in the case of multiple integrations in the host genome, these copies segregated in the next generation indicating their unlinked nature (De Block *et al.*, 1997). After its successful application for transformation, niacinamide treatment was patented (De Block, US Patent No. 6074876). Transient expression of a reporter gene was several-fold increased by performing bombardment in the presence of silver thiosulfate and calcium nitrate (Perl *et al.*, 1992). Lowering temperatures during co-cultivation with *Agrobacterium* (Li *et al.*, 2003a) and chilling during a regeneration step (Immonen, 1996) also improved transformation efficiency.

High transformation efficiency does not rely only on successful transformation *per se*, it also requires the establishment of optimal conditions for efficient regeneration of transgenic material from transformed tissues. In many cases, regeneration of transformed cells represents a major challenge in transformation of many agronomically important plants that are susceptible to T-DNA integration and recalcitrant to somatic embryogenesis (SE) (Takumi and Shimada, 1997; Li *et al.*, 2003b). To date, a number of chemicals promoting SE and diminishing negative effects during a tissue culture stage have been reported. Among the most interesting are the following: various methods of increasing cytosolic levels of calcium that can promote SE (Racusen and Schiavone, 1990; Chugh and Khurana, 2002; Malabadi and Staden, 2006); the application of silver that prevents cellular necrosis caused by ethylene (Dias and Martins, 1999; Sahrawat, *et al.,* 2003); promotion of SE in wheat by zinc deficiency (He *et al.*, 1991; Kothari, *et al.,* 2004) and various nitrogen sources (Immonen, 1996); promotion or prevention of shoot regeneration by cupric sulphate (Saharawat, *et al.,* 2003; Kothari, *et al.,* 2004) and high EDTA (Kothari, *et al.,* 2004). Similarly, supplementation of regeneration media with spermidine was shown to improve the recovery of wheat transformants by more than 3-fold (Khanna and Daggard, 2003). The application of a wide spectrum of rare earth elements during a tissue culture stage also holds much promise, as a positive effect of these elements on nitrogen metabolism has been documented (Weiping *et al.*, 2003 and references therein).

Ammonium and nitrate levels were reported to promote SE in various plant species (He *et al.,* 1989; Grimes and Hodges, 1990; Mordhorst and Lorz, 1993; Choi *et al.*, 1998; Jiménez, *et al.,* 2001; Kothari, *et al.,* 2004). High concentrations of ammonium

nitrate in regeneration media greatly improved SE and increased the yield of somatic embryos in ginseng (Choi *et al.*, 1998). In rice, the relative ratio of ammonium to nitrate could affect the sensitivity of immature embryos to auxin; the ratio of 1:1 produced the greatest insensitivity (Grimes and Hodges, 1990). In contrast, in barley microspore culture, it was shown that the ratio of two ions (except when it is extreme) had no influence upon SE yields (Mordhorst and Lorz, 1993). However, a link between the total concentration of inorganic nitrogen and the yield of somatic embryos has been established (Mordhorst and Lorz, 1993). In wheat, it was not possible to show a strong connection between either the ratio or the total content of nitrogen species and SE yield (Menke-Milczarek and Zimny, 2001). Interestingly, recent reports established a link between the activity of human Rad51 and ammonium-sulphate at the molecular level (Sigurdsson *et al.*, 2001; Liu *et al.*, 2004b; Shim *et al.*, 2006). It was demonstrated that ammonium-based salts induce conformational changes in hRad51 leading to an increase in its activity and therefore promoting recombination (Sigurdsson *et al.*, 2001; Liu *et al.*, 2004b; Shim *et al.*, 2006).

Overall, it has become evident that each species, cultivar, and even tissue has its own unique set of requirements for various salt combinations and concentrations (Maës *et al.*, 1996; He *et al.,* 1989). The foregoing supports the existing need for extensive studies directed on elucidating specific concentrations of macro and micro salts as well as physical conditions optimal for promoting T-DNA integration and SE in various economically important plant species.

3. IMMEDIATE AND TRANSGENERATIONAL EFFECTS OF SALT STRESS ON PLANT GENOME STABILITY AND STRESS ADAPTATION

3.1. MATERIALS AND METHODS

3.1.1. Plant cultivation

In the current work, transgenic *A. thaliana* line #11 plants were used. Plants were obtained from Friedrich Miescher Institute (Basel, Switzerland) and were previously described (Swoboda, *et al.*, 1994; Ilnytskyy, *et al.*, 2004).

3.1.1.1. Growing *Arabidopsis* plants for salt stress experiments

Arabidopsis seeds were surface-sterilized with 70% ethanol for 2 min, followed by a solution of 1% sodium hypochloride, 0.05% Tween-80 for 3 min, and washed twice with a large excess of sterile distilled water for 5 min each. Then seeds were planted on MS medium solidified with agar (8 g/L) (Murashige and Skoog, 1962) and contained in a 100 mm Petri. Plates were incubated at 4 °C in the dark to break dormancy, and then moved to a growth chamber (Enconair, Winnipeg, MB, Canada); light was provided by Octron T8 Fluorescent bulbs (Sylvania, Mississauga, ON, Canada).

After 3 weeks, on the 22nd day post germination (dpg), half of the *Arabidopsis* plants was transplanted to *All purpose potting soil* (Plant *Etc*; Lethbridge, AB, Canada) for propagation, and the rest of the plants were harvested for histochemical staining or snap frozen for further analysis. Once transplanted to soil, plants were grown in a growth chamber (Enconair, Winnipeg, MB, Canada), light provided by *Cool White* Fluorescent bulbs (Sylvania, Mississauga, ON, Canada) and *Longlife* Incandescent bulbs (Sylvania, Mississauga, ON, Canada).

Over the whole duration of the experiment, plants were grown in high light conditions (32.8 $\mu Em^{-2}s^{-1}$) at 22 °C in a 16 hour light regime and at 18 °C in a 8 hour dark regime, under a constant humidity of 65%.

3.1.1.2. Growth medium composition

A solid MS medium of standard composition (Murashige and Skoog, 1962) was used to grow plants under control conditions. For stress experiments, standard MS medium was supplemented with various quantities of NaCl or MMS as discussed below. All components of MS medium were prepared separately and combined together prior to use. 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. For experiments that tested effects of NaCl or MMS stresses on the plant genome, 1 M NaCl (filter-sterilized) or 10^6 ppm MMS solutions were added to the cooled medium after autoclaving.

3.1.2. Detection and analysis of homologous recombination events in *Arabidopsis*

3.1.2.1. Detection of homologous recombination events

Transgenic *A. thaliana* line #11 carries in the genome the *uidA* (GUS) reporter gene which serves as a HR substrate. It consists of two overlapping truncated non-functional parts of the *uidA* gene cloned in direct orientation under the control of the 35S cauliflower mosaic virus (CaMV) promoter (Figure 3.1.1A) (Swoboda, *et al.*, 1994; Ilnytskyy, *et al.*, 2004). Repair of DSB in the region of homology via HR results in a recombination event that restores the reporter gene, thereby activating *β–glucuronidase* (GUS) (Figure 3.1.1B).

3.1.2.2. Visualization of homologous recombination events using the GUS gene activity

Recombination events that yielded an intact functional copy of the GUS gene were visualized following a histochemical staining procedure as described by Jefferson (1987). Plants were harvested and vacuum infiltrated for 10 min in a sterile staining buffer containing 100 mg of a 5-bromo-4-chloro-3-indolyl glucuronide (X-glu) substrate (Rose Scientific LTD) in 200 mL of 100 mM phosphate buffer (pH 7.0), 0.1% NaN_3, 0.05% Tween-80, 1 mL dimethylformamide. Then, plants were incubated at 37 °C during 48 hours to allow cells containing an active GUS gene to cleave the X-glu substrate that yields an insoluble indigo coloured product. After 48 hours, plants were bleached with 70% ethanol at room temperature for 2 – 4 days. Stained plants appeared as translucent

with blue spots visible under a dissecting microscope which indicated the location of the GUS activity (Figure 3.1.1B).

3.1.2.3. Calculating recombination rate and frequency of homologous recombination

The RR was calculated by relating HRF to the number of haploid genomes per single plant. It represented the number of HR events per single haploid genome. The HRF was calculated by counting the number of HR events (sectors) in each plant separately, summing and then relating it to the number of plants in tested populations. The number of haploid genomes per single plant 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. To avoid a bias during DNA preparation, DNA was extracted by two different methods.

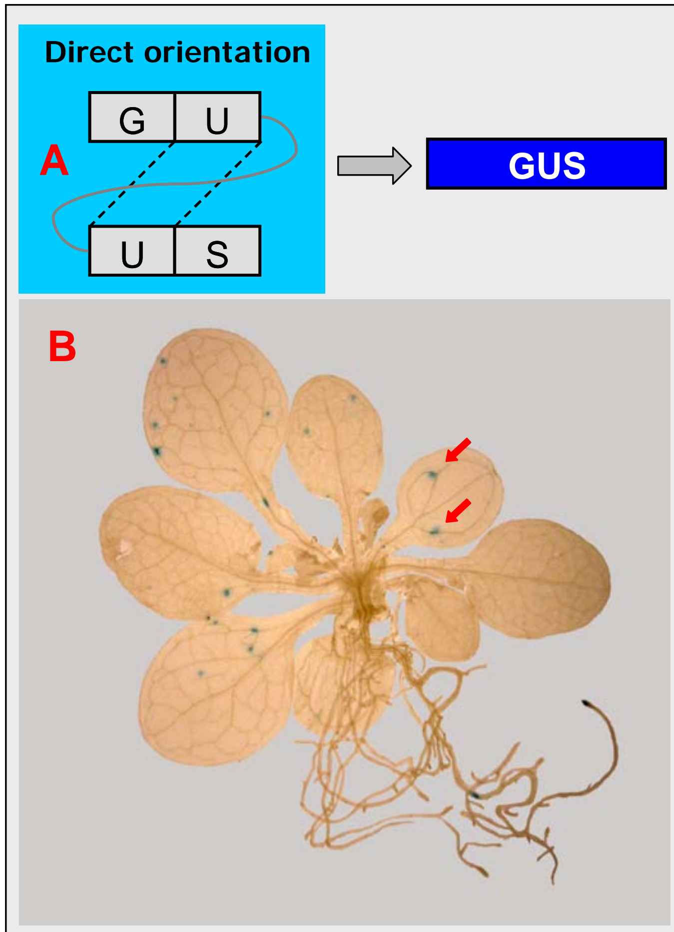

Figure 3.1.1. Detection of recombination events in *Arabidopsis* line #11 plants

A – the structure of the GUS-based reporter construct cloned in direct orientation. The construct served as a substrate for somatic HR; **B** – detection of recombination events using histochemical staining (an arrow indicates recombination event in leaf tissues).

3.1.3. Effects of salt stress on genome stability in *Arabidopsis*

3.1.3.1. Immediate effects of salt stress on genome stability

Arabidopsis line #11 plants were germinated and grown on standard solid MS medium (Murashige and Skoog, 1962) supplemented with 0, 50, 75 and 100 mM of NaCl (Merck KGaA). After 3 weeks, plants were harvested for histochemical staining, and tissues were taken and snap frozen for DNA and RNA preparation. HRF and RR were calculated and correlated with the level of DNA DSBs and transcriptional activity of the *AtRad51* and *AtKu70* genes.

3.1.3.2. Obtaining the first (G1) and the second (G2) generations of salt treated plants

To obtain the G1 generation, *A. thaliana* plants were germinated and grown on solid MS medium (Murashige and Skoog, 1962) supplemented with 0, 25 or 75 mM of NaCl (Merck KGaA). After 3 weeks, plants were transplanted to soil and propagated into the G1 generation. The progenies of plants exposed to 0, 25 and 75 mM NaCl were called PofC, PofS_25 and PofS_75, respectively. To obtain the G2 generation PofC, PofS_25 and PofS_75 plants were grown on MS medium (Murashige and Skoog, 1962) without NaCl. After 3 weeks, plants were transplanted to soil and propagated into the G2 generation. The progenies of PofC, PofS_25 and PofS_75 plants were called P2ofC, P2ofS_25 and P2ofS_75, respectively. G2 plants were grown for 3 weeks on standard MS medium (Murashige and Skoog, 1962) and harvested for histochemical staining (Figure 3.1.2). Three independent groups of progenies were generated for each experimental point using the same conditions, and the data obtained were averaged.

3.1.3.3. Transgenerational effects of salt stress on genome stability

Further molecular analysis and histochemical staining was done using tissues collected from 3-week-old plants immediately before transferring them from media to soil. The RR was calculated for G1 and G2 plants. It was correlated with the level of DNA DSBs and compared with the transcriptional activity of *AtRad51* and *AtKu70* genes. Then, the activity of HR reflected in RR was correlated with global genome methylation, methylation at pericentromeric regions and locus-specific methylation of the *AtAct2* gene promoter, the GUS gene promoter and GUS gene coding sequences, respectively.

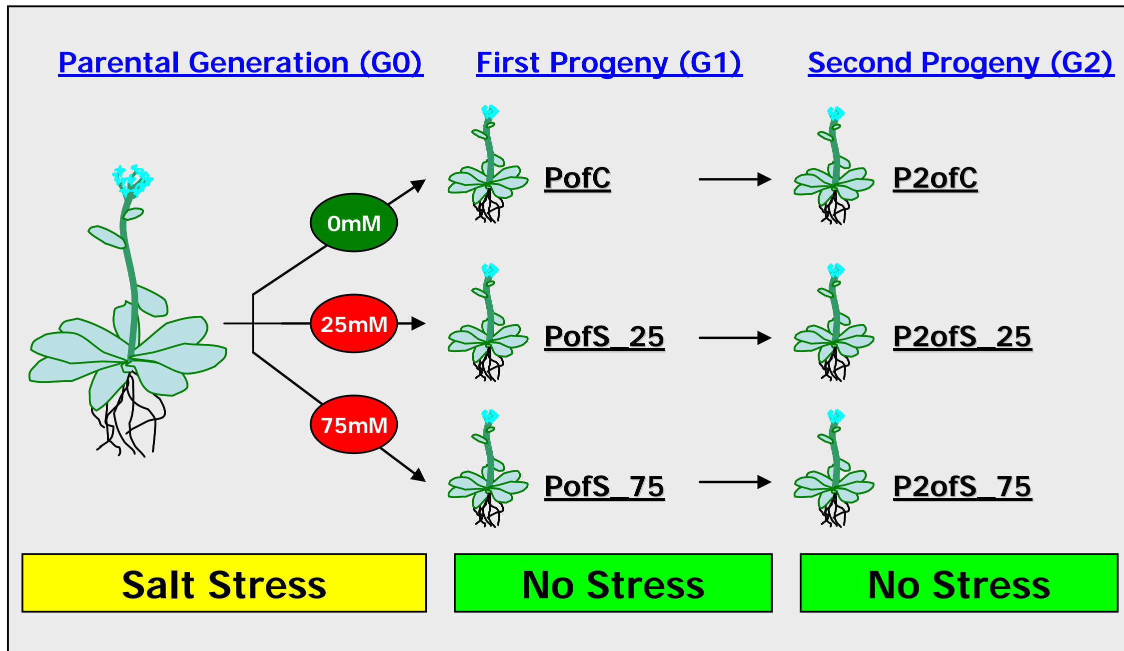

Figure 3.1.2. Generation of the first (G1) and second (G2) progenies of salt-treated plants

Arabidopsis plants were grown on solid MS medium (control) and media containing 25mM and 75mM of NaCl. After 22 days on media, they were transplanted to soil and propagated to the next generation. The first progenies (PofC, PofS_25 and PofS_75) were grown under control conditions. They were used to obtain the second after stress generation (P2ofC, P2ofS_25 and P2ofS_75).

3.1.4. Analysis of plant adaptation to stress

To analyze whether the progeny of stressed plants acquired higher tolerance to the same and different stresses, G1 and G2 plants were exposed to NaCl (Merck KGaA) and MMS (Sigma) stress. For salt treatment, plants were germinated and grown on solid standard MS medium in the presence of NaCl (Merck KGaA) concentrations ranging from 25 to 300mM. For MMS treatment, plants were germinated and grown on solid standard MS medium (Murashige and Skoog, 1962) containing 20 – 250 ppm of MMS (Sigma). Plant adaptation to stress was evaluated based on increased germination rates, decreased germination delays and displayed phenotypes. The germination rate was calculated by relating the number of germinated seeds to the total number of seeds planted. Additionally, RRs were calculated to reflect the HR activity under specific stress.

3.1.5. Description of molecular techniques and assays used for analysis

3.1.5.1. Total DNA, RNA and cDNA preparations

Total genomic DNA was prepared using the *Gene Elute Plant Genomic DNA Miniprep Kit* (Sigma) according to a manufacturer's protocol. On average, 25 plants were used for preparation of a single sample, and three independent DNA samples per each group were prepared. To avoid a bias during DNA preparation in the experiments calculating the number of genomes per plant, we used another protocol as was previously described (Boyko *et al.*, 2005). Despite some difference in the total DNA yield from two extraction protocols, the ratio between the amount of DNA in plants grown under different conditions was the same.

Total RNA was prepared using the *Trizol* reagent (Invitrogen) accordingly to a manufacturer's protocol. On average, 25 plants were used for preparation of a single sample, and three independent RNA samples per each group were prepared. cDNA was prepared using the *Revertaid H-Minus First Strand cDNA Synthesis Kit* according to manufacturer's protocol (Fermentas).

Aliquots of each sample were diluted to the final volume of 200 µl in TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) and quantified using the Ultrospec 1100 pro UV/Visible Spectrophotometer (Biochrom Ltd, Cambridge, UK) at a wavelength of 260 nm.

3.1.5.2. Evaluation of the *AtRad51* and *AtKu70* transcriptional activity

The transcriptional activity of *AtRad51* and *AtKu70* genes was measured using a semi-quantitative Real Time PCR. The genes were amplified from cDNA using the

following primers for the *AtRad51* gene: forward primer: 5'-TTGATGTAAAGAAGCTTAGGG-3' and reverse primer: 5'-TACAATCTTGTCAACCTTGGC-3'; for the *AtKu70* gene: forward primer: 5'-GAAGAAAGTGACTCAGGGGC-3' and reverse primer: 5'-TTCTTCCCGCTGACGAGGAG-3'. A *SmartCycler* (Cepheid, Sunnyvale, CA) was used to perform PCR cycles, and the fluorescence of SYBRGreen (Molecular Probes) was quantified against standards. Melting temperatures were estimated for every gene product. Equal loading of each amplified sample was determined by amplification of the control *Ubiquitin* PCR product: forward primer: 5'-TCAAATGGACCGCTCTTATCA-3' and reverse primer: 5'-GGACTCCAAGCATTCTTCAA-3'.

3.1.5.3. DSB measurement (the ROPS assay)

Quantification of 3'OH DNA breaks was performed using the random oligonucleotide primed synthesis (ROPS) assay as described (Basnakian and James, 1996). 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 to 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).

Immediately before the ROPS reaction, a 1 μg aliquot of plant DNA was heat-denatured at 100 °C for 5 min and then chilled on ice. The reaction mixture for one

sample contained: 1 µg of heat denatured DNA, 2.5 µl of 0.5 mM 3 dNTPs (dGTP, dATP and dTTP mix) (Fermentas), 2.5 µl of 10x *Klenow fragment* buffer (New England Biolabs), 5 units of the *Klenow* enzyme (New England Biolabs), 0.1 µl of [^{3}H]-dCPT (PerkinElmer; Boston, MA, USA). The reaction volume was adjusted to 25 µl with sterile distilled water. After incubation at 25 °C for 60 min, the reaction was stopped by the addition of 2.5 µl of 100 mM EDTA pH 8.0. After this, the reaction volume of each sample was pipetted to 25 mm DE-81 ion-exchanging filter paper (Whatman). Filter papers were allowed to dry at 70 °C, and then were washed with 500 mM sodium phosphate buffer (pH 7.0) for 10 min, repeated three times. Then, filters were thoroughly air-dried and transferred to vials containing 5 mL of scintillation cocktail each. Radiation levels, ^{3}H decays per minute (DPM), were detected using a scintillation counter (Beckman LS 5000CE; Fullerton, CA, USA). The assay was repeated three times, and readings were taken at least twice per each reaction.

3.1.5.4. Analysis of global genome methylation

The level of global genome methylation was measured using a cytosine extension assay (Pogribny, *et al.*, 1999). The assay is based on digesting of total genomic DNA with methylation-sensitive *Hpa*II restriction endonuclease (Fermentas) and incorporation of radioactively labeled nucleotides, [^{3}H]-dCPT, in resulting overhangs. Under strictly defined reaction conditions, the incorporation of [^{3}H]-dCPT will be proportional to the initial number of *Hpa*II recognition sites (C↓CGG) containing unmethylated cytosine. Hence, higher methylation is associated with a decrease in the digestion rate, and thus a lower incorporation [^{3}H]-dCTP.

One μg of total genomic DNA was digested with 10 units of methylation-sensitive *Hpa*II restriction endonuclease (Fermentas) and 1x enzyme buffer in a total volume of 20 μl overnight at 37 °C. After digestion, 10 μl of each reaction was used for a cytosine extension assay (Pogribny, *et al.*, 1999). The reaction mixture for each sample contained 1.5 mM $MgCl_2$, 0.5 units of *Tag* polymerase (Fisher Scientific), and 0.1 μl of [^{3}H]-dCPT (PerkinElmer; Boston, MA, USA) in a final volume of 25 μl. Samples were incubated at 56 °C for 1 hour. Following extension, a 25 μl reaction volume of each sample was applied to 25 mm DE-81 ion-exchanging filter paper (Whatman), dried at 70 °C, and washed with 500 mM sodium phosphate buffer (pH 7.0) for 10 min, repeated three times. Subsequently, filters were thoroughly air-dried and transferred to vials containing 5 mL of scintillation cocktail. Radiation levels, in DPM, were detected in a scintillation counter (Beckman LS 5000CE; Fullerton, CA, USA). As a control, each sample underwent initial incubation in the absence of *Hpa*II enzyme and was subjected to identical extension conditions. Incorporation of radioactivity was measured in DPM/μg of DNA. The data from treated samples were related to the data from control samples, with controls taken as 100%. The assay was repeated three times, and readings were taken at least twice per each reaction.

3.1.5.5. Analysis of methylation of pericentromeric DNA repeats

A methylation pattern was analysed within the 180 bp long highly repetitive element that is tandemly repeated in long, > 50 kbp arrays present at all five *A. thaliana* centromeres (Martinez-Zapater *et al.*, 1986; Maluszynska and Heslop-Harrison, 1991). The sequence of a 180 bp repeat was PCR-amplified from *Arabidopsis* genomic DNA

using primers: forward 5’-GCTTTCATGGTGTAGCCAAAGTCC-3’, reverse 5’-ACGCTTTGAGAAGCAAGAAGAACG-3’; and cloned into a *pGEM-T easy* vector (*pGEM-T easy vector system I*, Promega) for further work. The resulting clones were sequenced and compared with an originally reported sequence of that repeat (Martinez-Zapater *et al.*, 1986) to ensure that the right clone is used. No mismatches were found.

The methylation level at *Arabidopsis* pericentromeric repeats was analysed using the Southern (DNA) blot analysis as described (Vongs *et al.*, 1993; Boyko *et al.*, 2007). Total genomic DNA (10 μg) was digested for 24 h with the methylation sensitive enzyme *Hpa*II, separated in a 1% agarose (Bio-Rad) gel, transferred to a positively charged nylon membrane (Roche). Membranes were hybridized with a cloned 180 bp repeat and detected using the *DIG Luminescent Detection Kit* (Roche) accordingly to manufacturer’s protocol. The probe was DIG-labelled using the *PCR DIG Probe Synthesis Kit* (Roche) according to manufacturer’s protocol. The cloned 180 bp repeat served as a template for probe synthesis, and the same set of primers was used. The completeness of digestion of centromeric arrays detected by a 180 bp repeat probe depends on their methylation: the more restriction sites are methylated, the less completely DNA will be digested.

3.1.5.6. Analysis of locus-specific methylation using the combined bisulphite restriction analysis (COBRA)

The assay was performed as described before (Frommer *et al.*, 1992; Xiong and Laird, 1997). It consists of three consecutive steps: a bisulphite treatment that converts all unmethylated cytosines to thymines, a gene-specific PCR that amplifies a gene fragment to be analyzed, and a restriction digestion reaction that allows the identification of newly

created methylation-dependent restriction sites and methylation-dependent retention of pre-existing sites (Xiong and Laird, 1997). Methylation-dependent cytosine retention/conversion results in the creation/disappearance of a *Hpy*CH4IV recognition sequence (ACGT), and leads to the appearance of a differential digestion pattern.

For sodium bisulphite treatment, 2 μg of total genomic DNA was denatured by adding freshly prepared 3 M NaOH to a final concentration of 0.3 M in a final reaction volume of 20 μl. The reaction was incubated for 15 min at 37 °C. Following denaturation, sodium bisulphite stock (208 μl of a 3.6 M stock solution) (Sigma), and hydroquinone (12 μl of a 10mM stock solution) (Sigma) were added to denatured DNA in a final volume of 240 μl. Samples were incubated overnight at 55 °C in the dark for 16 hours. Next, free bisulphite was removed by passing a sample through a desalting column (*Wizard DNA Clean-Up System*, Promega) and DNA was eluted in 50 μl of sterile distilled water. DNA was again denatured by adding freshly prepared 3 M NaOH to a final concentration of 0.3 M and incubating a sample at 37 °C for 15 min. The reaction was neutralized by adding ammonium acetate (pH 7.0) to a final concentration of 3 M. DNA was then ethanol-precipitated and resuspended in sterile distilled water.

Bisulphite-treated DNA was PCR-amplified with *AtAct2* or GUS-specific primers and digested with the *Hpy*CH4IV (New England Biolabs) enzyme (Boyko *et al.*, 2007). The following primers were used for the promoter region of the *AtAct2* gene: forward primer: 5’-CCTCTCCGCTTTGAATTGTCTCG-3’, reverse primer: 5’-ACACCATGATGTCTTGGCCTACCA-3’; for the promoter region of the GUS gene: forward primer: 5’-ACAGTCTCAGAAGACCAAAGGGCA-3’, reverse primer: 5’-ACGTTCGTCACCTGCTTAGTCTGA-3’; and for the coding region of the GUS gene:

forward primer: 5'-AAGGGATGACGCACAATCCCACTA-3', reverse primer: 5'-TGTAGAGCATTACGCTGCGATGGA-3'.

One µg of PCR-amplified DNA was digested with 10 units of the *Hpy*CH4IV enzyme (New England Biolabs) and 1x enzyme buffer supplemented with BSA (New England Biolabs) in a final volume of 20 µl. Restriction was analysed in a 3% agarose (Bio-Rad) gel and intensity of an undigested fragment (769 nt in actin, 486 nt in the GUS promoter and 744 nt in the GUS coding sequence) from three independent experiments (25 plants from each group were pooled together for each experiment) was analyzed using *Image J* program (NIH, www.rsb.info.nih.gov/ij). In actin, complete restriction digestion results in two fragments of 551 and 218 nt; in the GUS promoter region, complete digestion creates five fragments: 239, 193, 40, 11, 3 nt; and in the GUS coding region, complete digestion yeilds four fragments: 219, 103, 187, 148 nt. Since sequences selected for the analysis contained only original enzyme recognition sites (no new sites could be created via bisulphite treatment), the amount of remaining undigested product reflected the number of original restriction sites that were demethylated prior to bisulphite treatment.

3.1.6. Statistical treatment of data

In all cases, the mean and standard error or standard deviation was calculated. Statistical significance of the experiment was confirmed by either the two-tailed paired Student's t-test with $\alpha=0.05$ or $\alpha=0.1$ (comparing data from two treatments), or the single factor ANOVA (comparing data from three or more treatments). Statistical analysis was performed using the *JMP 5.0* software (SAS Institute Inc).

3.2. RESULTS

3.2.1. Immediate effects of salt stress

To study the influence of NaCl on genome stability, we first performed a pilot experiment that intended to identify concentrations that do not change plant appearance. This is important, since concentrations of mutagens that interfere with plant physiology can trigger cell apoptosis, thus resulting in DNA damage that is higher than DNA damage caused by stress. Plants were germinated and grown in media supplemented with different concentrations of NaCl ranging from 0 to 300 mM. Our data revealed no significant phenotypic difference between plants germinated and grown for 3 weeks in the presence of 0 – 75 mM of NaCl (Figure 3.2.1). The first noticeable differences were observed in plants subjected to 100 mM of NaCl. The presence of higher amounts of salt in growth media led to more severe growth inhibition (Figure 3.2.1). Concentrations equal to or exceeding 250 mM completely inhibited plant germination. Further experiments were performed with plants grown in 25 – 100 mM of NaCl.

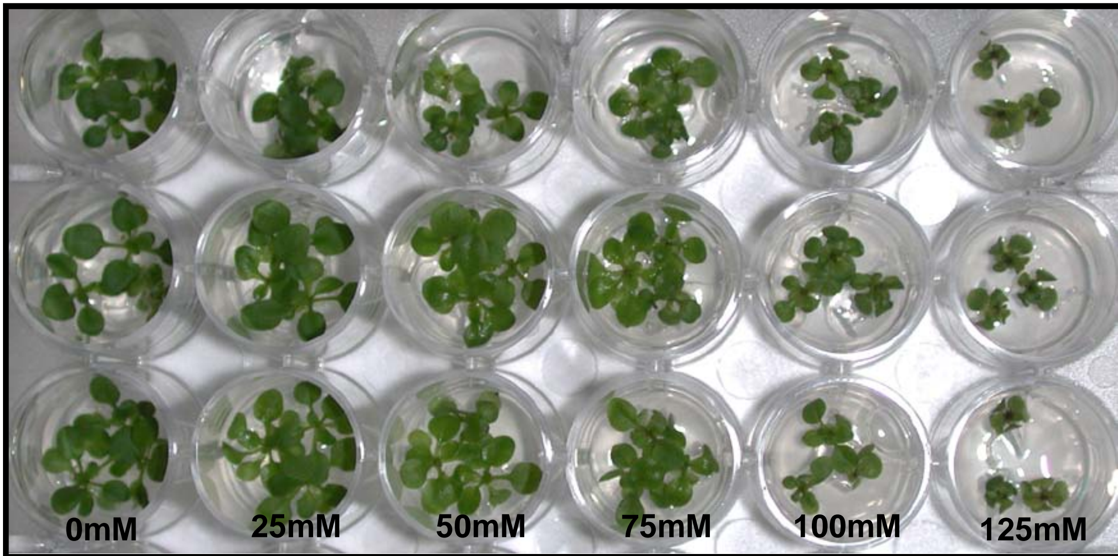

Figure 3.2.1. Sensitivity of *Arabidopsis* plants to different concentrations of NaCl

A. thaliana plants were germinated and grown on solid MS medium supplemented with different amounts of NaCl. Pictures were taken on the 22nd day post germination.

3.2.1.1. Salt stress causes DNA damage and increases recombination rates

Despite the fact that plants exposed to 25 and 75 mM NaCl were not phenotypically different from control plants (Figure 3.2.1), these plants showed a significant increase in the number of DSBs (Student's t-test, $\alpha=0.05$). Applications of 25 and 75 mM NaCl increased the number of DSBs found per single genome by 16% and 21%, respectively (Table 3.2.1). High levels of DNA damage resulted in an increase of RR by factors of 1.6 and 3.0, respectively, as compared to untreated control plants (Student's t-test, $\alpha=0.05$). Similarly, exposure of plants to 100 mM NaCl resulted in a significant 15% increase of DSBs (Student's t-test, $\alpha=0.05$), and a 3.9-fold increase in RR (Student's t-test, $\alpha=0.05$). A moderate positive correlation between the number of DSBs and RR was found ($r= 0.66$, $P<0.05$).

3.2.1.2. Exposure to salt up-regulates *AtRad51* transcription and down-regulates *AtKu70* transcription

An increase in RR could be a reflection of either an increase in strand breaks or a shift in the mechanism of repair of available breaks towards HR repair. We have already showed that salt treatment results in an increase in DSBs. Next, we tested whether salt exposure leads to the change of expression of genes involved in strand break repair. We analyzed the steady state levels of *AtRad51* and *AtKu70* mRNA as hallmarks of HR and NHEJ. Exposure of plants to 100 mM NaCl resulted in a significant 31% increase in *AtRad51* expression (Student's t-test, $\alpha=0.05$). At the same time, the transcriptional activity of the *AtKu70* gene was noticeably reduced in plants exposed to 75 mM (Table 3.2.1). No significant difference, however, was observed in the activity of *AtKu70* in

plants exposed to either 25 or 100 mM (Table 3.2.1). Up-regulation of the transcriptional activity of *AtRad51* was strongly and positively correlated with an increase of RR ($r = 0.92$, $P < 0.05$). This experiment suggests that the RR increase could be due to two factors: first, an increase in the number of strand breaks; second, a partial shift in the expression of repair genes.

Table 3.2.1. The number of DNA double strand breaks (*DPM ^{3}H*), the recombination rate (*RR*) and the transcriptional activity of *AtRad51* and *AtKu70* in *Arabidopsis* exposed to salt

	DPM ^{3}H, E+03	Fold	RR, E-08	Fold	*AtRad51* related to *Ubiquitin*	Fold	*AtKu*70 related to *Ubiquitin*	Fold
NaCl 0 mM	32.94±0.72 [a]	1	7.50±0.01 [a]	1	0.496±0.019 [b]	1	0.643±0.016 [ab]	1
NaCl 25 mM	38.34±1.62 [b]	1.16	12.29±0.18 [b]	1.64	0.526±0.029 [b]	1.06	0.604±0.027 [ab]	0.94
NaCl 75 mM	39.92±0.35 [b]	1.21	22.52±0.34 [c]	3	0.545±0.049 [ab]	1.1	0.577±0.017 [b]	0.9
NaCl 100 mM	37.98±0.64 [b]	1.15	29.57±0.82 [d]	3.93	0.647±0.038 [a]	1.31	0.673±0.043 [a]	1.05

Tissues were prepared from *A. thaliana* plants germinated and grown for 3 weeks in 0 – 100 mM NaCl. At least, two independent measurements were conducted for each data point. Values represent mean ± s.e. Values that are not connected by the same letter are significantly different. Student's t-test, $\alpha=0.05$: for DSBs, $t=2.03$; for RR, $t=2.78$; for *AtRad51*, $t=2.18$; for *AtKu70*, $t=2.18$. ANOVA: for DSBs, $P<0.01$; for RR $P<0.01$; for *AtRad51*, $P<0.05$; for *AtKu70*, $P>0.1$. Fold was calculated by relating data obtained from plants grown in the presence of 25, 75 and 100 mM NaCl to data obtained from plants grown in 0 mM.

3.2.2. Transgenerational effects of salt stress

Previous publications suggested that various stresses result in an increase of HRF in the progeny of stressed plants (Molinier *et al.*, 2006; Boyko *et al.*, 2007). Hence, the progeny of plants exposed to stress exhibits an elevated HRF, even if plants are grown under normal, non-stressful conditions. To analyze whether exposure to salt has similar effects, we collected seeds of plants grown in 25 and 75 mM NaCl (Figure 3.1.2). While 25 mM NaCl was the lowest concentration tested in the experiment, 75 mM was the highest concentration that did not change plant appearance (Figure 3.2.1). The progeny of these plants were called the “progeny of salt” (PofS_25 and PofS_75) treated plants. To analyze whether changes in the G1 progeny are maintained when plants are propagated without stress, we grew PofS plants without stress.

3.2.2.1. Exposure to salt results in an increase in recombination rates in the progeny

As expected, we did not observe a significant difference in plant phenotypes; all plants looked healthy and were phenotypically comparable with the progeny of control (PofC) untreated plants. The analysis of recombination events showed that both the G1 and G2 progenies of salt treated plants had higher RRs, when grown under normal conditions. A 2-fold difference in RR was significant for the G1 PofS_25 progeny (Student’s t-test, $\alpha=0.05$), in comparison to the G1 PofC progeny (Figure 3.2.2). Similarly, a 1.6-fold difference was found, when G1 PofS_75 plants were compared to the PofC. Intriguingly, when the G2 progeny of salt treated plants were compared to G2 PofC plants, no significant difference was found, although the trend toward an increase

was noticed (Figure 3.2.2). The elevated spontaneous RR observed in the G1 progeny of salt treated plants greatly decreased when these plants were propagated into the next generation. The spontaneous RRs in the G2 PofS_25 and PofS_75 plants remained 1.4- and 1.3-fold higher as compared to the G2 PofC progeny (Figure 3.2.2).

Furthermore, RRs remained different if G1 PofC and PofS plants were grown in media supplemented with NaCl in the same amounts that were used to obtain PofS plants (Figure 3.2.3). RRs increased by 2.6- and 4.7-fold in PofC plants exposed to 25 mM and 75 mM, respectively. In contrast, RRs increased by 23.2-fold in PofS_25 plants exposed to 25 mM and by 19.6-fold in PofS_75 plants exposed to 75 mM NaCl (Figure 3.2.3).

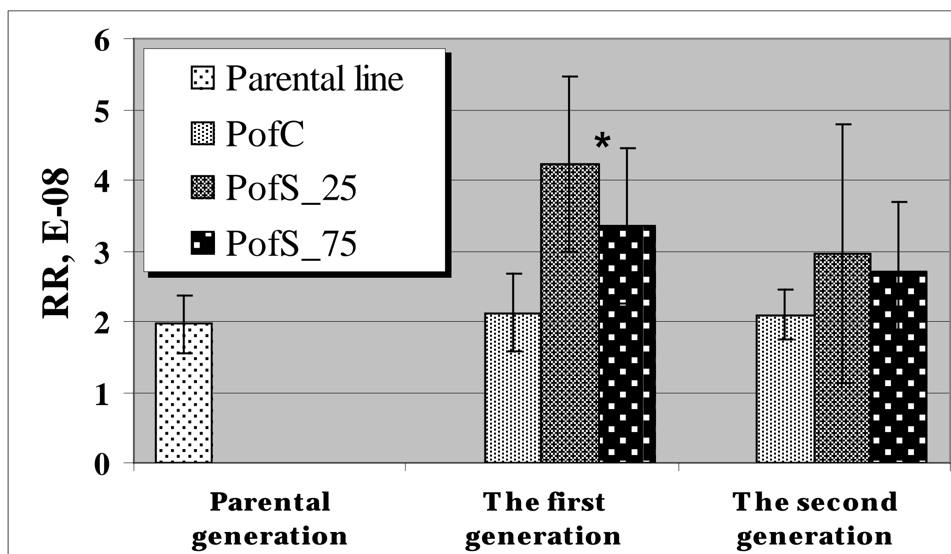

Figure 3.2.2. Spontaneous recombination rates in a parental line and the G1 and G2 progenies of control and salt treated plants

Plants were grown on solid MS medium for 3 weeks. The data represent three independent experiments. For each independent experiment, each data point was replicated twice, and each progeny was generated using at least 100 plants. Values represent mean ± s.d. Asterisks shows a statistically significant difference compared to PofC plants. Student's t-test: $\alpha=0.05$, $t=2.45$. ANOVA: $P<0.1$.

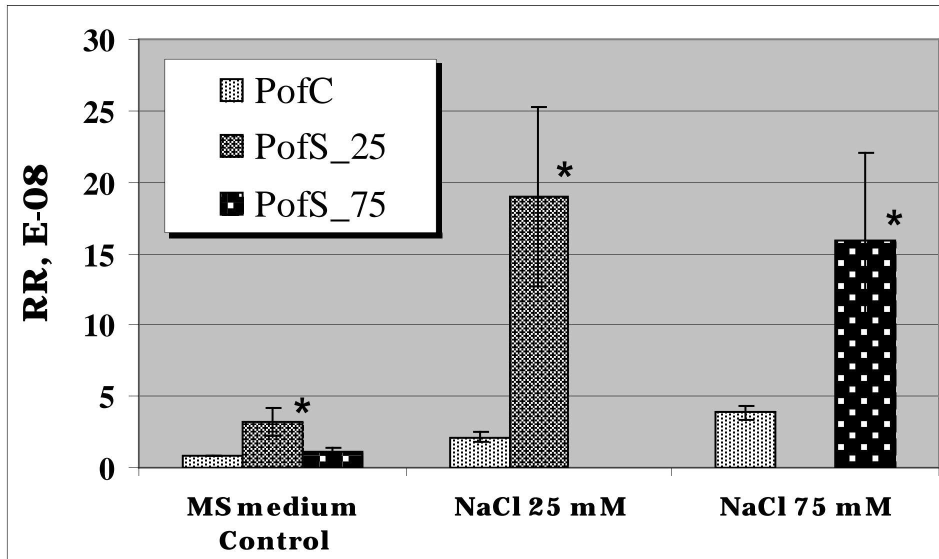

Figure 3.2.3. Spontaneous and stress-induced recombination rates in the G1 progeny of control and salt-treated plants

Plants were grown for 3 weeks on solid MS medium without NaCl (PofC, PofS_25 and PofS_75), and in the presence of 25 mM (PofC and PofS_25) and 75 mM (PofC and PofS_75) NaCl. Two experiments were performed. For each independent experiment, each data point was replicated three times, and each progeny was generated using at least 100 plants. Values represent mean ± s.d. Asterisks show a statistically significant difference compared to PofC plants, with the Student's t-test at $\alpha=0.05$ of $t=2.17$ and $t=2.78$ for plants grown on MS medium control and in 25 or 75 mM NaCl, respectively. ANOVA: for plants grown on MS medium control, in 25 and 75 mM NaCl, $P<0.05$.

3.2.2.2. The elevated recombination rate in the G1 progeny of salt treated plants is mediated by up-regulated *AtRad51* transcription and down-regulated *AtKu70* transcription

The elevated RR in the progeny of salt-stressed plants could be due to the higher level of strand breaks or due to the more frequent involvement of HR enzymes in the repair of generated strand breaks. To reveal the possible nature of this phenomenon, we have determined the level of DSBs as a main substrate for HR. To our surprise, the level of DNA DSBs was comparable between PofC and PofS plants (Table 3.3.2). In contrast, the semi-quantitative Real Time PCR analysis showed a significant 17% increase in the transcriptional activity of the *AtRad51* gene in PofS_25 plants as compared to PofC plants (Student's t-test, $\alpha=0.05$). Moreover, a significant 9% decline in the transcriptional activity of the *AtKu70* gene was also observed in PofS plants (Student's t-test, $\alpha=0.05$) (Table 3.3.2). Hence, the elevated spontaneous RR observed in PofS plants was possibly due to a partial shift in DNA repair gene expression, which was inherited by the G1 progeny.

Table 3.2.2. The number of DNA double strand breaks (*DPM ^3H*) and the transcriptional activity of *AtRad51* and *AtKu70* in the G1 progeny of stressed plants

	DPM ^{3}H, E+03	Fold	*AtRad51* related to *Ubiquitin*	Fold	*AtKu70* related to *Ubiquitin*	Fold
PofC	33.04±0.71 ^{a}	1	0.577±0.016 ^{b}	1	0.666±0.015 ^{a}	1
PofS_25	33.85±1.00 ^{a}	1.02	0.676±0.018 ^{a}	1.17	0.608±0.013 ^{b}	0.91
PofS_75	31.77±0.74 ^{a}	0.96	0.635±0.002 ^{ab}	1.1	0.635±0.009 ^{b}	0.91

Tissues were prepared from *A. thaliana* plants germinated and grown for 3 weeks on solid MS medium. At least two independent measurements were conducted for each data point. Values represent mean ± s.e. Values that are not connected by the same letter are significantly different. Student's t-test at $\alpha=0.05$ was $t=1.99$, $t=3.18$, $t=2.45$ for DSBs, *AtRad51*, *AtKu70*, respectively. ANOVA: for DSBs, $P>0.1$; for *AtRad51*, $P<0.05$; for *AtKu70*, $P<0.05$. A fold difference was calculated by relating data from PofS_25 and PofS_75 plants to data from PofC plants.

3.2.3. Plant adaptation to stress

Elevated spontaneous and stress-induced RRs displayed by PofS could produce a potential benefit to plants, if they are exposed to genotoxic stresses resulting in extensive DNA damage. Next, we tested this hypothesis by growing G1 and G2 plants in the presence of high concentrations of NaCl and MMS; the latter is a well-known DNA damaging agent.

3.2.3.1. Acquired tolerance to NaCl stress in the progeny of salt treated plants correlates with high recombination rates

Consistently with our previous findings, G1 PofS plants grown under high salt conditions displayed higher stress-induced RRs, as compared to PofC (Student's t-test, $\alpha=0.05$) (Figure 3.2.4). The diminishing differences in RR induction observed among the G2 progenies were consistent with the previously shown reversion of elevated spontaneous RR in G2 PofS plants (Figure 3.2.2). In fact, all the G2 plants, except PofS_25 grown in 100 mM (Student's t-test, $\alpha=0.1$), had insignificantly higher stress-induced RRs (Figure 3.2.3). A less pronounced response of G2 PofS plants to stress suggests that they have partially lost their ability to respond to stress.

The elevated activity of HR can positively contribute to stress-induced DSB repair. This potentially could increase stress tolerance. To test this hypothesis, we analyzed the sensitivity of PofC and PofS plants to NaCl stress. Both G1 progenies of salt treated plants exhibited higher tolerance to salt stress, as reflected by increased germination rates in G1 progenies grown in media with the NaCl concentration over 100 mM (Figure 3.2.5A, B). Germination rate was measured twice, at 9 and 18 dpg. At 9 dpg, germination was not observed on plates with 175 and 200 mM NaCl. At this time point,

the most pronounced and statistically significant differences between PofC and either of PofS groups were observed in plants grown in 125 (Student's t-test, $\alpha=0.05$) and 150 mM NaCl (Figure 3.2.5A, C). At 18 dpg, germination was observed in all groups (Figure 3.2.5B). At this time point, statistically significant differences between PofC and PofS groups were observed for 125 (Student's t-test, $\alpha=0.05$) and 150 mM (Student's t-test, $\alpha=0.1$) (Figure 3.2.5B). Importantly, the germination rate of G1 PofS_25 plants remained significantly higher, if germinated in the presence of NaCl in concentrations as high as 200 mM (Student's t-test, $\alpha=0.05$) (Figure 3.2.5B). It is noteworthy that stress tolerance of the G2 progeny diminished dramatically (Figure 3.2.5D).

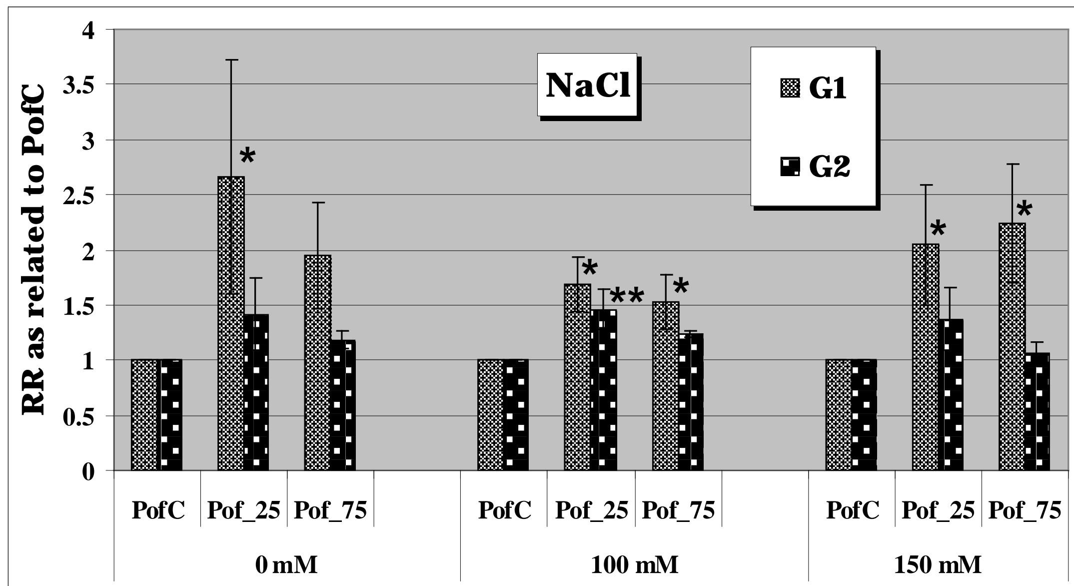

Figure 3.2.4. Increased recombination rates in the progeny of salt-treated plants grown in media supplemented with NaCl

The figure shows a fold increase of RR in in PofS_25 and PofS_75 plants in comparison to PofC. Plants were grown on standard MS medium and on MS medium supplemented with 100 and 150 mM NaCl. Three independent experiments, each containing two replicates, were performed, and data were averaged. Values represent mean ± s.d. Asterisks show a statistically significant difference as compared to PofC plants. One asterisk shows the Student's t-test at $\alpha=0.05$ of $t=2.07$, $t=2.06$, $t=2.05$ for PofS_25 G1 plants grown in 0 mM NaCl, PofS_25 and PofS_75 G1 plants grown in 100 or 150mM, respectively. Two asterisks show the Student's t-test at $\alpha=0.1$ of $t=1.71$ for PofS_25 G2 plants grown in 100 mM NaCl. ANOVA: $P<0.1$, $P<0.05$ and $P<0.1$ for plants grown in 0 mM, 100 mM and 150 mM NaCl, respectively.

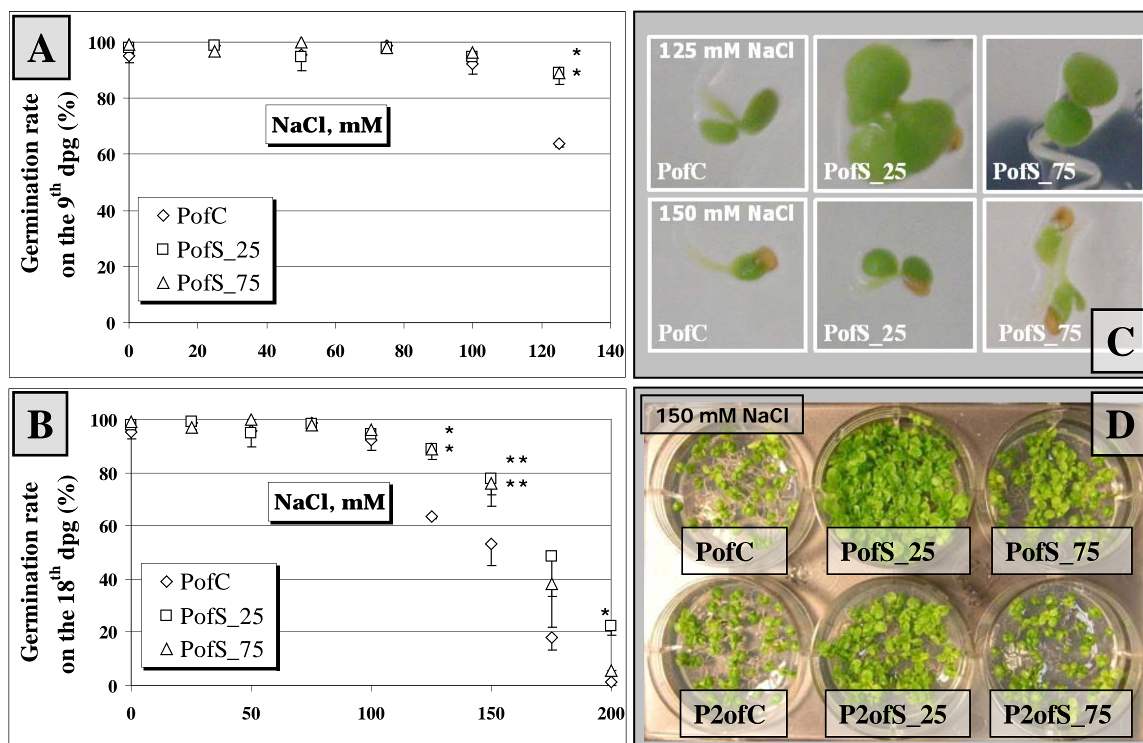

Figure 3.2.5. Acquired salt stress tolerance in the progeny of salt-treated plants

A and **B** – Germination rates in PofC, PofS_25 and PofS_75 plants germinated and grown on solid MS medium supplemented with 0 – 200 mM NaCl. Germination was scored twice, on 9th (**A**) and 18th (**B**) day post germination. Two independent experiments were performed. Values (percent of the total number of seeds plated) represent mean ± s.e. Asterisks show a statistically significant difference compared to PofC plants. One asterisk shows the Student's t-test at $\alpha=0.05$ of $t=2.45$ for PofS_25 and PofS_75 plants grown in 125 mM and for PofS_25 plants grown in 200 mM NaCl. Two asterisks show the Student's t-test at $\alpha=0.1$ of $t=1.94$ for PofS_25 and PofS_75 plants grown in 150 mM NaCl. ANOVA: for plants grown in 125 mM NaCl, $P<0.05$; and in 150 mM NaCl, $P<0.1$. **C** – Phenotype of the G1 progeny plants germinated and grown on solid MS medium supplemented with 125 and 150 mM NaCl, respectively. Pictures were taken on the 9th day post germination. **D** – A phenotype of G1 and G2 plants grown in 150 mM NaCl. Pictures were taken on the 22nd day post germination.

3.2.3.2. Acquired tolerance to MMS stress in the progeny of salt treated plants correlates with high recombination rates

To investigate the extent of acquired elevated tolerance to stress, we germinated and grew G1 and G2 plants in the presence of high concentrations of MMS, a substance known to generate DSBs. Overall, PofS plants responded to MMS in the same manner as for high NaCl concentrations stress. The stress-induced RR was significantly higher in both G1 PofS plants grown in the presence of 140 ppm MMS (Student's t-test, $\alpha=0.1$) and G1 PofS_75 plants exposed to 100 ppm MMS (Student's t-test, $\alpha=0.05$) (Figure 3.2.6). Similarly to high salt stress, the difference in the stress-induced RR diminished in the G2 generation between PofC and PofS plants (Figure 3.2.6).

As described above, acquired tolerance to high salt stress in PofS plants was similar to that observed to MMS stress (Figure 3.2.7). PofS plants grown in the presence of various concentrations of MMS displayed more resistant phenotypes as compared to PofC plants (Figure 3.2.7A). Both PofS groups had a better developed root system and more leaves per plant on average. In fact, on the 16th dpg, there was a 1.6-fold difference between G1 PofS_75 and PofC plants grown in 100 ppm MMS (Student's t-test, $\alpha=0.05$) (Figure 3.2.7B). Similarly, 30-day old G1 PofS_25 (Student's t-test, $\alpha=0.05$) and PofS_75 (Student's t-test, $\alpha=0.1$) plants grown in the presence of 150 ppm MMS had longer roots than PofC plants by factors of 2.2 and 1.8, respectively (Figure 3.2.7A, C). Additionally, a significant 2.0-fold difference in root length was found between PofS_75 and PofC G1 plants grown in 130 ppm MMS (Student's t-test, $\alpha=0.05$) (Figure 3.2.7A, C). In general, these findings suggest that G1 progenies of salt treated plants acquired a broad genotoxic stress tolerance not restricted to NaCl.

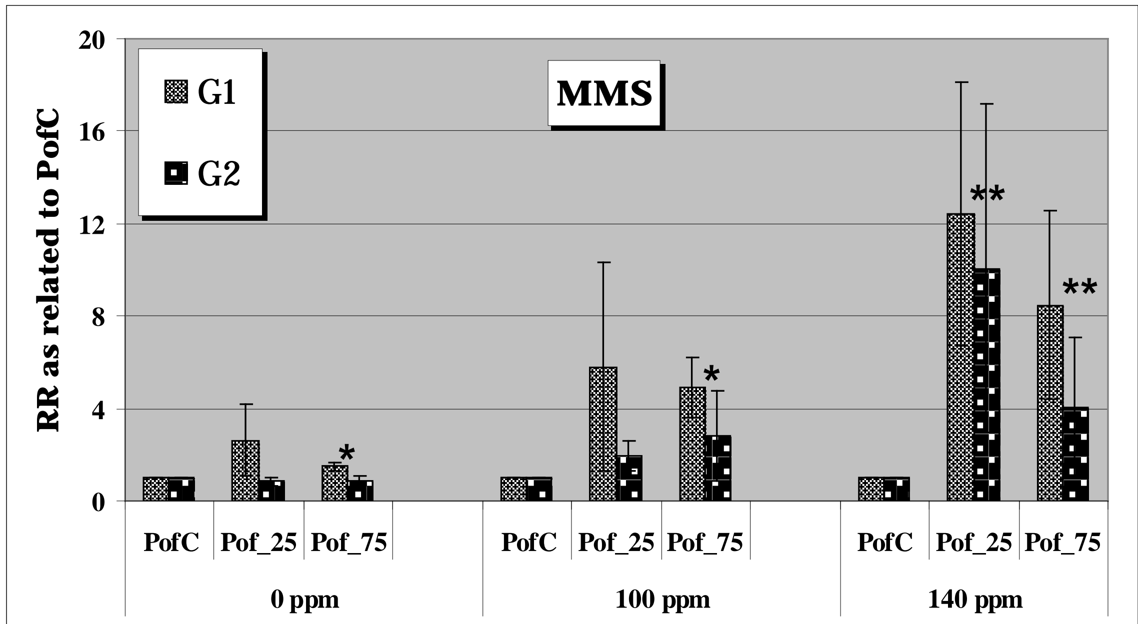

Figure 3.2.6. Increased recombination rates in the progeny of salt-treated plants grown in media supplemented with MMS

The figure shows a fold increase of RR in in PofS_25 and PofS_75 plants in comparison to PofC. Plants were grown on standard MS medium and on MS medium supplemented with 100 and 140 ppm MMS. Three independent experiments, each containing two replicates, were performed, and data were averaged. Values represent mean ± s.d. Asterisks show a statistically significant difference compared to the PofC plants. One asterisk shows the Student's t-test at $\alpha=0.05$ of $t=2.18$ and $t=2.16$ for PofS_75 G1 plants grown in 0 ppm MMS and 100 ppm MMS, respectively. Two asterisks show the Student's t-test at $\alpha=0.1$ of $t=1.70$ and $t=1.75$ for PofS_25 and PofS_75 G1 plants grown in 140 ppm MMS, respectively. ANOVA: $P<0.05$, $P<0.1$ and $P<0.1$ for plants grown in 0 ppm, 100 ppm and 140 ppm MMS, respectively.

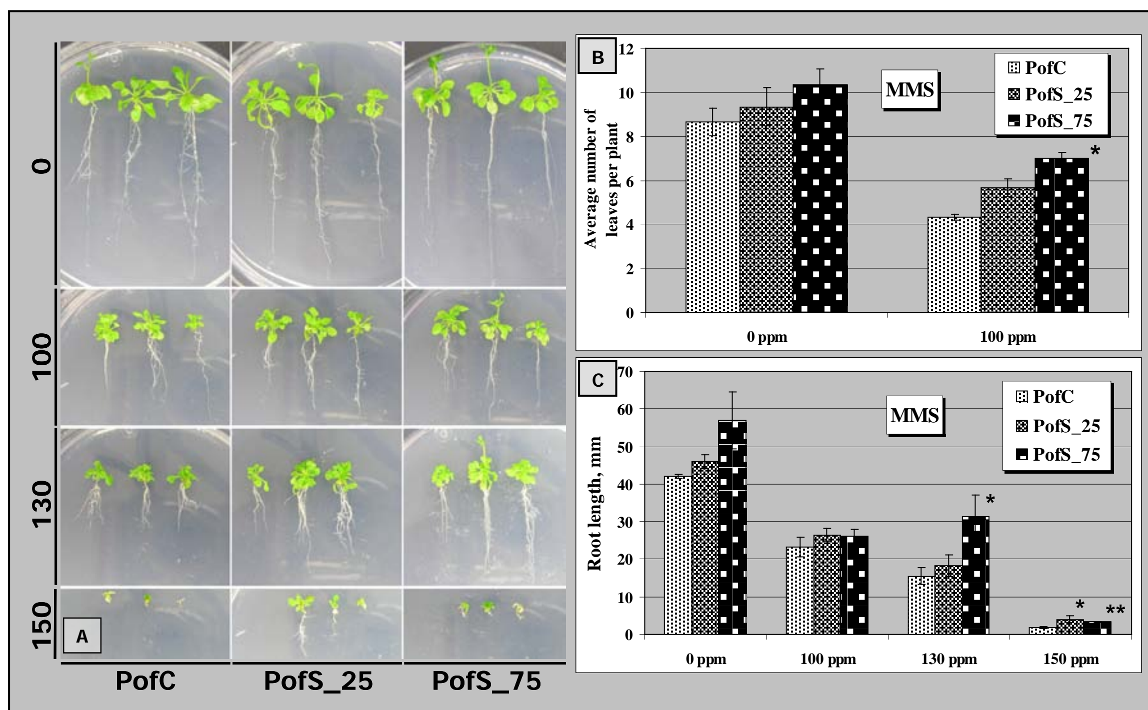

Figure 3.2.7. Acquired tolerance to MMS stress in the G1 progeny of salt-treated plants

A – A phenotypic appearance of the G1 progeny of salt-treated plants germinated on solid MS medium and then transplanted for growth on solid MS medium supplemented with 0, 100, 130 and 150 ppm of MMS; pictures were taken on the 30th day post germination. **B** – The number of leaves per single plant in the G1 progeny of control and salt-treated plants grown on solid MS medium and on MS medium supplemented with 100 ppm of MMS. Leaves were counted on the 16th day post germination. Two independent experiments were performed. Values represent mean ± s.e. Asterisks show a statistically significant difference in the PofC plants. Student's t-test, $\alpha=0.05$, $t=2.18$. ANOVA: for plants grown in 100 ppm MMS, $P<0.05$. **C** – The average root length in the G1 progeny of control and salt-treated plants grown on solid MS medium and on MS medium supplemented with various concentrations of MMS. Roots were measured on the 30th day post germination. Two independent experiments were performed. Values represent mean ± s.e. Asterisks show a statistically significant difference compared to the PofC plants. One asterisk shows the Student's t-test at $\alpha=0.05$ of $t=2.45$ for PofS_75 G1 plants grown in 130 ppm MMS and PofS_25 G1 plants grown in 150 ppm MMS. Two asterisks show the Student's t-test at $\alpha=0.1$ of $t=1.94$ for PofS_75 G1 plants grown in 150 ppm MMS. ANOVA: $P<0.05$ and $P<0.1$ for plants grown in 130 ppm and 150 ppm MMS, respectively.

3.2.4. Genome-wide and locus-specific changes in DNA methylation in the progeny of salt treated plants

Our results demonstrate that exposure of plants to stress can generate a signal capable of changing HRF in the next after stress generation. Interestingly, when the G1 PofS plants are propagated into the G2 generation without stress exposure, the elevated spontaneous RR reverts back to the level close to the one observed in control PofC plants. Similarly, acquired tolerance of G1 PofS plants to NaCl and MMS stresses diminishes in the G2 generation. Overall, these findings may suggest the epigenetic nature of the adaptation to the stress.

Changes in global genome methylation may represent one of the primary signs indicating the occurrence of epigenetic DNA modifications (reviewed in Boyko and Kovalchuk, 2008a). Indeed, we found that PofS plants have hypermethylated genomes, as compared to PofC plants. There was a significant 7% decrease in the number of unmethylated cytosines found in PofS_25 plants (Student's t-test, $\alpha=0.05$) (Figure 3.2.8A). At the same time, the methylation status of G2 PofS plants had a tendency to return to a methylation level found in PofC (Figure 3.2.8A).

Methylation at repetitive elements is usually rather high. Changes in their methylation status would influence global genome methylation. To analyze whether an increase in global genome methylation observed in G1 PofS plants was paralleled by changes in repetitive element methylation, we compared a methylation pattern of centromeric repeats in the G1 and G2 generation of PofC and PofS plants. Overall, no significant difference among these groups was found, which indicates that changes in DNA methylation were targeted to other than centromeric repeat regions of the genome

(Figure 3.2.8B). These regions could include coding sequences; and their epigenetic modification could promote plant adaptation to stress and an elevated HR response observed in the G1 progeny.

To investigate whether changes in DNA methylation were directed to coding sequences, we analysed the methylation status at the promoter of the *AtAct2* gene using the COBRA analysis. The *AtAct2* gene was chosen as a representative for a group of housekeeping genes. Additionally, to insure that the increased activity of HR observed in PofS plants was not due to methylation of a transgene locus that was lower than in PofC, we performed the COBRA analysis of promoter and coding regions of the GUS transgene.

We found that the *AtAct2* gene promoter in PofS_25 plants was hypermethylated as compared to a PofC group. A nearly complete restriction digestion of PCR product corresponding to the *AtAct2* promoter in PofS plants by the *Hpy*CH4IV enzyme demonstrated the higher number of methylated CpG sites present at this locus in the progeny of stressed plants (Figure 3.2.9A). The percentage of methylated cytosines was higher in the G1 PofS_25 plants, as compared to the G1 PofC plants, 66.8% and 50.5%, respectively (Student's t-test, $\alpha=0.05$) (Figure 3.2.9A, D). Importantly, we observed a reduced and insignificant difference in CpG sites methylation between PofC and PofS_25 plants in the G2 generation (Figure 3.2.9A, D).

In contrast, no significant difference between PofC and PofS plants in the number of methylated CpG sites was found at the GUS promoter and GUS coding loci (Figure 3.2.9B, C). This demonstrated that the elevated HRF observed in PofS plants was not caused by changes in methylation in the reporter transgene sequence.

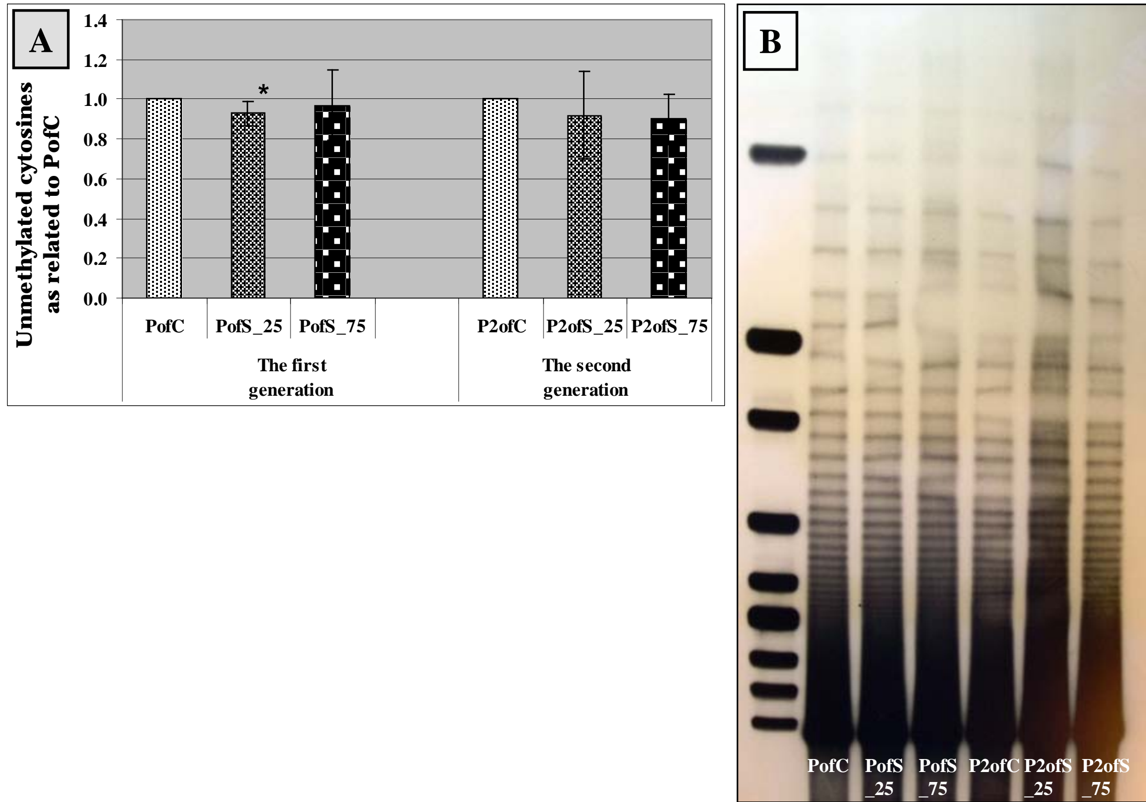

Figure 3.2.8. Global genome methylation and methylation of pericentromeric regions in the G1 and G2 progenies of salt-exposed plants

A – Decrease in the total number of unmethylated cytosines in the genome of 1 and G2 PofS plants as compared to the respective PofC plants. The number of unmethylated cytosines in PofC and P2ofC was standardized to 1.0. Global genome methylation in the progeny of salt-treated plants (PofS_25, P2ofS_25, PofS_75 and P2ofS_75) shows an increase in methylation in folds as related to the respective PofC plants. The assay was repeated three times, and readings were taken at least twice per each reaction. Values represent the mean ± s.d. Asterisks show a statistically significant difference compared to PofC plants. Student's t-test: $\alpha=0.05$, $t=2.31$. ANOVA: $P<0.05$. **B** – The level of DNA methylation at pericentromeric regions of all five chromosomes remained unchanged, as shown using the Southern blot analysis of genomic DNA digested with the methylation sensitive *Hpa*II enzyme and hybridized to 180 bp centromeric repeat sequence.

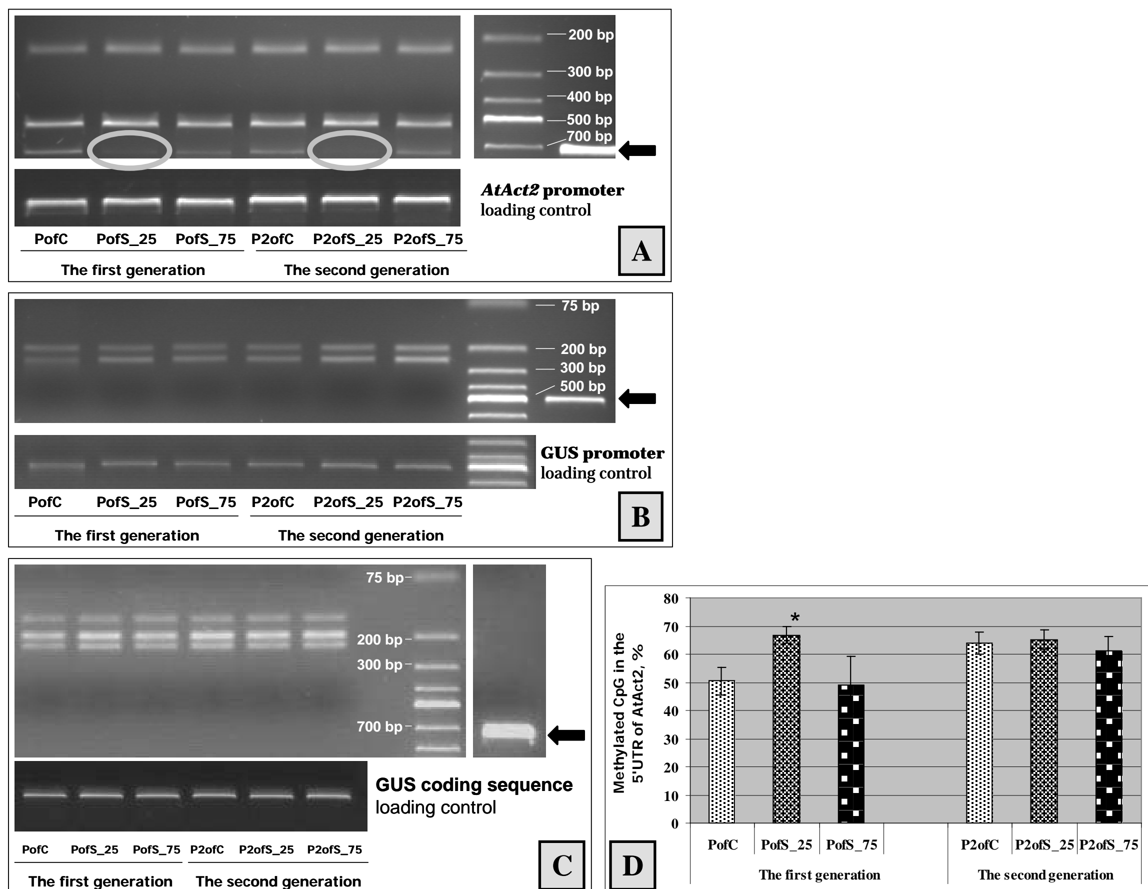

Figure 3.2.9. Locus-specific DNA methylation analysis of the G1 and G2 progenies of salt-exposed plants

A, **B**, and **C** – Gel images show the *Hpy*CH4IV restriction enzyme digestion of PCR fragments representing *AtAct2* promoter, GUS promoter and GUS coding sequences, respectively. The amount of remaining undigested product reflects the number of non-methylated CpG sites within a sequence of interest. Arrows point at undigested PCR product; circles indicate the almost complete digestion of *AtAct2* promoter PCR product in PofS_25 and P2ofS_25 plants. **D** – Percent of methylated CpG sites of the total number of CpG sites (methylated and non-methylated) available within the 5'UTR of *AtAct2* sequence. The percent of methylated CpG sites was determined by relating the intensity of a 551 nt restriction fragment to the total intensity of DNA bands (the remaining undigested PCR product and two bands resulted from restriction with *Hpy*CH4IV). The higher intensity of a 551 nt fragment reflects the higher number of *Hpy*CH4IV recognition sites that were methylated prior to bisulphite conversion and remained unchanged. DNA samples from three independent experiments were analyzed, and restriction analysis was replicated twice. Values represent the mean ± s.d. Asterisks show a statistically significant difference compared to the PofC plants. Student's t-test: $\alpha=0.05$, $t=2.78$. ANOVA: for the G1 progeny $P<0.05$.

3.3. DISCUSSION

In this study, we analyzed the influence of salt stress on plant genome stability and investigated the impact of stress exposure on the development of stress tolerance. We have found that a) NaCl results in a dose dependent RR increase; b) exposure to 25-75 mM NaCl leads to a 15-20% increase of DSBs; c) salt exposure leads to an increase in *AtRad51* and decrease in *AtKu70* transcriptional activity; d) the progeny of salt exposed plants have an elevated RR that correlates with an increase in the *AtRad51* transcription; e) the progeny of salt exposed plants have higher tolerance to salt stress and to MMS; f) the progeny of salt treated plants have a modified DNA methylation pattern, which suggests an epigenetic nature of a signal that changes HR; g) a signal that changes HR is stress-inducible and capable of transmission to the next generation.

3.3.1. Effects of salt stress on genome stability

In our study, we estimated possible genotoxic effects of salt exposure on plants. One of the earliest reports by Puchta *et al.* (1995) suggested that plant exposure to salt is potentially genotoxic. Exposure of plants to 100 mM NaCl resulted in a 2.2 – 2.7 increase in HRF. Unfortunately, the authors did not analyze a range of concentrations that changes HRF, and they did not uncover possible mechanisms of recombination induction. In contrast, our work showed that exposure to NaCl concentrations as low as 25 mM increased recombination.

One of the possible mechanisms of an increase in RR is an elevated level of DNA damage caused by salt stress. Osmotic and ionic stresses that result from exposure to NaCl are complemented with secondary oxidative stress (Niu *et al.*, 1995, Zhu *et al.*,

1997, Hasewaga *et al.*, 2000, Zhu, 2000) that leads to the accumulation of a wide range of ROS including hydrogen peroxide, hydroxyl radicals, superoxide anion radicals, and singlet oxygen (Smirnoff, 1998; Bartels, 2001; Apel and Hirt, 2004). The accumulation of ROS affects DNA integrity and generates a number of different DNA lesions including DNA DSBs (Breimer, 1990; Chatgilialoglu and O'Neill, 2001) that can be lethal to a cell (Evans *et al.*, 1993). Our experiments showed a significant increase in the level of DSBs in plants grown in 25 – 100 mM NaCl. A higher level of DSBs resulted in a higher RR. Intriguingly, increased ROS production triggered by salt stress may also have a positive impact on plant salt tolerance induction via mediation of mRNA stability for SOS1-like genes that are crucial for the ion homeostasis maintenance under salt stress (Chung *et al.*, 2008).

Homologous recombination is one of two major DSB repair pathways, with NHEJ being a major alternative mechanism (Salomon and Puchta, 1998; Kirik *et al.*, 2000). Cells can choose one of these two pathways based on a variety of factors including repair template availability, cell cycle phases, proliferation rate, and cell type-specific functions (reviewed in Shrivastav *et al.*, 2008). Since we did not find a drastic increase in the level of strand breaks, we hypothesized that exposure to NaCl leads to an increase in RR via differential regulation of NHEJ and HR pathways. As there was no comparable system similar to the GUS-based transgenic recombination assay for scoring NHEJ events, we used a semi-quantitative Real Time PCR approach to compare transcriptional activity of two DNA repair genes, *AtRad51* and *AtKu70*, which function exclusively in HR and NHEJ repair (Sonoda *et al.*, 2006). These genes are plant homologues of yeast *RAD51* (Doutriaux *et al.*, 1998) and *KU70* (Tamura *et al.*, 2002). Rad51 plays a critical role in

HR via the formation of a Rad51/ssDNA nucleofilament from 3' overhangs of DSB and promotion of strand exchange reactions (Bleuyard *et al.*, 2006 and references therein). Ku70 forms a heterodimer with Ku80. The KU heterodimer represents a part of DNA-PK and binds various types of DNA ends, including single-stranded gaps. It also helps protect them and forms a bridge between two ends of a break, thereby contributing to their juxtaposition (Bleuyard *et al.*, 2006 and references therein). Consequently, both Rad51 and Ku70 proteins are critical for HR and NHEJ repair pathways (Gorbunova and Levy, 1999; Ray and Langer, 2002).

Previous studies demonstrated the existence of correlation between levels of DNA damage such as DSBs and expression of DSB repair enzymes (Kovalchuk *et al.*, 2004b; Boyko *et al.*, 2006d). Indeed, we found the steady state RNA level of *AtRad51* increased by 30% in plants exposed to 100 mM NaCl. In contrast, the *AtKu70* RNA level was reduced by 5-10%, suggesting that there indeed was a shift in strand break repair towards HR. Stress regulation of DSB repair balance in plants represents a novel finding. Importantly, exposure to radiation was reported to cause similar up-regulation of HR proteins (as the one reported here) and down-regulation of NHEJ proteins in mice (Kovalchuk *et al.*, 2004b; Koturbash *et al.*, 2006).

Our findings partially supported the suggestion made by Ray and Langer (2002) that a higher activity of HR requires low levels of NHEJ proteins. Studies of meiotic mice cells (Goedecke *et al.*, 1999) and meiosis-competent yeast cells (Valencia *et al.*, 2001) indicated that a low activity of NHEJ is necessary for HR to occur. Moreover, a shift from NHEJ toward HR during transition from G1 to S/G2 phase of the cell cycle is accompanied by increased RAD51 expression in mammals (Chen *et al.*, 1997). Enhanced

HR activity observed during the early neural development in mice may be mediated via active suppression of NHEJ (Orii *et al.*, 2006).

To date, it is not clear what mechanisms regulate a choice of a pathway to be used for generated DSB repair. The presence of DNA-PKcs in higher eukaryotes may play a critical role in NHEJ regulation and can account for its prevalence over HR (reviewed in Shrivastav *et al.*, 2008). A distinctly different role of HR in DSB repair in yeast and higher eukaryotes may also reflect their differences in genome size and coding sequence content (Beaton and Cavalier-Smith, 1999). Finding of a homologous repair template in large genomes may represent a significant challenge for the DNA repair machinery (Sonoda *et al.*, 2001; Sonoda *et al.*, 2006). In fact, the selection of a wrong repair template may result in gene translocation or duplication events, and may generate large-scale deletions, if intrachromosomal recombination occurs within a DNA region containing multiple repeats (Swoboda, *et al.*, 1994; Orel *et. al.*, 2003; Dudas and Chovanec, 2004; Puchta, 2005). Hence, taking into consideration a large amount of repetitive DNA present in the plant genome (Flavell, 1985), using HR as a dominant mode of DNA repair may pose a threat to genome stability. In contrast, the high content of non-coding DNA found in higher eukaryote genomes allows an easy accommodation of mistakes generated by the NHEJ pathway.

3.3.2. Transgenerational effects of salt stress

Several recent reports demonstrated that the progeny of plants exposed to pathogen (Boyko *et al.*, 2007) or UV-C (Molinier *et al.*, 2006) stresses display the elevated spontaneous HRF. To study the possible transgenerational influence of salt stress on genome stability of the immediate progeny of salt exposed plants, we analyzed the progenies of 25 and 75 mM treated plants (PofS_25 and PofS_75, respectively) and compared them to the progeny of untreated control plants (PofC). The G1 and G2 PofS_25, PofS_75 and PofC plants grown without NaCl showed no difference in the level of DSBs. Similarly, no difference was observed in the plant phenotype. This indicated that low salt stress conditions applied to the paternal generation did not result in significant physiological changes in their progeny.

Genome stability was changed and appeared to be different between the G1 PofC and PofS plants. A strong increase in RR in PofS plants as compared to PofC plants was observed in the G1 generation, suggesting that exposure to stress can modify the spontaneous RR in a single generation. In contrast, the spontaneous RR of the G2 plants progeny of the G1 PofS plants grown and propagated under normal conditions had reverted back to the level found in control PofC plants. This may indicate that the maintenance of heritable changes in RR requires certain stress-induced signals. These signals may establish a new inherited gene regulation pattern that defines the elevated level of HR. Consequently, removal of stress eliminates these signals and causes this pattern to revert to normal conditions, thus returning RR back to normal in the G2 generation.

To reveal molecular mechanisms leading to the increased HRF, we analyzed steady state RNA levels of the *AtRad51* and *AtKu70* genes. We found that the increased RR was mirrored at a molecular level by higher transcriptional activity of *AtRad51* and lower transcriptional activity of *AtKu70* in PofS plants. This was consistent with previous studies of Goedecke *et al.* (1999), Valencia *et al.* (2001) and Orii *et al.* (2006). They indicated that low activity of the NHEJ pathway is an important determinant for high levels of HR. Besides, the modification of DSB repair enzyme expression and the shift in DSB repair toward NHEJ was reported before. It was found that HR was developmentally regulated. HRF decreased gradually, while the level of breaks remained unchanged in growing *Arabidopsis* plants (Boyko *et al.*, 2006d). Overall, it can be hypothesized that salt stress produced an opposite shift in DSB repair, from NHEJ towards HR. This shift was inherited by the progeny.

However, our data contradict the study of Molinier *et al.* (2006) that demonstrated a continuous HRF increase in at least four generations following a single exposure to stress. A biological meaning of a persistent HR increase in the absence of stress exposure remains unclear. It is possible that UV-C, an artificial environmental stress factor, triggered a response different from that in plants exposed to natural stresses like temperature, light or salinity. In contrast, our study used salinity, a natural environmental stress. Plants exposed to NaCl could reveal responses normally triggered by stress exposure.

3.3.3. Plant adaptation to stress

An increase in the spontaneous RR in PofS plants might reflect other major changes in these plants. It can be hypothesized that a salt stress-induced shift in DSB repair from NHEJ towards HR is a part of an adaptation process (Rocha *et al.*, 2002; Molinier *et al.*, 2006). DSB repair via the NHEJ pathway generates deletions and insertions of various sizes (Gorbunova and Levy, 1997). It can be assumed that even a small shift in the balance would increase the number of breaks repaired via HR and thus decrease the number of deletions/insertions upon NHEJ repair. Hence, it is possible that a higher contribution of HR to DSB repair may lead to an increased plant tolerance to genotoxic stresses.

To test this hypothesis, we analyzed the response of PofS plants to the same (NaCl) or different stresses (MMS). Importantly, we found that PofS_25 and PofS_75 plants exhibited similar elevated tolerance to both stresses. Increased plant stress tolerance was correlated with increased RRs. The G1 PofS plants germinated better on media containing high concentrations of NaCl, and they formed longer roots when exposed to MMS. Our data were consistent with previous reports that demonstrated stress-mediated fast acclimation to drought, cold and freezing, high temperature, high light and UV-B radiation stresses (reviewed in Chalker-Scott and Scott, 2004; Turunen and Latola, 2005; Caldwell *et al.*, 2007). Our observations are further supported by studies of Blödner *et al.* (2007). They demonstrated that plants growing under low temperatures can increase tolerance of their immediate progeny to chilling and freezing conditions. Moreover, Boyko *et al.* observed the delayed appearance of disease

symptoms in the progeny of pathogen-challenged plants infected with a virus (Boyko *et al.*, data not published).

Co-tolerance to two different stresses observed in PofS plants is consistent with Levitt's theory of stress cross-protection (Levitt, 1980), which suggests that plant exposure to one type of stress can result in acquired tolerance to another stress to which this plant had no previous exposure. Indeed, exposure to UV-B can increase plant tolerance to subsequent freezing (Dunning *et al.*, 1994; Richer and Hoddinott, 1997; Binder and L'-Hirondelle, 1999; Mendez *et al.*, 1999; Chalker-Scott and Scott, 2004), high temperatures (Teklemariam and Blake, 2003), drought and high light (Poulson *et al.*, 2002). It can also increase a plant's defenses against insect herbivory (Roberts and Paul, 2006) and fungal attack (Raviv and Antignus, 2004). Both NaCl and MMS increase the level of DNA DSBs, suggesting that an elevated activity of the HR machinery can play an active role in the formation of elevated tolerance to these stresses.

Importantly, acquired stress tolerance that was observed in the G1 PofS plants diminished in the G2 generation, in which reversion of the spontaneous RR to PofC levels was observed. This supports the idea that there is a link between the elevated spontaneous RR and stress tolerance in G1 PofS plants. Furthermore, decreased acquired stress tolerance in the G2 plants demonstrates that the establishment of prolonged tolerance requires more than one generation of stress exposure. This hypothesis is consistent with the high metabolic cost required for the maintenance of new gene expression patterns conferring resistance to stress (Thomashow, 1999; Cook *et al.*, 2004; Kaplan *et al.*, 2004; Larkindale *et al.*, 2005; Kotak *et al.*, 2007; Zhu *et al.*, 2007a). Overall, the development of acquired tolerance by the progeny of plants exposed to a

certain stress may represent an important stress tolerance mechanism in plants as sedentary organisms.

3.3.4. Genome-wide and locus-specific changes in DNA methylation in the progeny of salt treated plants

Our study demonstrated that plant exposure to stress results in producing a certain signal that can be transmitted to the next generation and change a gene regulation pattern leading to an increased HRF and acquired tolerance to stress. Reversion of these changes upon growing and propagation of the G1 progenies in the absence of stress suggests the epigenetic nature of the signal that mediated the establishment of the aforementioned changes. Indeed, Molinier *et al.* (2006) crossed stressed plants with non-stressed plants and found that all progeny plants had higher HRF, which supported the hypothesis of an epigenetic regulation of stress response.

Changes in DNA methylation represent one of the primary mechanisms for epigenetic regulation (reviewed in Boyko and Kovalchuk, 2008a). Furthermore, DNA methylation can be actively modified in response to stress (Steward *et al.*, 2000; Henderson and Dean, 2004; Wada *et al.*, 2004; Dyachenko *et al.*, 2006), using various smRNA molecules as a guiding force for various DNA modifying enzymes (Bender 2004; Matzke *et al.*, 2004; Chan *et al.*, 2006). In fact, a number of smRNAs reported to date were shown to be regulated by a broad range of abiotic stresses including mechanical stress, dehydration, salinity, cold, abscisic acid and nutrient deprivation (Sunkar and Zhu, 2004; Borsani *et al.*, 2005; Lu *et al.*, 2005; reviewed in Sunkar *et al.*, 2007). This might allow directing methylation to specific loci by producing stress-specific smRNAs (Matzke *et al.*, 2007). The resulting stress-induced changes in a pattern of DNA methylation may have an important adaptive function. Dyachenko *et al.* (2006) demonstrated that increased methylation in satellite DNA of common ice plants exposed

to high salinity conditions resulted in a switch-over from C3-photosynthesis to C4-type carbon dioxide assimilation. In yet another report, an age-dependent increase in methylation was shown to confer resistance to the blight pathogen *X. oryzae* in rice (Sha *et al.*, 2005).

We hypothesized that stress-generated signals might establish a new DNA methylation pattern in plant reproductive tissue. Such changes should thus be reflected in the next generation resulting in elevated RR and stress tolerance. Indeed, we found that genomes of G1 PofS plants were hypermethylated compared to PofC plants. Importantly, the most significant methylation difference found in G1 PofS plants correlated with the highest spontaneous RR and tolerance to salt stress observed among the G1 progenies. Consistently with our hypothesis, the difference in methylation patterns decreased in the G2 generation obtained from the G1 nonexposed plants. It is noteworthy that no significant difference was found in methylation patterns at pericentromeric regions, which might indicate that changes in DNA methylation occurred primarily within coding or other repetitive sequences.

The hypermethylation status of the G1 PofS plants was confirmed by analyzing the methylation level of actin gene loci; the most pronounced difference was observed in the G1 PofS_25 plants. Taking into consideration an inverse correlation between HRF and the methylation status of a given locus (Bassing *et al.*, 2002; Wada *et al.*, 2004), increased methylation at the *actin2* gene locus may represent a protective mechanism that prevents extensive rearrangements from occurring at housekeeping gene loci (Rizwana and Hahn, 1999). Importantly, similar changes in methylation patterns were observed in the progeny of virus infected plants (Boyko *et al.*, 2007). This demonstrated that the

elevated spontaneous RR correlated with increased methylation at the actin gene locus in the progeny of virus infected tobacco plants. In contrast to the actin gene locus, the *R-gene*-like loci were hypomethylated and displayed an increased frequency of rearrangements (Boyko *et al.*, 2007). Considering that the *R-gene*-like loci carry homology to the *N-gene* which was absent in plants used for infection, it was suggested that locus-specific changes in methylation and rearrangements could be a plant strategy of creating an active *R-gene*. Studies of Meyers *et al.* (2005) suggested that the evolution of plant *R-genes* involved gene duplication and recombination events. Hence, it is possible that the elevated spontaneous RR observed in PofS plants also represented one of the early responses involved in stress-directed genome evolution.

Finally, to insure that an increase in RR was not due to changes in the methylation status at a transgene locus, we analyzed methylation at GUS promoter and GUS coding regions. No significant difference was found among progenies, suggesting that the increased HRF was not caused by changes in methylation of a reporter transgene. These data also support a previously published report which suggested that changes in the epigenetic status of the transgene are not responsible for a transgenerational increase in HRF (Molinier *et al.*, 2006). Overall, our findings support the epigenetic nature of a signal that mediated inheritance of a high somatic HRF and development of stress tolerance by the progeny of stress treated plants.

3.4. SUMMARY

Our study demonstrated that continuous exposure to even low concentrations of NaCl can trigger substantial changes in plant genome stability, including an increased level of DNA DSBs and an elevated frequency of genome rearrangements. Our data represent a novel finding that the balance of DSB repair in plants can be controlled by stress; and it complements recent reports demonstrating that changes in the balance between HR and NHEJ under physiological conditions can be mediated via modification of the activity of factors involved in DSB repair.

Our study provided evidence that the effect of salt stress on plants is not restricted to the exposed generation, and it can result in the elevated spontaneous RR in the progeny. A strong increase in RR in the G1 PofS plants suggests that exposure to stress can modify the spontaneous RR in a single generation. Furthermore, growing and propagating the G1 PofS progenies in the absence of stress changed the elevated spontaneous RR in the G2 generation back to a level of control plants. These findings suggest the existence of a certain stress-inducible signal that may establish a new heritable gene regulation pattern defining an elevated level of HRF.

We found that plants exposed to stress can establish a memory of stress exposure, leading to acquired tolerance of their progeny to the same or different stress. We believe that the establishment of stress memory is epigenetically mediated. Changes in a methylation pattern in the progeny of plants exposed to salt correlated with increased spontaneous and stress-induced RRs and elevated tolerance to NaCl and MMS.

A stress-induced shift in DSB repair from NHEJ toward HR can be seen as a part of an adaptation process, since higher fidelity of HR repair may be advantageous for

plants experiencing genotoxic stress. Besides, an increased frequency of rearrangements may represent one of the early responses involved in stress-directed genome evolution, as it was recently described for the progeny of plants challenged with a pathogen. Overall, our findings support the epigenetic nature of a stress-induced signal mediating the establishment of stress memory and inheritance of the elevated HRF. This can represent an important stress tolerance mechanism employed by plants.

3.5. FUTURE DIRECTIONS

Our study demonstrated that plant exposure to salt stress results in a heritable increase in HRF that is mediated by a stress-induced signal that has an epigenetic nature. It would be important to reveal the molecular nature of this signal and demonstrate the importance of gene-specific epigenetic changes in the establishment of acquired tolerance to stress and the elevated spontaneous RR observed in the progeny of stress treated plants.

We believe that the establishment of sequence-specific changes in DNA methylation and their inheritance by progenies can be at least partially mediated by smRNAs. It would be interesting to conduct similar experiments using plants impaired in various aspects of smRNA biogenesis. In addition, using single, double and triple *Arabidopsis* dicer mutants could help to elucidate what part of the smRNA biogenesis pathway is involved in producing transgenerational changes in genome stability and stress tolerance. Besides, extracting a smRNA fraction from stress treated plants and injecting it into control plants could be a fast and simple way to confirm the importance of stress-induced smRNAs in this process.

Furthermore, it is important to correlate elevated tolerance to stress observed in the progeny of stress exposed plants with changes in methylation and expression of genes involved in the development of salt tolerance. This could be achieved by direct bisulfite sequencing of loci of interest followed by the comparison of analyzed sequences between PofC and PofS plants. Alternatively, the ChIP-on-chip analysis can be applied here to perform the genome-wide methylation analysis.

Exploring transgenerational effects of other stresses and comparing them with the effect of salt stress described here could help to reveal major mechanisms triggering memory of stress and leading to stress adaptation in plants.

4. EFFECTS OF AMMONIUM NITRATE ON THE FREQUENCY OF *AGROBACTERIUM*-MEDIATED TRANSFORMATION IN *NICOTIANA TABACUM*

4.1. MATERIALS AND METHODS

4.1.1. Plant cultivation

In the current work, *Nicotiana tabacum* cultivar Big Havana wild type plants and transgenic *A. thaliana* line #11 plants were used. All plants were obtained from Friedrich Miescher Institute (Basel, Switzerland) and were previously described (Swoboda, *et al.*, 1994; Ilnytskyy, *et al.*, 2004).

4.1.1.1. Growing *Arabidopsis* plants for recombination analysis

Arabidopsis seeds were surface-sterilized, grown and harvested as previously described (see Section 3.1.1.1. Growing *Arabidopsis* plants for salt stress experiments). Surface-sterilized seeds were plated on control or modified solid MS medium containing various quantities of ammonium nitrate (Table 4.1.1).

4.1.1.2. Growing *N. tabacum* plants for transformation experiments

4.1.1.2.1. Growing *N. tabacum* plants for transformation with a 35S::GUS T-DNA construct

Seeds of wild type *N. tabacum* cultivar Big Havana were surface-sterilized with 1% sodium hypochloride, 0.05% Tween-80 solution for 3 min and then rinsed twice with sterile distilled water for 5 min each. Next, seeds were placed in 100 mm Petri dishes on sterile Whatman paper submersed in 4 ml of control or modified liquid MS medium containing various quantities of ammonium nitrate (Table 4.1.2). Following that, seeds were transferred to a growth chamber (Enconair, Winnipeg, MB, Canada) for germination. Once germinated, wild type plants were grown for one week at 22 °C /18

°C, 16/8 hours light/dark regime under high light conditions (32.8 $\mu Em^{-2}s^{-1}$) with light provided by Octron T8 Fluorescent bulbs (Sylvania, Mississauga, ON, Canada), and a constant humidity of 65%. One week old seedlings were harvested from Petri dishes for transformation with *Agrobacterium*.

4.1.1.2.2. Growing *N. tabacum* plants for transformation with a N::LUC T-DNA construct

Seeds of wild type *N. tabacum* cultivar Big Havana were surface-sterilized and grown as previously described (see Section 4.1.1.2.1. Growing *N. tabacum* plants for transformation with a 35S::GUS T-DNA construct). Surface-sterilized seeds were placed in 100 mm Petri dishes on sterile Whatman paper submersed in 4 ml of control or modified liquid MS medium containing various quantities of ammonium nitrate (Table 4.1.2).

Three to five one week old *N. tabacum* wild type plants were transferred to a single sterile 250 ml glass flask containing 15 ml of sterile control or modified liquid MS medium. Next, flasks were installed on an orbital shaker, and plants were continuously grown under the aforementioned growth conditions at 50 – 75 rpm. The growth medium was replaced weekly with 25 ml of fresh medium. Three weeks later, plants were removed from flasks, and 2-3 pairs of fully developed 2-4 cm long fresh leaves were harvested (cut from plants) for transformation with *Agrobacterium*.

Following transformation, regenerated transgenic plants were grown and propagated on soil into the next generation using the same growth conditions as described for *Arabidopsis* (see Section 3.1.1.1. Growing *Arabidopsis* plants for salt stress

experiments). Once transformants became well-developed on soil, plant leaf tissues were harvested and snap frozen for further analysis.

Table 4.1.1. Various modifications of chemical composition of standard solid MS medium for studying the effect of ammonium nitrate on recombination in *Arabidopsis*

MS macro components	Final concentrations in MS medium, mM	NH_4NO_3 gradient, all final concentrations listed in mM			
	Control	NH_4NO_3 0.3x	NH_4NO_3 1x	NH_4NO_3 2.5x	NH_4NO_3 5x
NH_4NO_3	20.6	6.18	20.6	51.5	103
KNO_3	18.8	-	-	-	-
$CaCl_2$	3	3	3	3	3
$MgSO_4$	1.5	1.5	1.5	1.5	1.5
KH_2PO_4	1.25	1.25	1.25	1.25	1.25
K_2SO_4	-	9.4	9.4	9.4	9.4

Table 4.1.2. Various modifications of chemical composition of standard liquid MS medium for studying the effect of ammonium nitrate on transformation efficiency in *N. tabacum*

MS macro components	Final concentrations in MS medium, mM	Experimental media compositions, all final concentrations listed in mM				
		NH_4NO_3 0.3x	NH_4NO_3 1x	NH_4NO_3 2x	NH_4NO_3 3x	NH_4NO_3 5x
NH_4NO_3	20.6	6.18	20.6	41.2	61.8	103
KNO_3	18.8	-	-	-	-	-
$CaCl_2$	3	3	3	3	3	3
$MgSO_4$	1.5	1.5	1.5	1.5	1.5	1.5
KH_2PO_4	1.25	1.25	1.25	1.25	1.25	1.25
K_2SO_4	-	9.4	9.4	9.4	9.4	9.4

4.1.2. Composition of experimental MS medium for testing effects of ammonium nitrate on transformation efficiency

The effect of various chemicals on HRF was tested using *Arabidopsis* line #11 plants that carried in the genome a GUS-based recombination substrate (Swoboda, *et al.*, 1994; Ilnytskyy, *et al.*, 2004). This experimental system allowed the identification of ammonium nitrate as the best candidate for *N. tabacum* transformation.

4.1.2.1. Growing *Arabidopsis* plants for recombination analysis

Pilot trials were performed with *Arabidopsis* line #11 plants germinated and grown on control or modified solid MS medium containing various amounts of ammonium nitrate (Table 4.1.1). To get ammonium nitrate as a single source of nitrogen in the medium, potassium nitrate originally present in MS medium was substituted with potassium sulfate (Table 4.1.1). The effect of potassium sulfate on HRF was shown to be negligible (Boyko and Kovalchuk, 2008b). Standard solid MS medium (Murashige and Skoog, 1962) was used as a control. Following 3 weeks, plants were harvested for histochemical staining, and HRF was calculated.

4.1.2.2. Growing *N. tabacum* plants for transformation experiments with a N::LUC T-DNA construct

For transformation experiments, wild type *N. tabacum* plants were germinated and grown in control or modified liquid MS medium supplemented with various quantities of ammonium nitrate (Table 4.1.2). To get ammonium nitrate as a single source of nitrogen in the medium, potassium nitrate originally present in MS medium was substituted with

potassium sulfate (Table 4.1.2). Modified MS medium containing 20.6 mM of ammonium nitrate (the amount naturally present in standard MS medium) (Murashige and Skoog, 1962) was used as a control (Table 4.1.2). Once grown (on the cotyledons or fully developed leaf stages), plants were used for transformation with *Agrobacterium*.

4.1.3. Detection and analysis of homologous recombination events in *Arabidopsis*

Detection and analysis of HR was performed as previously described (see Section 3.1.2. Detection and analysis of homologous recombination events in *Arabidopsis*).

4.1.4. Visualization of the *luciferase* reporter gene activity in *N. tabacum*

Constitutive expression of the *luciferase* gene in stable transformants regenerated from tobacco leaves that were transformed with a N::LUC T-DNA construct was visualized using a CCD camera (Gloor Instruments; Basel, Switzerland). When transplanted to soil, regenerated plants were topically treated with a 0.5 mM beetle luciferine (Promega), a 0.05% Tween-80 solution and incubated in the dark for 30-45 min. Following incubation, plants were photographed using a CCD camera. Plants containing the *luciferase* gene were able to cleave the luciferine. This resulted in the ATP-dependent production of light.

4.1.5. *Agrobacterium*-mediated gene transfer to *Nicotiana tabacum*

4.1.5.1. *Agrobacterium* strains used in experiments

Two *Agrobacterium* GV3101 strains used for transformation were obtained from Friedrich Miescher Institute (Basel, Switzerland). One strain carried T-DNA containing the active GUS gene driven by the 35S CaMV promoter and the *barnase* gene as a herbicide resistance marker. Another strain carried a T-DNA cassette containing the active *luciferase* (LUC) gene driven by the *N-gene* promoter and the *hph* gene that confers resistance to antibiotic hygromycin as a selection marker. The 35S driven GUS containing T-DNA construct was used to analyze efficiency of transient and stable transformation. The *N-gene* promoter driven LUC containing a T-DNA construct was used for stable transformation analysis only. All antibiotics and plant hormones used in the experiments were ordered from Sigma.

4.1.5.2. Bacteria culture

Two *Agrobacterium* GV3101 strains were streaked on plates containing a solid YEP medium (An *et al.*, 1988) supplemented with antibiotics. The following antibiotics were used for the GUS strain: spectinomycin (50 mg/ml), rifampicin (25 mg/ml) and gentamicin (25 mg/ml). For the LUC strain we used kanamycin (50 mg/ml), rifampicin (25 mg/ml) and gentamicin (25 mg/ml), respectively. The plates were incubated at 28 °C overnight, and then, a single colony was used to start a small 3 ml liquid culture (YEP supplemented with aforementioned antibiotics). The bacterial culture was incubated overnight at 28 °C and 190-200 rpm, and then it was used to inoculate the main 150 ml culture that was grown overnight under the same conditions. The next day, bacteria were

harvested (5000 rpm, 5 min) and resuspended in ½ stringency liquid standard MS medium (Murashige and Skoog, 1962) to a final optical density of 0.6 measured at 600 nm. The resulting bacterial suspension was supplemented with 100 mM acetosyringone (Sigma) solution at a final concentration of 100 µM and then incubated for at least 30 min to stimulate bacteria. Following incubation with acetosyringone, bacteria were used for transformation.

4.1.5.3. Transformation of *N. tabacum* plants with a 35S::GUS T-DNA construct

Traces of a growth medium were removed via blotting of one-week old cotyledons on sterile filter paper. Next, cotyledons were submersed into a resuspended *Agrobacterium* culture and vacuum-infiltrated for 10 min under sterile conditions. Once infiltrated, they were blotted dry and placed upside-down in solid standard MS medium (Murashige and Skoog, 1962); there they were incubated for 3 days. Incubation was done in the dark at 22 °C. Next, cotyledons were removed from plates, well rinsed with sterile distilled water, and blotted dry. At this stage, they were either harvested for histochemical staining for the evaluation of transient transformation efficiency, or transferred to solid MS medium (Murashige and Skoog, 1962) containing IAA (0.8 mg/L) kinetin (2 mg/L) for calli induction and regeneration and a combination of ticarcillin (100 mg/L) with potassium clavulanate (3 mg/L) to control *Agrobacterium* growth. Active selection conditions were achieved by supplementing the regeneration medium with phosphinothricin (PPT) (5 mg/L). The efficiency of transient transformation was evaluated based on GUS expression, visualized using a previously described histochemical staining procedure (see Section <u>3.1.2.2. Visualization of homologous</u>

recombination events using the GUS gene activity). Stable integration events were PCR-confirmed using the following primers for the GUS gene, forward primer: 5'-CAGACTCAGACTAAGCAGGTG-3' and reverse primer: 5'-GATCAATTCCACAGTTTTCGCG-3'.

4.1.5.4. Transformation of *N. tabacum* plants with a N::LUC T-DNA construct

To remove traces of a growth medium, leaves were blotted on sterile filter paper and then submersed into a Petri dish laid out with Whatman paper and containing resuspended *Agrobacterium*. Once upside-down and completely submersed, the leaf surface was incised using a sharp surgical blade. Incisions were made in parallel to side veins. The distance between two parallel incisions was 5 – 7 mm. The main vein and leaf margins were left intact. When cutting was completed, leaves were allowed to be submersed for 10 min, then they were blotted dry and placed upside-down in solid standard MS medium (Murashige and Skoog, 1962). After 3 days of incubation in the dark at 22 °C, leaves were removed from plates, well rinsed with sterile distilled water, blotted dry, and transferred to solid standard MS medium (Murashige and Skoog, 1962) containing IAA (0.8 mg/L), kinetin (2 mg/L) for calli induction and regeneration, and the combination of ticarcillin (100 mg/L) with potassium clavulanate (3 mg/L) to control *Agrobacterium* growth. Selection conditions were obtained by supplementing a regeneration medium with hygromycin (25 mg/L). Non-selective conditions were used for studying effects of ammonium nitrate on calli regeneration efficiency.

Following 3 – 4 weeks on callus inducing medium, developed shoots were excised from calli and transferred to the root inducing solid standard MS medium

(Murashige and Skoog, 1962) containing NAA (0.5 mg/L), ticarcillin (100 mg/L) and potassium clavulanate (3 mg/L). After 1 – 2 weeks of root induction, plantlets were transplanted to soil and checked for *luciferase* gene expression using a CCD camera as previously described (see Section 4.1.4. Visualization of the *luciferase* reporter gene activity in *N. tabacum*). *Luciferase* gene expressing plants were counted as stable transformants, and their number was related to the total number of incisions made in leaf during transformation to obtain a stable transformation frequency (STF). Shoots produced on callus inducing medium were scored, and their number was related to the total number of incisions made in leaf during transformation to obtain a callus regeneration efficiency (CRE).

4.1.5.5. T-DNA segregation analysis

Seeds of self-pollinated transgenic *N. tabacum* plants regenerated after *Agrobacterium* transformation with a N::LUC T-DNA containing construct were germinated and grown for 3 weeks in solid standard MS medium (Murashige and Skoog, 1962) containing hygromycin (25 mg/L). Each plate contained wild type and hygromycin positive plants for a negative and positive control, respectively. Following 3 weeks, plants showing an antibiotic resistance phenotype were scored, and a segregation ratio was calculated. Statistical significance of calculated segregation ratios was confirmed using *Chi square* statistic with $\alpha=0.05$.

4.1.6. Description of molecular techniques and assays used for analysis

4.1.6.1. Total DNA preparation

Total genomic DNA was prepared and analyzed as previously described (see Section 3.1.5.1. Total DNA, RNA and cDNA preparations).

4.1.6.2. DSB measurement (the ROPS assay)

Quantification of 3'OH DNA breaks was performed as previously described (see Section 3.1.5.3. DSB measurement (the ROPS assay).

4.1.6.3. Cloning DNA sequences flanking T-DNA insertions in transgenic *N. tabacum* plants

Cloning DNA sequences flanking T-DNA insertions was done by using an improved PCR-Walking technique (Siebert *et al.*, 1995; Cottage *et al.*, 2001). The technique is based on ligation of specially designed asymmetric adapters to the ends of DNA fragments generated by digestion of genomic DNA with blunt-end yielding endonucleases. Following adaptor ligation, DNA was used as a template for PCR using an adaptor and gene specific primers allowing the amplification of unknown genomic regions flanking a T-DNA insertion site. The presence of an amine group on the exposed 3'end of the adaptor prevents non-specific amplification between adaptor-specific primers. Following the first PCR, the second PCR round was performed using a set of nested primers; diluted product of the first PCR was used as a template. The resulting PCR products could be cloned and sequenced.

Total genomic DNA (2.5 µg) extracted from *N. tabacum* plants that carry T-DNA

insertions in unknown locations was digested with 80 units of restriction endonuclease overnight at 37 °C. The *Dra*I (New England Biolabs) enzyme was used for restriction digestion of DNA samples for cloning left T-DNA border flanking sequences. Following digestion, samples were extracted with an equal volume of chloroform-isoamyl alcohol (24:1 v/v), ethanol precipitated and resuspended in 20 µl of sterile distilled water. Ten µl of the genomic DNA digest was ligated to 1 µl of annealed adaptors using 10 units of *T4* DNA ligase (New England Biolabs) and 1x *T4* DNA ligase buffer (New England Biolabs) in a final volume of 20 µl. Adaptor sequences, adaptor annealing conditions and the sequence of adaptor-specific primers were previously described (Siebert *et al.*, 1995; Cottage *et al.*, 2001). Following heat inactivation of ligase at 65 °C for 10 min, a 180 µl volume of TE buffer was added to the ligation mix completing an adaptor library.

Primary PCR amplification was performed using 1 µl of adaptor library as a template, an adaptor- and T-DNA-specific primer for 35 cycles. The second PCR was performed using 1 µl of diluted (1:100) primary PCR as a template, an adaptor- and T-DNA-nested primer for 20 cycles. Both primary and secondary PCR were done using 0.63 units of *Takara Ex Taq™ DNA Polymerase* (Takara Bio USA), *1x Ex Taq™ Buffer* (contains 2 mM $MgCl_2$) (Takara Bio USA), *dNTP Mixture* (2.5 mM each dNTP) (Takara Bio USA) and 10 pmol of each primer in a final volume of 25 µl. Cycle parameters were as follows: 2 min of initial denaturation followed by a cycle event of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, elongation at 68 °C for 4 min, and a final extension for 10 min. The sequence of the T-DNA specific-nested primer pair was as follows: T-DNA left border specific primer 5’-TACGAGGTCGCCAACATCTTCTTCTG-3’ and T-DNA left border nested primer 5’-

GCGTATATGCTCCGCATTGGTCTTGA-3'. PCR products were cloned into a *pGEM-T easy* vector (*pGEM-T easy vector system I*, Promega) and sequenced. Resulting sequences were compared to the original T-DNA sequence for detection of possible alterations in a transgene sequence. The cloned genomic DNA sequences were used for searching possible matches in the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and the Tobacco Genome Initiative (TGI) (http://tgi.ncsu.edu/) genome databases, thereby allowing the identification of T-DNA integration sites in the *N. tabacum* genome.

4.1.7. Statistical treatment of data

The statistical analysis of the data was performed as previously described (see Section 3.1.6. Statistical treatment of data).

4.2. RESULTS

4.2.1. Selection of growth medium components influencing homologous recombination frequency

In our study, we attempted to manipulate the host before transformation to increase its susceptibility to *Agrobacterium* infection. This would result in the higher number of stable transformants being produced during a single round of transformation. The main emphasis was on the use of various modified growth medium compositions that would not affect host physiology negatively but could result in an increased activity of HR at the time of transformation. Our pilot experiments indicated that changing a final concentration of several macrosalts present in the standard MS medium originally (Murashige and Skoog, 1962) could drastically affect the HRF in plants (Boyko A, MSc Thesis, 2004). Based on these preliminary data, we selected ammonium nitrate as the best candidate for growth medium modification experiments.

4.2.1.1. High concentrations of ammonium nitrate increase recombination rates in *Arabidopsis*

Transgenic *Arabidopsis* line #11 plants represent a sensitive and reliable system that allows the measurement of the effects of various factors on HRF (Ilnytskyy, *et al.*, 2004). We used this transgenic line for the evaluation of ammonium nitrate effects on HRF. We germinated and grew *Arabidopsis* plants on solid control or modified MS medium in the presence of various quantities of ammonium nitrate. To have ammonium nitrate as a single source of nitrogen in all modified media, we substituted potassium

nitrate for potassium sulfate (Table 4.1.1). The control medium composition was not changed.

Our results demonstrated that increasing the concentration of ammonium nitrate in the growth medium can significantly increase RR in *Arabidopsis*. The presence of 51.5 and 94 mM ammonium nitrate comprising 250 and 500% of its amount in standard MS medium (Murashige and Skoog, 1962) resulted in 2.2- and 4.1-fold increases of RR, as compared to plants grown on control unmodified MS medium (Student's t-test, $\alpha=0.05$) (Figure 4.2.1). Consistently, a depletion of ammonium nitrate in the growth medium to 30% of its concentration in the control medium decreased the RR by a factor of 1.3 (Figure 4.2.1). Importantly, only a minor difference of 9% was observed during a comparison of RRs between plants grown in modified medium containing 20.6 mM of ammonium nitrate and control plants (Figure 4.2.1) This suggests that a substitution of potassium nitrate for potassium sulfate in all modified media did not significantly affect the RR. Overall, a strong positive correlation between RR and the amount of ammonium nitrate present in a growth medium was found ($r=0.99, P<0.05$).

It is noteworthy that plants grown in the medium containing 2.5x ammonium nitrate look healthier, grew better and showed a higher chlorophyll content (Figure 4.2.2). In contrast, plants grown in the medium containing 5x ammonium nitrate displayed growth inhibition and showed a lower chlorophyll content (Figure 4.2.2). This indicated a negative effect of 103 mM ammonium nitrate on plant physiology, and suggested that active concentrations of this chemical should be within a range of 41.2 and 61.8 mM. These concentrations were 2.0- and 3.0-fold higher as compared with the concentration of ammonium nitrate in control MS medium.

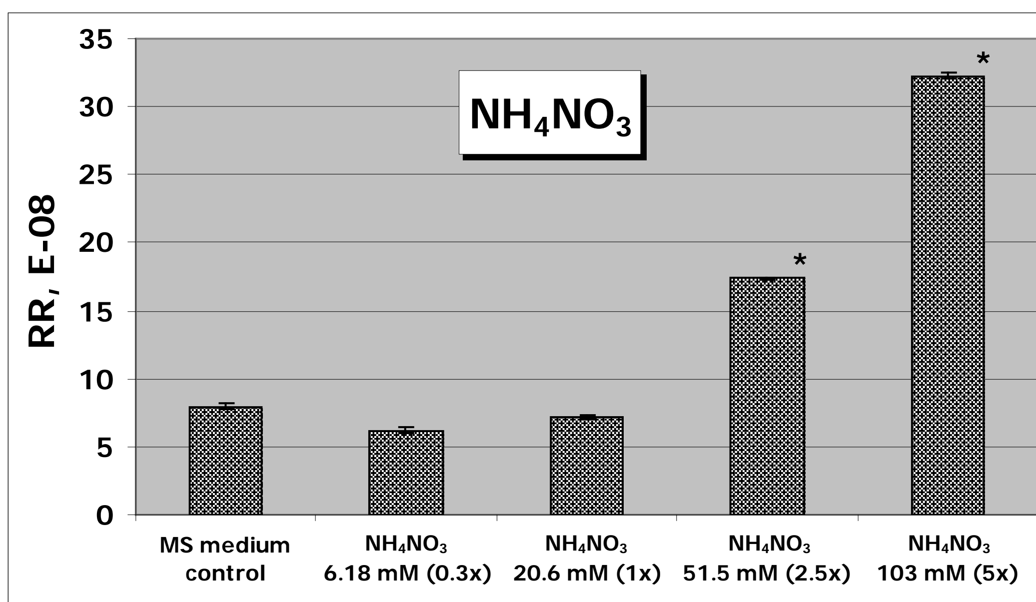

Figure 4.2.1. Recombination rates in *Arabidopsis* plants grown on solid MS medium supplemented with various amounts of ammonium nitrate

RRs were measured in plants germinated and grown for 3 weeks on MS medium (control) and modified medium containing 6.18 (0.3x), 20.6 (1x), 51.5 (2.5x) and 103 (5x) mM ammonium nitrate. 'x' – stands for a concentration of ammonium nitrate in standard MS medium. Two independent experiments were performed. Values represent the mean ± s.d. Asterisks show a statistically significant difference as compared with control MS medium. Student's t-test: $\alpha=0.05$, $t=2.57$. ANOVA: $P<0.05$.

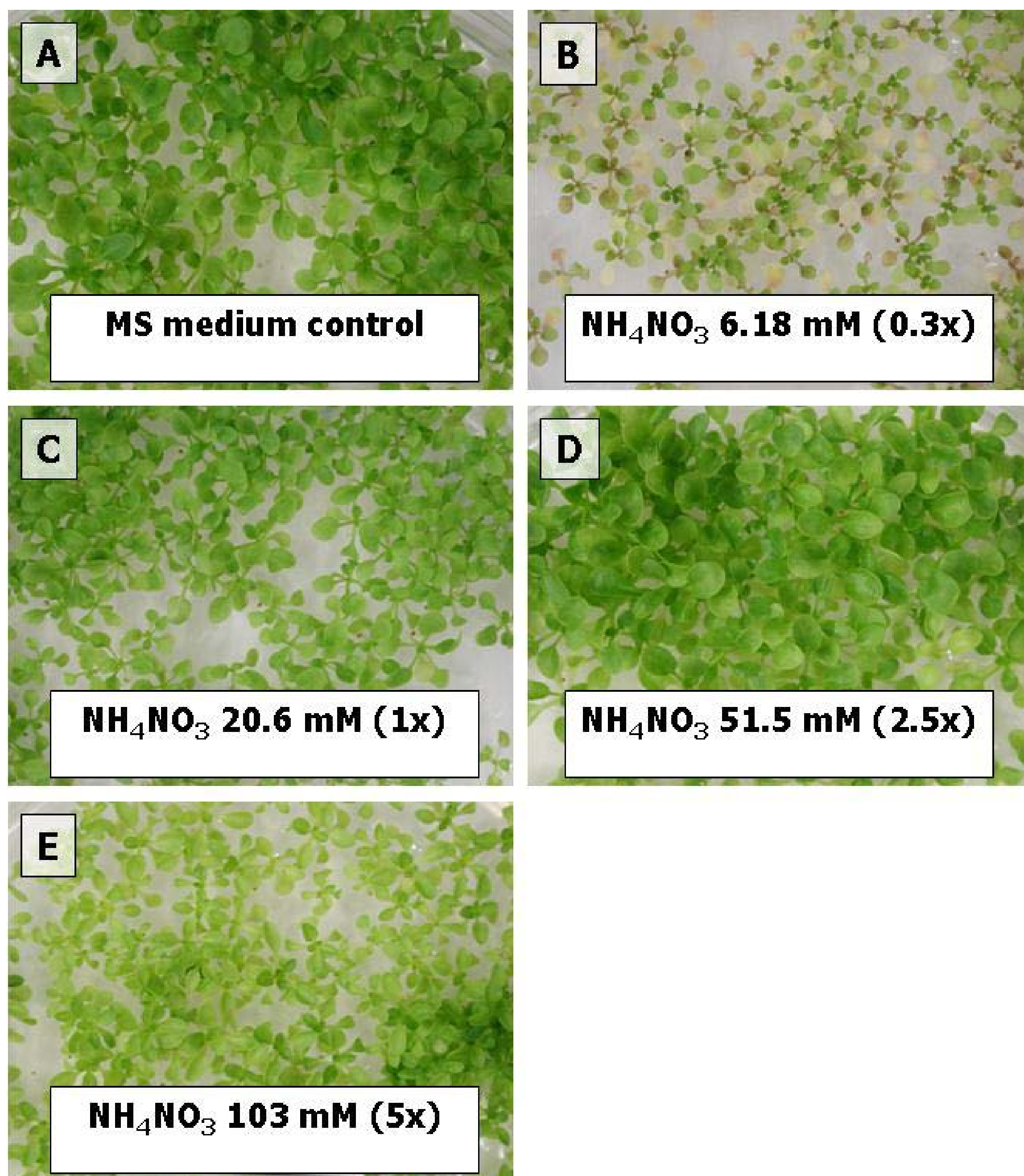

Figure 4.2.2. Phenotypic appearance of *Arabidopsis* plants grown on solid MS medium supplemented with various amounts of ammonium nitrate

A, **B**, **C**, **D** and **E** – *Arabidopsis* plants germinated and grown for 3 weeks on MS medium (control) and modified medium containing 6.18 (0.3x), 20.6 (1x), 51.5 (2.5x) and 103 (5x) mM of ammonium nitrate. 'x' – stands for a concentration of ammonium nitrate in standard MS medium.

4.2.1.2. High concentrations of ammonium nitrate do not increase DNA double strand breaks

A dose-dependent increase of RR observed in plants grown in the presence of high amounts of ammonium nitrate can be due to a higher level of DNA DSBs. It also can reflect a higher involvement of HR enzymes in strand break repair. To investigate a possible genotoxic effect of growth media enriched in ammonium nitrate, we measured DSB levels in plants grown on modified solid MS medium containing 20.6, 41.2 and 61.8 mM ammonium nitrate. Importantly, we found no significant differences in strand break levels in plants grown in the presence of 20.6 and 41.2 mM (Figure 4.2.3). Moreover, a significant (Student's t-test, $\alpha=0.05$) 1.2-fold decrease in the number of DSBs was found, while comparing plants grown in 61.8 mM with those grown in 20.6 mM (Figure 4.2.3). Overall, our data supported a positive effect of ammonium nitrate on the HR activity, and confirmed that the presence of this chemical in plant growth medium has no DNA damaging effect.

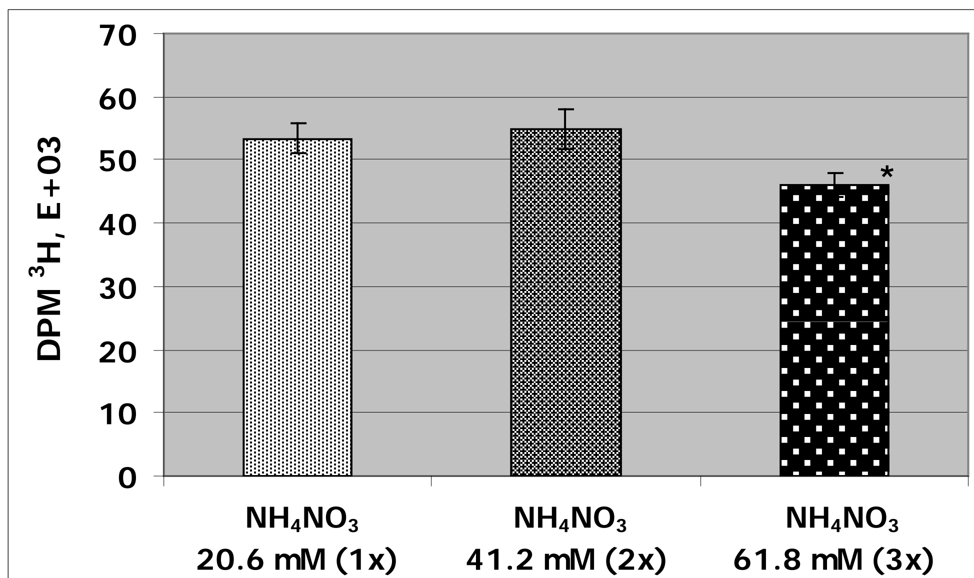

Figure 4.2.3. Levels of DNA double strand breaks in *N. tabacum* plants grown on solid MS medium supplemented with various amounts of ammonium nitrate

The figure shows DSB levels (radioactive counts, DPM ^{3}H) in plants germinated and grown for 4 weeks in the presence of 20.6 (1x), 41.2 (2x) and 61.8 (3x) mM ammonium nitrate. The higher the radioactive count, the more DSBs are present in the genome. 'x' – stands for a concentration of ammonium nitrate in standard MS medium. Two independent experiments were performed, and radioactivity of each sample was counted twice. Values represent the mean ± s.d. Asterisks show a statistically significant difference compared with control 20.6 mM (1x) medium. Student's t-test, $\alpha=0.05$, $t=2.05$.

4.2.2. Effects of ammonium nitrate on *Agrobacterium*-mediated transient transformation of *N. tabacum*

Our previous experiments demonstrated that enrichment of a plant growth medium with ammonium nitrate to a level of 200 or 300% of its original concentration has a positive effect on HR and does not exert a negative influence on plant physiology in general. Next, we investigated whether an increase in RR can improve the efficiency of plant genetic transformation with *Agrobacterium*. We evaluated the efficiency of transient plant transformation using plants grown in media enriched with ammonium nitrate. Overall, we found a significant difference in GUS transgene expression levels in plants derived from various growth media. The highest GUS expression was observed in plants grown in the medium containing 61.8 mM ammonium nitrate (Figure 4.2.4). Consistently, the second highest GUS expression was found in plants grown in the presence of 41.2 mM ammonium nitrate (Figure 4.2.4). Finally, plants grown in the presence of 20.6 mM ammonium nitrate displayed the lowest transgene expression, indicating that enrichment of a growth medium with ammonium nitrate could improve the transformation efficiency (Figure 4.2.4).

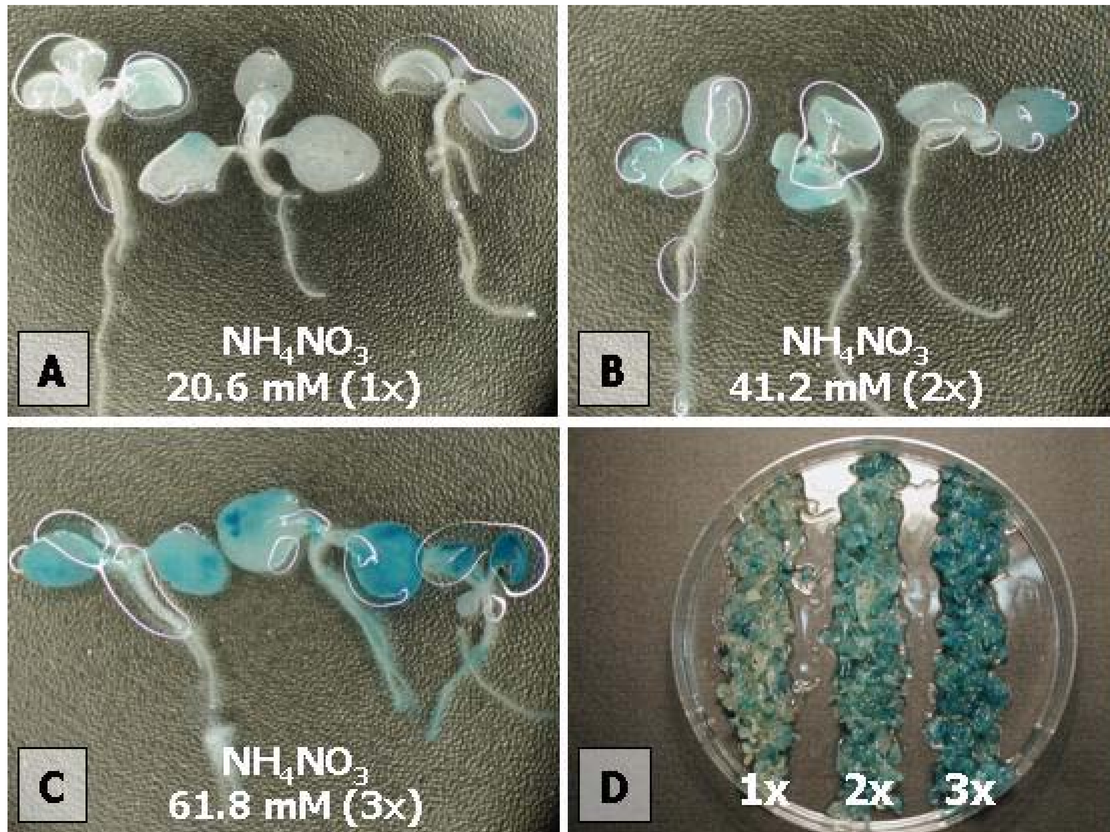

Figure 4.2.4. The efficiency of transient transformation of *N. tabacum* plants grown in liquid MS medium supplemented with various quantities of ammonium nitrate

A, **B** and **C** – GUS expression in transiently transformed tobacco seedlings germinated and grown in the medium supplemented with 20.6 (1x), 41.2 (2x) and 61.8 (3x) mM ammonium nitrate. 'x' – stands for a concentration of ammonium nitrate in standard MS medium. *N. tabacum* plants were transformed with *Agrobacterium* T-DNA containing the GUS reporter gene under control of the 35S CaMV promoter. Transient transformation efficiency was evaluated on the 3rd day after a transformation event. Three independent experiments were performed. **D** – Head-to-head comparison of GUS expression in transformed plants grown in the presence of different quantities of ammonium nitrate before transformation.

4.2.3. Effects of ammonium nitrate on stable *Agrobacterium*-mediated transformation of *N. tabacum*

Since a high content of ammonium nitrate resulted in an increase in transient plant transformation, we hypothesized that it could also increase stable transformation. To elucidate the role of ammonium nitrate for the improvement of plant transformation efficiency, we used media containing 6.18, 20.6 (control), 41.2, 61.8 and 103 mM ammonium nitrate.

We found that enrichment of growth media with ammonium nitrate before transformation with *Agrobacterium* significantly increased CRE (Figure 4.2.5A). Transforming plants grown in 41.2 and 61.8 mM ammonium nitrate resulted in CRE that was 1.9- and 2.7-fold higher than that of plants grown in the presence of 20.6 mM ammonium nitrate (Student's t-test, $\alpha=0.05$) (Figure 4.2.5A, B, C). In contrast to its positive effect on RR, the presence of 103 mM ammonium nitrate in the growth medium inhibited CRE which constituted 46% of CRE in plant tissues obtained from control medium (Student's t-test, $\alpha=0.05$) (Figure 4.2.5A). Inhibition of CRE was consistent with a previously observed negative effect that very high concentrations of this chemical had on plant physiology. Finally, a depletion of ammonium nitrate in the growth medium had an insignificant effect on CRE, suggesting that the decreased HR activity does not affect overall transformation efficiency (Figure 4.2.5A). Importantly, increasing the amount of ammonium nitrate from 41.2 to 61.8 mM resulted in a significant 1.5-fold increase of CRE (Student's t-test, $\alpha=0.05$). This was consistent with a previously observed dose-dependent increase of RR (Figure 4.2.5A, B, C). Overall, a strong positive correlation

between CRE and a concentration of ammonium nitrate in the medium was observed ($r=0.96, P<0.05$).

Consistent with its positive effect on CRE, a high content of ammonium nitrate also stimulated STF (Figure 4.2.6A, B, C). The presence of 41.2 and 61.8 mM ammonium nitrate in media led to a significant 1.7- (Student's t-test, $\alpha=0.1$) and 2.5-fold (Student's t-test, $\alpha=0.05$) increase in the number of transgenic plants obtained in a single round of transformation, as compared to control media containing 20.6 mM ammonium nitrate (Figure 4.2.6A). Similarly to its effect on CRE, exposure to 103 mM ammonium nitrate drastically reduced STF: it comprised 38% of STF observed in plant tissues obtained from control medium (Student's t-test, $\alpha=0.05$) (Figure 4.2.6A). A decrease in the concentration of ammonium nitrate to 6.18 mM had no significant effect on STF (Figure 4.2.6A). Importantly, a comparison of STF between tissues obtained from plants grown in the presence of 41.2 and 61.8 mM ammonium nitrate yielded a significant 1.5-fold difference (Student's t-test, $\alpha=0.05$) (Figure 4.2.6A). Overall, a strong positive correlation between STF and ammonium nitrate concentration in the medium was observed ($r=0.97, P<0.05$).

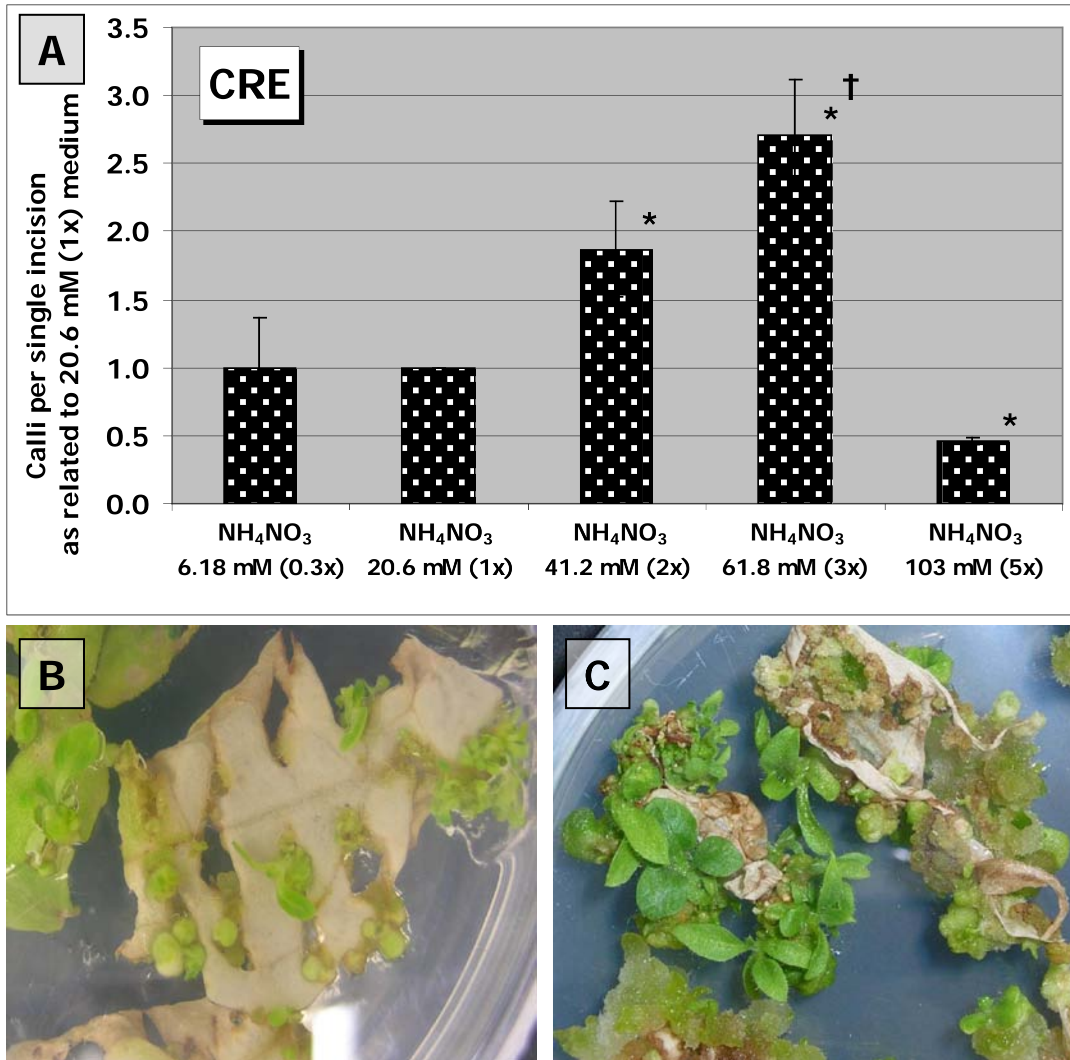

Figure 4.2.5. Callus regeneration efficiency in *N. tabacum* plants grown in liquid MS medium supplemented with various amounts of ammonium nitrate

A – Leaf tissues for transformation with *Agrobacterium* were harvested from *N. tabacum* plants grown in ammonium nitrate-modified liquid MS. 'x' – stands for a concentration of ammonium nitrate in standard MS medium. Calli were regenerated under selective conditions (hygromycin, 25 mg/L). Callus regeneration efficiency (CRE) represents the total number of calli (transgenic and non-transgenic) regenerated per single incision made in a leaf during transformation. CRE of plants grown in the presence of 20.6 mM (1x) ammonium nitrate was standardized to 1.0. CRE of plants grown in other types of growth medium shows fold changes in CRE as related to the medium containing 20.6 mM (1x) ammonium nitrate. Five independent experiments were performed using two T-DNA constructs carrying different reporter and selection marker genes (GUS and *barnase* genes, *luciferase* and *hph* genes, respectively). Values represent the mean ± s.d. Asterisks show a statistically significant difference as compared to control 20.6 mM (1x) medium. Student's t-test, $\alpha=0.05$, $t=2.26$. † - shows a statistically significant difference compared with 41.2 mM (2x) ammonium nitrate containing medium. Student's t-test, $\alpha=0.05$, $t=2.26$. ANOVA: $P<0.01$.
B and **C** – Representative pictures showing a difference in callus regeneration efficiency in host tissues grown in the presence of 20.6 (1x) and 61.8 mM (3x) ammonium nitrate.

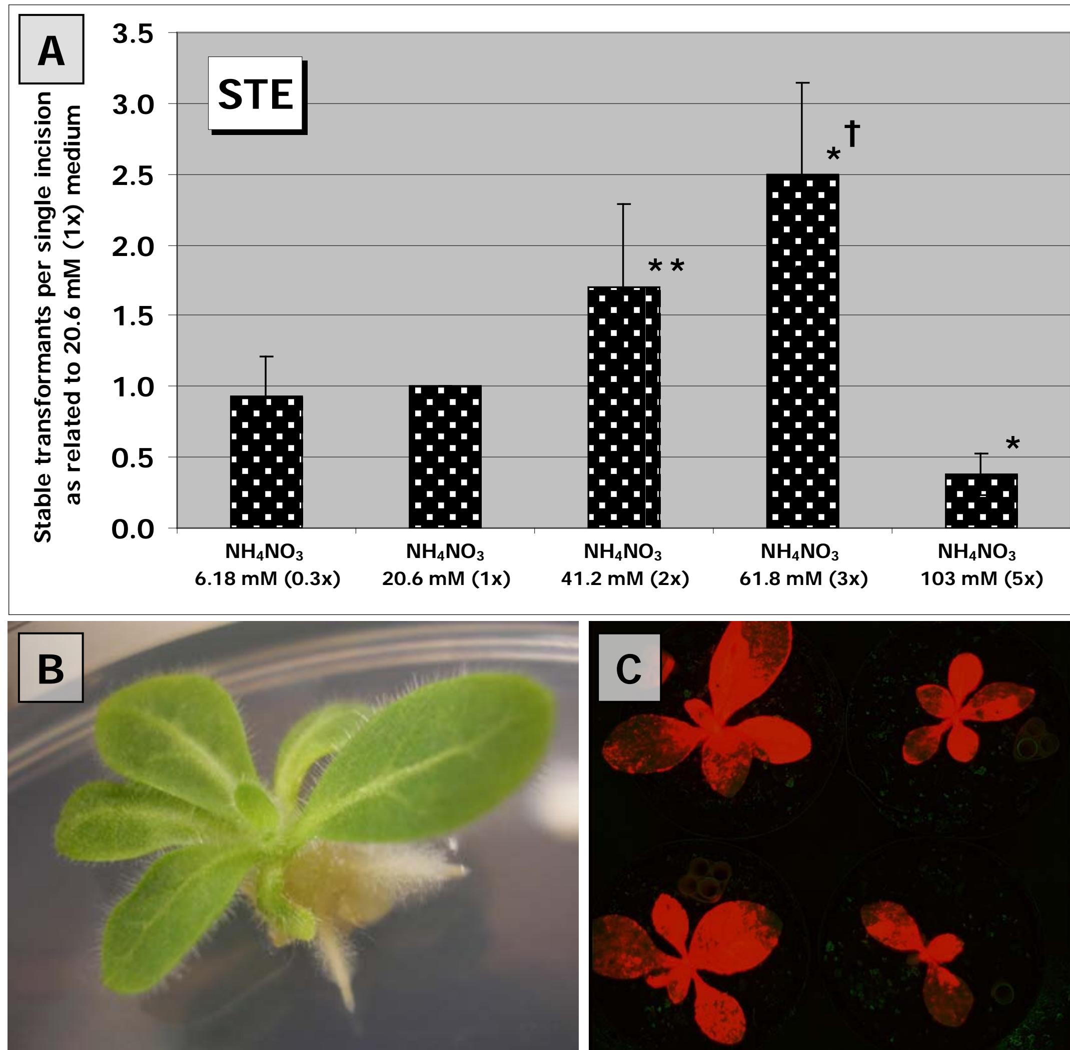

Figure 4.2.6. Stable transformation frequency in *N. tabacum* plants grown in liquid MS medium supplemented with various concentrations of ammonium nitrate

A – Leaf tissues for transformation with *Agrobacterium* were harvested from *N. tabacum* plants grown in modified liquid MS medium in the presence of various concentrations of ammonium nitrate. 'x' – stands for a concentration of ammonium nitrate in standard MS medium. Stable transformation frequency (STF) represents the total number of transgenic plants (expressing GUS or *luciferase* gene) regenerated per single incision made in a leaf during transformation. STF in plants grown in the presence of 20.6 mM (1x) ammonium nitrate was standardized to 1.0. STF in plants grown in other types of growth medium shows fold changes as related to the medium containing 20.6 mM (1x) ammonium nitrate. Five independent experiments were performed using two T-DNA constructs carrying different reporter and selection marker genes (GUS and *barnase* genes, *luciferase* and *hph* genes, respectively). Values represent the mean ± s.d. Asterisks show a statistically significant difference compared with control 20.6 mM (1x) medium. One asterisk – Student's t-test, $\alpha=0.05$: for the medium containing 61.8 mM (3x) and 103 mM (5x) ammonium nitrate, $t=2.26$. Two asterisks – Student's t-test, $\alpha=0.1$: for the medium containing 41.2 mM (2x) ammonium nitrate, $t=1.83$. † - shows a statistically significant difference compared with the medium containing 41.2 mM (2x) ammonium nitrate. Student's t-test, $\alpha=0.1$, $t=1.83$. ANOVA: $P<0.01$. **B** – Regenerated plants grown in the root inducing medium before being transplanted to soil, and before conducting a reporter gene expression test. **C** – a *luciferase* gene expression test in regenerated plants transplanted to soil (red color indicates transgene expression).

4.2.4. Comparing the effects of ammonium nitrate on callus regeneration and frequency of stable T-DNA integration events in *N. tabacum*

A positive effect of ammonium nitrate on the total number of stable transformants could be either due to an enhanced callus regeneration capacity or a higher frequency of transgene integrations. Previous experiments did not allow us to distinguish between these two effects, since active selection conditions were used. To differentiate between the effects of ammonium nitrate on the callus regeneration capacity and transgene integration frequency, we regenerated transformed plants under non-selective conditions. This allowed for equal survival of both transgenic and non-transgenic calli.

The absence of selection pressure greatly increased the total number of regenerated plants. However, no drastic difference was observed in the number of calli regenerated from transformed leaves harvested from plants grown in the presence of various concentrations of ammonium nitrate (Table 4.2.1). Growth media containing 41.2 and 61.8 mM ammonium nitrate yielded CRE that was 9 and 18% of that yielded from the control medium containing 20.6 mM (Table 4.2.1). Lowering the ammonium nitrate concentration to 6.18 mM decreased CRE by 11% only (Table 4.2.1). Overall, the data suggested a negligible influence of ammonium nitrate on a callus regeneration capacity of plant material used for transformation.

In contrast to a negligible effect on CRE, high concentrations of ammonium nitrate greatly increased the frequency of transgene integration events, as reflected by the increased STF (Table 4.2.1). The presence of 41.2 and 61.8 mM ammonium nitrate resulted in STF that was 212 and 316% of that obtained from plants grown in 20.6 mM

(Table 4.2.1). Consistently with previous experiments, the depletion of ammonium nitrate to 6.18 mM led to a minor 27% reduction of STF (Table 4.2.1). These findings are also consistent with the data on the influence of high concentrations of ammonium nitrate on HRF.

Table 4.2.1. Callus regeneration efficiency (CRE) and stable transformation frequency (STF) in *N. tabacum* plants grown in liquid MS medium supplemented with various amounts of ammonium nitrate, transformed with a LUC-containing T-DNA construct and regenerated under non-selective conditions

Type of growth medium	Number of incisions made	The total number of calli regenerated	Number of transgenic (LUC "+") calli regenerated	CRE	CRE, fold	STF	STF, fold
NH_4NO_3 6.18 mM (0.3x)	205	483	11	2.36	0.89	0.05	0.73
NH_4NO_3 20.6 mM (1x)	190	503	14	2.65	1.00	0.07	1.00
NH_4NO_3 41.2 mM (2x)	173	498	27	2.88	1.09	0.16	2.12
NH_4NO_3 61.8 mM (3x)	193	602	45	3.12	1.18	0.23	3.16

Leaf tissues for transformation with *Agrobacterium* were harvested from *N. tabacum* plants grown in modified liquid MS medium supplemented with various amounts of ammonium nitrate. 'x' – stands for a concentration of ammonium nitrate in standard MS medium. Transformed tobacco leaves were regenerated under non-selective conditions, equally allowing for surviving of transgenic and non-transgenic calli. Callus regeneration efficiency (CRE) represents the total number of calli regenerated (transgenic and non-transgenic) per single incision made in leaf disks during transformation. Stable transformation frequency (STF) represents the total number of transgenic *luciferase* gene expressing calli regenerated per single incision made in leaf disks. Fold was calculated by relating the data from plants grown in the medium containing 6.18 (0.3x), 41.2 (2x) and 61.8 (3x) mM ammonium nitrate to the data from the medium containing 20.6 mM (1x) ammonium nitrate.

4.2.5. Segregation analysis

Higher transformation efficiency could be associated with an increase in the number of simultaneous integration events targeted to various genomic loci. In general, single copy and single locus T-DNA integration events can be considered as a perfect transformation outcome. To compare the number of genomic loci targeted by integration events in plants grown in the presence of a standard and increased amount of ammonium nitrate, we performed segregation analysis of the T1 progeny of self-pollinated T0 plants (Figure 4.2.7).

Segregation analysis showed that most integration events are single locus integration events (Figure 4.2.7A). A comparison between 1x and higher concentrations of ammonium nitrate did not show any changes in the percentage of plants with single locus T-DNA integration events (Figure 4.2.7A). This suggests that increased transformation efficiency was achieved without compromising the quality of transgenic plants produced.

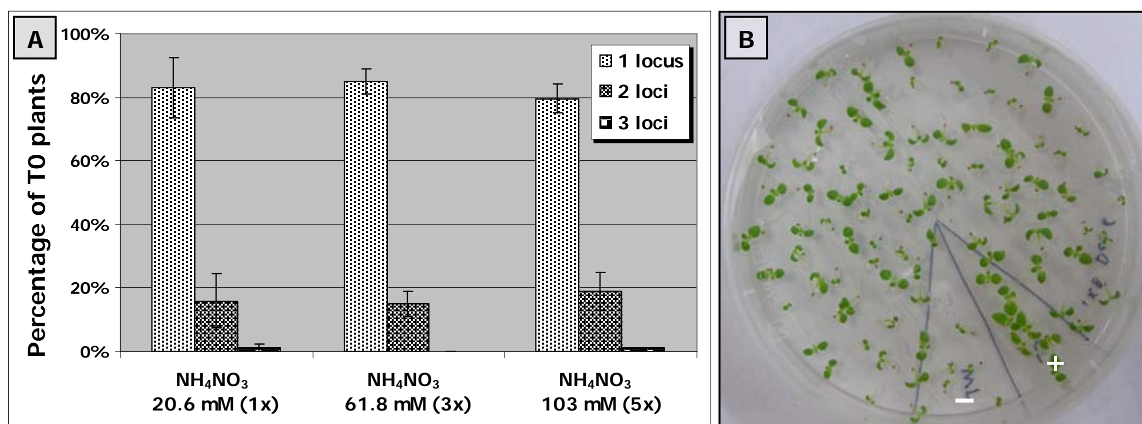

Figure 4.2.7. The number of genomic loci containing integrated T-DNA, as determined using segregation analysis of the T1 progeny of self-pollinated tobacco plants

A – The number of genomic loci containing integrated T-DNA, as determined by segregation analysis of the T1 progeny of transformed plants grown in medium containing 20.6 (1x), 61.8 (2x) and 103 (3x) mM ammonium nitrate before transformation. 'x' – stands for a concentration of ammonium nitrate in standard MS medium. The total number of integration events (transgenic plants) obtained from each treatment was taken to be 100%. Segregation analysis was performed on solid MS medium supplemented with hygromycin (25 mg/L). Each plate contained wild type and hygromycin positive plants for a negative and positive control, respectively. A segregation ratio was calculated based on antibiotic resistant phenotypes at 3 weeks post germination. Three independent experiments were performed. Values represent the mean ± s.e. The statistical significance of segregation ratios calculated was confirmed using the *Chi square* statistic for $\alpha=0.05$. **B** – Antibiotic resistant and sensitive phenotypes of T1 plants grown in MS medium containing hygromycin (25 mg/L); "+" and "-" – stand for positive (hygromycin resistant) and negative (wild type) selection controls, respectively.

4.2.6. Sequence analysis of T-DNA/plant DNA junctions

Transgene integration in the plant genome is frequently associated with modifications in transgene and genomic DNA. This could affect transgene expression and change the overall performance of genetically modified plants. Since a majority of T-DNA sequence truncations occur within the left T-DNA border (Kim *et al.*, 2007), we analysed intactness of left T-DNA border/plant DNA junctions. Considering that during T-DNA processing from a Ti plasmid, it is being nicked by the VirD2 protein between nucleotides 3 and 4 (Kim *et al.*, 2007), intact integration events should contain the T-DNA left border sequence starting from nucleotide 4. A majority of the left border T-DNA/plant DNA junctions analysed had T-DNA truncations, varying in size from 1 to 295 nucleotides (Table 4.2.2). The analysis of T-DNA integration events in transformants derived from plants grown in the medium containing 20.6 mM ammonium nitrate showed that 40% of junctions analyzed have 0 – 10 nt truncations, 20% have 11 – 100 nt truncations, and the remaining 40% have 200 – 300 nt truncations. Similarly, transformants obtained from plants grown in the medium containing 61.8 mM ammonium nitrate showed that 50% of junctions have 0 – 10 nt truncations, and 50% have 200 – 300 nt truncations. Overall, the average truncation size was comparable between these two media, and consisted of 143 and 125 nt for media containing 61.8 and 20.6 mM ammonium nitrate, respectively. Finally, using plant DNA sequences flanking the T-DNA left border, we were able to identify transgene integration sites. All integration events analysed were targeted to coding DNA sequences. In summary, high concentrations of ammonium nitrate resulted in higher STF and a quality of transgene integration that could be compared to that of plants from control medium.

Table 4.2.2. Summary of sequence analysis of T-DNA/plant DNA junctions

Growth medium	Transgenic line and T1 segregation ratio	T-DNA left border deletion size, nt	Genomic DNA sequence flanking T-DNA left border	
			Tobacco Genome Initiative (TGI) database	NCBI genome database
NH_4NO_3 20.6 mM (1x)	1x2 3 : 1 ($\chi2$ =0.041)	7	**CHO_SL007xj04f1.ab1** Identities = 20/26 (76%)	**gb|AY570725.1|** Nicotiana tabacum trehalose-phosphate phosphatase mRNA Identities = 447/448 (99%), Gaps = 1/448 (0%)
			CHO_SL014xc18f1.ab1 Identities = 16/20 (80%)	**gb|AY619947.1|** Nicotiana tabacum ADH-like UDP-glucose dehydrogenase mRNA Identities = 654/656 (99%), Gaps = 1/656 (0%)
	1x7 3 : 1 ($\chi2$ =0.237)	273	**CHO_SL005xo17f1.ab1** Identities = 24/27 (88%)	**dbj|D85912.1|** TOBTCAP Nicotiana tabacum mRNA for cytosolic ascorbate peroxidase Identities = 267/271 (98%), Gaps = 3/271 (1%)
	1x12 3 : 1 ($\chi2$ =0.070)	68	**CHO_SL025xo05f1.ab1** Identities = 12/12 (100%)	**gb|AF154640.1|** Nicotiana tabacum clone PR15 mRNA sequence Identities = 392/398 (98%)
				gb|U60490.1| NTU60490 Nicotiana tabacum actin (Tob71) pseudogene, partial sequence Identities = 233/236 (98%), Gaps = 0/236 (0%)
	1x15 3 : 1 ($\chi2$ =0.000)	274	**CHO_SL003xe01f1.ab1** Identities = 25/32 (78%)	**gb|DQ021457.1|** Nicotiana langsdorffii x Nicotiana sanderae clone 051E09 beta-amylase 1 (BAM1) mRNA Identities = 678/716 (94%), Gaps = /716 (0%)
			CHO_SL024xd22f1.ab1 Identities = 31/42 (73%)	**gb|AF401689.1|** Nicotiana tabacum genomic sequence and retrotransposon Tnt1 s11tr long terminal repeat Identities = 85/85 (100%), Gaps = 0/85 (0%)
	1x19 3 : 1 ($\chi2$ =0.067)	1	**CHO_SL021xd14f1.ab1** Identities = 19/25 (76%)	**gb|AF154641.1|** Nicotiana tabacum clone PR16 mRNA sequence Identities = 372/438 (84%), Gaps = 14/438 (3%)
				gb|AF440271.1| Nicotiana tabacum aquaporin (NtPIP1;1) mRNA Identities = 166/198 (83%), Gaps = 8/198 (4%)

Table 4.2.2. Summary of sequence analysis of T-DNA/plant DNA junctions (*continued*)

Growth medium	Transgenic line and T1 segregation ratio	T-DNA left border deletion size, nt	Genomic DNA sequence flanking T-DNA left border	
			Tobacco Genome Initiative (TGI) database	NCBI genome database
NH_4NO_3 61.8 mM (3x)	3x20 3 : 1 ($\chi 2$ =0.007)	295	**CHO_SL011xb21f2.ab1** Identities = 119/195 (61%)	**gb|U89604.1|** NTU89604 Nicotiana tabacum SAR8.2m mRNA Identities = 470/484 (97%), Gaps = 5/484 (1%)
				dbj|AB040408.1| Nicotiana tabacum EIG-B39 mRNA for elicitor inducible protein Identities = 458/468 (97%), Gaps = 1/468 (0%)
		4	**CHO_SL026xo23f1.ab1** Identities = 72/120 (60%)	**gb|AY962601.1|** Nicotiana tabacum nucleoside diphosphate kinase mRNA Identities = 307/327 (93%), Gaps = 6/327 (1%)
		274	**CHO_SL004xp03f1.ab1** Identities = 33/46 (71%)	**gb|AF159699.1|** AF159699 Nicotiana tabacum putative host factor mRNA Identities = 440/472 (93%), Gaps = 7/472 (1%)
	3x90 3 : 1 ($\chi 2$ =0.224)	0	**CHO_SL024xf07f1.ab1** Identities = 30/39 (76%)	**emb|X66942.1|** NTPRB1B N.tabacum prb-1b gene Identities = 37/37 (100%), Gaps = 0/37 (0%)

Sequences of *N. tabacum* genomic DNA flanking the left border of T-DNA insertion and the T-DNA part adjacent to its left border were cloned and sequenced. The cloned sequence of the T-DNA left border was compared with the original T-DNA sequence, allowing for detection of small deletions and truncations within the original left border site. The cloned tobacco genomic DNA sequence flanking the T-DNA left border was used to localize T-DNA insertion sites using Tobacco Genome Initiative (TGI) and National Center for Biotechnology Information (NCBI) databases. Cloned tobacco DNA sequences were used to find matches in the TGI database; matching sequences from the TGI database were used to identify integration sites using the NCBI genome database.

4.3. DISCUSSION

In this study, we analyzed effects of high concentrations of ammonium nitrate in a growth medium on the HR activity and evaluated the influence of this chemical on stable transformation efficiency with *Agrobacterium*. We have found that a) the presence of ammonium nitrate results in a dose-dependent increase in RR; b) elevated concentrations of ammonium nitrate have a positive effect on plant growth and phenotypic appearance; c) exposure to high concentrations of ammonium nitrate does not increase DSB levels in plants; d) exposure of plants to high concentrations of ammonium nitrate increases the frequency of transient transformation with *Agrobacterium*; e) plants grown on media enriched with ammonium nitrate before transformation exhibit significantly increased CRE and STF; f) a positive effect of ammonium nitrate on plant transformation is mainly mediated via an increased frequency of transgene integrations in the host genome; g) plants regenerated from ammonium nitrate-rich media exhibit normal segregation and transgene integration patterns.

4.3.1. Ammonium nitrate increases recombination rate and decreases the level of DNA double strand breaks

One of the ways to improve transformation efficiency is to make the host more susceptible to T-DNA integrations. Since T-DNA integration in the plant genome is highly dependent on the activity of host DNA repair factors (Citovsky *et al.*, 2007), we hypothesized that growing plants under conditions inducing the HR activity before transformation could improve transformation efficiency. The main emphasis was made

on the selection of growth media compositions and factors that could significantly increase HRF without affecting host physiology negatively.

Nitrogen is one of the major macronutrients necessary for plant growth and development. Plants can uptake nitrogen from media predominantly in the form of NH_4^+ and NO_3^- ions. The uptake of these two ions is under control of systemic signals related to the total nitrogen status in a whole plant (Ruffel *et al.*, 2008). However, the presence of NO_3^- ions usually results in higher levels of nitrogen intake and consequently a higher total nitrogen content in plants, compared to the presence of NH_4^+ ions (Ruffel *et al.*, 2008). Our preliminary experiments demonstrated that the depletion of ammonium nitrate in a growth medium has the most pronounced effect on HRF.

To study the effect of high ammonium nitrate concentrations in plant growth media on the HR activity, we supplemented MS medium with various amounts of this chemical. Our data revealed a strong positive correlation between the amount of ammonium nitrate present in the medium and RR in plants. Furthermore, consistently with the important role ammonium nitrate plays in plant metabolism, development and nutrition intake (Crawford, 1995; Stitt, 1999; Miller *et al.*, 2007), increased concentrations of ammonium nitrate improved plant growth. It is also possible that a positive effect on plant physiology was mainly mediated by NO_3^- ions that are known to stimulate lateral root growth, root initiation and elongation (Forde, 2002a, 2002b). Similarly, NO_3^- ions not only induce factors involved in NO_3^- assimilation but also positively regulate enzymes of the pentose phosphate pathway and carboxylic acid metabolism (Ruffel *et al.*, 2008).

Effects of inorganic nitrogen sources on the DNA repair activity still remain unclear in contrast to their well-documented influence on plant physiology. It is noteworthy that recent studies demonstrated a link between the activity of human Rad51 and ammonium-sulphate concentrations at a molecular level (Sigurdsson *et al.*, 2001; Liu *et al.*, 2004b; Shim *et al.*, 2006). Ammonium-based salts were shown to induce conformational changes in hRad51 leading to an increase in its activity and therefore promoting recombination (Sigurdsson *et al.*, 2001; Liu *et al.*, 2004b; Shim *et al.*, 2006). In this study, we hypothesised that ions of both NH_4^+ and NO_3^- positively influence the HR activity and plant growth, thus leading to growth- and recombination-stimulating effects. Consistently with this idea, the transition from moderate to high concentrations of ammonium nitrate in media significantly inhibited plant growth, but it still resulted in an increase of HRF. A depletion of ammonium nitrate in growth media led to drastic changes in plant appearance, but in contrast, only a minor decline in HRF was observed.

A positive dose-dependent effect of increased ammonium nitrate concentrations on HRF could result from elevated DNA damage caused by high salt concentrations. To check this possibility, we measured DNA DSB levels in plants grown in media enriched with ammonium nitrate. No significant increase in the DSB level was found, suggesting that it was not DNA damage that increased HR. Intriguingly, a medium containing 61.8 mM ammonium nitrate yielded larger plants with a significantly lower level of DNA DSBs as compared to plants obtained from control medium. These combined data allow us to speculate that ammonium nitrate can directly stimulate the activity of plant factors involved in the HR DNA repair pathway.

Pre-treatment of plants with higher concentrations of ammonium nitrate before transformation represents a promising strategy for the improvement of transformation. The fact that we could identify an active concentration range that did not lead to increased DNA damage makes the application of ammonium nitrate a more favourable factor, compared to other factors that may increase DNA damage. Finally, using conditions promoting HRF could substantially improve genetic engineering and allow the development of GT technology in plants (Vergunst and Hooykaas, 1999; Puchta 2002; Reiss 2003; Hanin and Paszkowski, 2003; Lida and Terada, 2004).

4.3.2. Effects of ammonium nitrate on callus regeneration and frequency of transgene integration events during *Agrobacterium*-mediated plant transformation

During the past decade, *Agrobacterium*-mediated genetic transformation became one of the most favourable tools used for plant transgenesis. However, the improvement of efficiency and precision of transgene integration is still necessary. A high dependence of *Agrobacterium* on host replication, transcription and DNA repair factors during T-DNA integration suggests that manipulating the activity of DSB repair pathways could help solve these problems. A majority of DSBs and T-DNA integration events are processed via NHEJ which affects intactness of integrated transgene sequences (Gorbunova and Levy, 1999; Puchta, 2005). Increasing the typically low activity of HR could possibly allow a better quality of integration events as well as generation of site-specific insertions and GT (Vergunst and Hooykaas, 1999; Puchta 2002; Reiss 2003).

In our study, we tested whether ammonium nitrate enrichment of growth medium could increase the frequency of stable transformation events. The preliminary evaluation of ammonium nitrate effects on plant transformation showed an increase in transient transformation efficiency.

However, transient transformation does not reflect the actual number of integration events, since both integrated and non-integrated T-DNA sequences present in the host nucleus can be expressed (Janssen and Gardner, 1989). Thus, we conducted a series of experiments directed toward regeneration of plants containing stable integration events in the genome. Our studies demonstrated that growing plants on media enriched with ammonium nitrate before transformation can significantly improve the total number

of regenerated calli and plants containing stable integration events. Our findings were consistent with recent reports indicating a positive effect of increased HRF on plant transformation (Shaked *et al.*, 2005). Indeed, expression of the yeast *RAD54* gene involved in the HR repair pathway increased the GT frequency in plants by one to two orders of magnitude (Shaked *et al.*, 2005). Similarly, hypersusceptibility of *Arabidopsis fas1* and *fas2* mutants to *Agrobacterium* transformation was attributed to enhanced transcription of the *AtRad51* and *AtRad54* genes (Endo *et al.*, 2006). In addition, growing wheat calli on a medium containing niacinamide, a PARP inhibitor, for four days just before bombardment significantly increased the number of low-copy transgene integration events (De Block *et al.*, 1997). Since PARP facilitates the NHEJ repair pathway, an inhibitor promotes the HR pathway.

The increased number of transgenic plants obtained from modified media can be due to increased regeneration capacity of plant tissues derived from growth media enriched with ammonium nitrate. Alternatively, it is possible that ammonium nitrate promotes transgene integration. To compare the effect of ammonium nitrate on these two parameters separately, we regenerated plants under non-selective conditions. This experiment allowed us to conclude that ammonium nitrate basically enhanced the frequency of transgene integrations. Its effect on tissue regeneration was minor.

It is noteworthy that a number of reports suggest a positive effect of elevated concentrations of ammonium nitrate on SE. Similarly to our study, Choi *et al.* (1998) compared the effects of five different macrosalts present in standard MS medium (Murashige and Skoog, 1962) on the frequency of somatic embryo formation, and they found that an increased concentration of ammonium nitrate was the most effective for SE.

In fact, the concentration of ammonium nitrate (Choi *et al.*, 1998) that resulted in the best embryo response was similar to that which resulted in the highest STF in our experiment. Consistently, Menke-Milczarek and Zimny (2001) proposed a similar concentration of ammonium nitrate as an optimal one for enhancing wheat SE. Furthermore, He *et al.* (1989) compared effects of concentrations of each of five MS macrosalts on the induction and morphology of embryogenic callus from immature embryo of wheat and found that elevated levels of ammonium nitrate were very effective for callus induction. Similarly, doubling the concentration of all MS salts in the induction medium led to a significant enhancement of regeneration frequency in wheat scutella (Maës *et al.*, 1996). Importantly, using compounds containing ammonium (Rijven, 1958; Halperin, 1966; Walker and Sato; 1981; He *et al.*, 1989; Choi *et al.*, 1998; Menke-Milczarek and Zimny, 2001) and nitrate (Choi *et al.*, 1998; Kothari *et al.*, 2004; Menke-Milczarek and Zimny, 2001) as a source of inorganic nitrogen in the medium appeared to be highly effective for SE.

In our studies, we applied a different approach. We used ammonium nitrate only before transformation rather than for regeneration of embryos. A minor stimulating influence of ammonium nitrate on CRE was observed in the experiment in which calli were regenerated under non-selective conditions. This may be due to residual effects caused by the presence of an increased amount of ammonium nitrate in growth media used before transformation.

Considering an important role of nitrogen for plant growth and development, the following model explaining effects of ammonium nitrate on plant transformation with *Agrobacterium* can be proposed. The long-distance nitrogen signalling hypothesis of

Forde and Sakakibara suggests that nitrogen is transduced to cytokinin via the enhanced isopentenyltransferase (*ipt*) activity in the roots and is translocated up the shoot with the subsequent promotion of leaf/bud outgrowth (Cline *et al.*, 2006). Indeed, nitrogen-dependent accumulation of cytokinins and stimulating effects of nitrogen fertilization on plant growth are well-documented, and they are consistent with plant phenotypes observed in our study. Takei *et al.* (2001) demonstrated that after four hours of nitrogen resupply, the levels of cytokinins in maize leaves significantly increase and remain elevated for at least 24 hours. Stimulation of cell division by cytokinins increases the number of cells entering the S/G2 phase of the cell cycle, thus promoting higher expression of HR proteins such as Rad51 (Chen *et al.*, 1997). Moreover, it is still possible that elevated levels of ammonium nitrate can also directly stimulate the Rad51 activity in plants via an unknown mechanism in a similar manner to that previously described for human Rad51 (Sigurdsson *et al.*, 2001; Liu *et al.*, 2004b; Shim *et al.*, 2006).

Furthermore, cells undergoing active division are more likely to be targeted by a stable T-DNA integration event. Indeed, an absolute requirement for the S-phase for transfer and/or T-DNA integration in *Petunia hybrida* was previously demonstrated (Villemont *et al.*, 1997). Similarly, transformation of synchronized tobacco protoplasts during S–M phase resulted in increased recovery of selection-resistant colonies (Meyer *et al.*, 1985; Okada *et al.*, 1986). An important role of active cell division in plant transformation was also supported by higher transformation efficiency observed in maize cells expressing a modified version of the viral replication-associated protein (RepA) that stimulates cell division (Gordon-Kamm *et al.*, 2002). It can thus be hypothesized that

effects of ammonium nitrate on plant transformation are based on its combined influence on HR activity and cell division.

Despite the remaining ambiguity surrounding the nature of ammonium nitrate mediated effects on plant transformation, it is clear that an increase in concentration of this chemical in growth media can lead to a significant improvement of transformation efficiency. Furthermore, the quality of integration events in plants regenerated from media with high ammonium nitrate concentrations was comparable with that from control media. All cloned T-DNA insertions were found within gene coding regions, which is consistent with the analysis of T-DNA integration sites performed by Alonso *et al.* (2003). Since we used active selection conditions in our experiments, we can not exclude that plants with T-DNA insertions in loci with low transcriptional activity were lost during selection (Francis and Spiker, 2005; Kim *et al.*, 2007). To summarize, our study allowed us to develop the plant growth medium composition that enhances HRF and improves the frequency of transgene integration in the plant genome (Boyko and Kovalchuk, US Patent No. 11/466184).

4.4. SUMMARY

Our study demonstrated a great potential to manipulate growth media composition for improving plant transformation efficiency with *Agrobacterium*. Our approach was based on using a well-described transgenic plant system to evaluate the genotoxic influence of various chemical compounds present in the plant growth environment. This system made it possible to identify chemicals which can increase HRF.

The low activity of HR in plant cells represents one of the major obstacles in the improvement of plant transformation quality and development of efficient GT protocols. We hypothesized that activation of host DNA repair factors should increase the frequency of transgene integration.

Our data demonstrated that an increased amount of ammonium nitrate in growth medium can substantially increase the activity of HR. Ammonium nitrate represents one of the macrosalts originally present in the growth medium, and increasing its concentration within physiological limits can enhance plant growth and metabolism. The application of this chemical does not increase DSB levels in the plant genome. Moreover, induction of HRF by high concentrations of ammonium nitrate can significantly reduce levels of DNA damage and thus improve the quality of plant material used for transformation with *Agrobacterium*.

Transformation of plant tissues derived from plants grown in media enriched in ammonium nitrate yields the higher number of regenerated calli and increases a total yield of stable transformants obtained in a single transformation round. This improvement was primarily due to an increased frequency of stable transgene integrations in the host genome. However, a minor positive influence of this chemical on

callus regeneration was also observed. The quality of integration events was comparable between control and ‘ammonium nitrate enriched’ transformations.

Overall, the developed composition of plant growth medium enhances HRF and improves a total transformation yield without negatively affecting plant physiology. However, the mechanism of action of ammonium nitrate still remains to be specified.

4.5. FUTURE DIRECTIONS

Our study demonstrated that manipulating the amount of ammonium nitrate present in growth media can improve the efficiency of *Agrobacterium*-mediated genetic transformation. Considering that ammonium nitrate is one of five macrosalts present in standard growth medium, it is important to determine what other factors normally present in growth medium are critical for efficient plant transformation. This knowledge could help us develop an optimal growth medium for enhancing plant transformation, improve transformation of recalcitrant species, and thus reduce total costs required for plant transgenesis.

Each plant species, cultivar and even plant tissue, has its own unique set of requirements for various salts and their amounts. With that in mind, further studies should be directed to the development of individualized growth media promoting T-DNA integration and SE in various economically important crops. This strategy represents a better alternative to the use of a common growth medium composition.

Our transformation experiments generated a large number of integration events that require further characterization. The analysis of intactness of T-DNA/plant DNA junctions that we started in this study should be continued to get a final conclusion about the effects of the newly developed medium on the quality of transgene integration. Moreover, the stability of transgene expression in generated transgenic lines should be monitored for at least several generations to determine if silencing of T-DNA insertions can occur. Besides, some recent reports have generated controversy regarding either preferential or non-specific T-DNA integration into transcriptionally active loci of the

host genome. It might be of keen interest to determine and compare integration sites in the transformants obtained under selective and non-selective conditions.

Further studies should be directed to elucidating mechanisms that mediate ammonium nitrate's influence on HR. This could help us gain a better understanding of plant physiological aspects necessary for efficient genetic transformation, and thus indicate what other factors or conditions can be successfully applied for plant transgenesis.

It would be interesting to test whether the effects of ammonium nitrate on plant transformation are mediated by the influence of high concentrations of ammonium nitrate on both the HR activity and cell division. This would require: to measure cytokinin levels in leaves, to conduct the flow-cytometric analysis of nuclear DNA content for determining a prevalent phase of the cell cycle, and to determine the activity of major HR proteins that can be involved in transgene integration. Overall, the knowledge obtained will be beneficial for both theoretical and applied sciences, specifically for plant transgenesis.

5. PROSPECTS FOR APPLICATION OF POTASSIUM CHLORIDE AND RARE EARTH ELEMENTS TO IMPROVE PLANT TRANSFORMATION

5.1. MATERIALS AND METHODS

5.1.1. Plant cultivation

In the current work, *Nicotiana tabacum* cultivar Big Havana wild type plants, transgenic *Nicotiana tabacum* line #LU2, and transgenic *A. thaliana* line #11 plants were used. All plants were obtained from Friedrich Miescher Institute (Basel, Switzerland) and were previously described (Swoboda, *et al.*, 1994; Gorbunova *et al.*, 2000; Ilnytskyy, *et al.*, 2004).

5.1.1.1. Growing *Arabidopsis* plants for recombination analysis

Arabidopsis seeds were surface-sterilized, grown and harvested as previously described (see Section 3.1.1.1. Growing *Arabidopsis* plants for salt stress experiments). Surface-sterilized seeds were plated on control or modified solid MS medium containing various quantities of potassium chloride (Table 5.1.1).

5.1.1.2. Growing *N. tabacum* plants for recombination analysis

Seeds of transgenic *N. tabacum* line #LU2 plants were surface-sterilized and grown as previously described (see Section 4.1.1.2.1. Growing *N. tabacum* plants for transformation with a 35S::GUS T-DNA construct). Surface sterilized seeds were plated on control or modified solid MS medium containing various quantities of cerium (III) and/or lanthanum (III) chlorides, two rare earth elements chosen for the experiment.

5.1.1.3. Growing *N. tabacum* plants for transformation experiments with a N::LUC T-DNA construct

Seeds of wild type *N. tabacum* plants were surface sterilized, grown and harvested as previously described (see Section 4.1.1.2.2. Growing *N. tabacum* plants for transformation with a N::LUC T-DNA construct). Surface sterilized seeds were grown in control or modified liquid MS medium containing various quantities of potassium or cerium (III) chlorides (Table 5.1.2).

Table 5.1.1. Various modifications of chemical composition of standard solid MS medium for studying the effect of potassium chloride on recombination in *Arabidopsis*

MS macro components	Final concentrations in MS medium, mM	KCl gradient, all final concentrations listed in mM		
	Control	**KCl 1x**	**KCl 2.5**	**KCl 5x**
NH_4NO_3	20.6	41.2	41.2	41.2
KNO_3	18.8	-	-	-
$CaCl_2$	3	3	3	3
$MgSO_4$	1.5	1.5	1.5	1.5
KH_2PO_4	1.25	-	-	-
KCl	-	18.8	47	94
$NH_4H_2PO_4$	-	1.25	1.25	1.25

Table 5.1.2. Various modifications of chemical composition of standard liquid MS medium for studying the effect of potassium and cerium (III) chlorides on transformation efficiency in *N. tabacum* and comparing it with the effect of ammonium nitrate

MS macro components	**Final concentrations in MS medium, mM**	**Experimental media compositions, all final concentrations listed in mM**					
		NH_4NO_3 1x	**NH_4NO_3 2x**	**NH_4NO_3 3x**	**$CeCl_3$ 0.3**	**$CeCl_3$ 1.0**	**KCl 50**
NH_4NO_3	20.6	20.6	41.2	61.8	20.6	20.6	20.6
KNO_3	18.8	-	-	-	-	-	-
$CaCl_2$	3	3	3	3	3	3	3
$MgSO_4$	1.5	1.5	1.5	1.5	1.5	1.5	1.5
KH_2PO_4	1.25	1.25	1.25	1.25	1.25	1.25	1.25
K_2SO_4	-	9.4	9.4	9.4	9.4	9.4	9.4
KCl	-	-	-	-	-	-	50
$CeCl_3$	-	-	-	-	0.0003	0.001	-

5.1.2. Composition of experimental MS medium for testing effects of potassium chloride and rare earth elements on transformation efficiency

The effect of various chemicals on HRF was tested using transgenic *Arabidopsis* line #11 (Swoboda, *et al.*, 1994; Ilnytskyy, *et al.*, 2004) and *N. tabacum* line #LU2 (Gorbunova *et al.*, 2000; Ilnytskyy, *et al.*, 2004) plants that carried in their genome a GUS- and LUC-based recombination substrate, respectively. Based on our previous studies on salt stress in plants (Boyko and Kovalchuk, 2008b), we introduced potassium chloride as another possible candidate for transformation experiments. Although previous studies showed a dramatic increase in HRF in plants exposed to NaCl, we substituted Na^+ with K^+ to avoid well documented toxic effects of Na^+ (Hasewaga *et al.*, 2000; Zhu, 2000, 2002). The Cl^- ion was retained, as it was reported to be a major factor that increased HRF under salt stress (Boyko and Kovalchuk, 2008b). Additionally, we tested the effects of two rare earth elements, cerium (III) and lanthanum (III) chlorides, which were reported to have a positive influence on nitrogen metabolism, plant growth and differentiation (Wang, 1988; Dong *et al.*, 1992; Bai and Ma, 1993; Chen *et al.*, 1999; Fashui *et al.*, 2002; Weiping *et al.*, 2003). Using this experimental system, we have selected potassium and cerium (III) chlorides as potential candidates for *N. tabacum* transformation.

5.1.2.1. Composition of experimental MS medium for testing effects of potassium chloride on recombination

Pilot trials were performed with *Arabidopsis* line #11 plants germinated and grown on control or modified solid MS medium containing various amounts of potassium chloride (Table 5.1.1). To get potassium chloride as a single source of potassium in the medium, potassium nitrate and potassium dihydrogenphosphate that were originally present in standard MS medium were replaced by ammonium dihydrogenphosphate. To compensate a total loss of nitrate, the concentration of ammonium nitrate was increased proportionally (Table 5.1.1). Standard solid MS medium (Murashige and Skoog, 1962) was used as a control. Following 3 weeks, plants were harvested for histochemical staining, and HRF was calculated.

5.1.2.2. Composition of experimental MS medium for testing effects of rare earth elements on recombination

Pilot trials were performed with *N. tabacum* line #LU2 plants germinated and grown on standard solid MS medium (Murashige and Skoog, 1962) supplemented with 0.1, 0.3, 1.0, 3.0 and 5.0 μM of either cerium (III) or lanthanum (III) chlorides, or both salts simultaneously. Standard solid MS medium (Murashige and Skoog, 1962) was used as a control. Following 4 weeks since germination, HR events were detected and scored using a CCD camera.

5.1.2.3. Composition of experimental MS medium for testing effects of potassium and cerium (III) chlorides on transformation in *N. tabacum* plants

For transformation experiments, wild type *N. tabacum* plants were germinated and grown in control or modified liquid MS medium supplemented with various quantities of potassium or cerium (III) chlorides (Table 5.1.2). To compare the effect of these two salts with that of ammonium nitrate, plants germinated and grown in modified liquid MS medium supplemented with 20.6, 41.2 and 61.8 mM of ammonium nitrate were used (Table 5.1.2). To get ammonium nitrate as a single source of nitrogen in the medium, potassium nitrate that was originally present in standard MS medium was substituted for potassium sulfate (Table 5.1.2). Modified MS medium containing 20.6 mM of ammonium nitrate, an amount that is naturally present in standard MS medium (Murashige and Skoog, 1962) was used as a control (Table 5.1.2). Once grown (at a fully developed leaf stage), plants were used for transformation with *Agrobacterium*.

5.1.3. Detection and analysis of homologous recombination events

5.1.3.1. Detection of homologous recombination events in *Arabidopsis*

Detection and analysis of HR events in transgenic *A. thaliana* line #11 was performed as previously described (see Section 3.1.2.1. Detection of homologous recombination events).

5.1.3.2. Detection of homologous recombination events in *N. tabacum*

N. tabacum line #LU2 transgenic plants carried a *luciferase* based substrate for HR which consisted of two overlapping truncated non-functional copies of the *luciferase* gene cloned in direct orientation under the 35S CaMV promoter (Gorbunova *et al.*, 2000; Ilnytskyy, *et al.*, 2004) (Figure 5.1.1A). Repair of DSBs in a region of homology via HR results in a recombination event that restores the reporter gene, thereby activating the *luciferase* gene (Figure 5.1.1B).

5.1.3.3. Visualization of homologous recombination events

5.1.3.3.1. Visualization of the GUS gene activity

Recombination events that yielded an intact functional copy of the GUS gene were visualized using a previously described procedure (see Section 3.1.2.2. Visualization of homologous recombination events using the GUS gene activity)

5.1.3.3.2. Visualization of the LUC gene activity

Recombination events occurring in *N. tabacum* line #LU2 plants that yielded a functional copy of the *luciferase* gene were visualized using a CCD camera (Gloor

Instruments; Basel, Switzerland) as previously described (see Section 4.1.4. Visualization of the *luciferase* reporter gene activity in *N. tabacum*). Cells containing the recombined functional LUC gene were able to cleave luciferine resulting in the ATP-dependent production of light. Superimposing a light-exposed image (5 s exposure under white light) on two dark-exposed images (10 min exposure with no background light to distinguish *luciferase* expressing cells) using the *AnaliSIS* program (Soft Imaging Systems, Muntser, Germany) allowed the detection of recombination events (Figure 5.1.1B).

5.1.3.4. Calculating recombination rates and frequency of homologous recombination

HRF and RR were calculated for *Arabidopsis* line #11 and *Nicotiana tabacum* line #LU2 plants as previously described (see Section 3.1.2.3. Calculating recombination rate and frequency of homologous recombination).

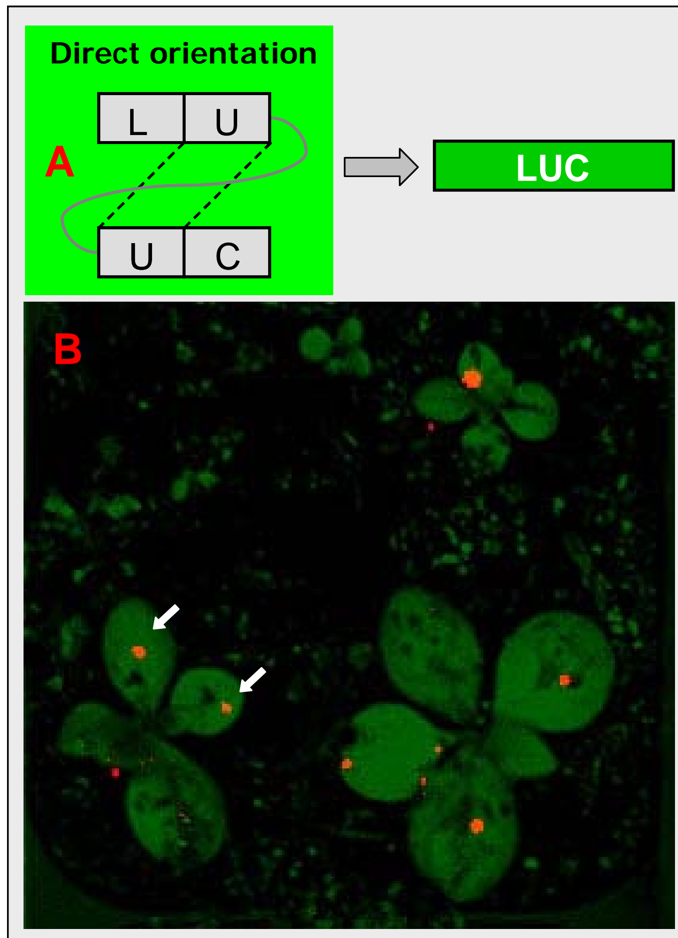

Figure 5.1.1. Detection of recombination events in *N. tabacum* line #LU2 plants

A – the structure of the LUC-based reporter construct cloned in direct orientation. The construct served as a substrate for somatic HR; **B** – detection of recombination events using a CCD camera (an arrow indicates recombination event in leaf tissues).

5.1.4. Visualization of the *luciferase* reporter gene activity in *N. tabacum*

Constitutive expression of the *luciferase* gene in stable transformants regenerated from tobacco leaves that were transformed by a N::LUC T-DNA construct was visualized as previously described (see Section 4.1.4. Visualization of the *luciferase* reporter gene activity in *N. tabacum*).

5.1.5. *Agrobacterium*-mediated gene transfer to *Nicotiana tabacum*

5.1.5.1. *Agrobacterium* strain used in experiments

The *Agrobacterium* GV3101 strain used for transformation was obtained from Friedrich Miescher Institute (Basel, Switzerland). The strain carried a T-DNA cassette containing the active *luciferase* gene driven by the *N-gene* promoter and the *hph* gene that confers resistance to antibiotic hygromycin as a selection marker. All antibiotics and plant hormones used in experiments were ordered from Sigma.

5.1.5.2. Bacteria culture

Agrobacterium was cultured and prepared for transformation as previously described (see Section 4.1.5.2. Bacteria culture), using the following antibiotics: kanamycin (50 mg/ml), rifampicin (25 mg/ml) and gentamicin (25 mg/ml).

5.1.5.3. Transformation of *N. tabacum* plant with a N::LUC T-DNA construct

Transformation of plant leaf tissues with *Agrobacterium* was carried out using a previously described procedure (see Section 4.1.5.4. Transformation of *N. tabacum* plant with a N::LUC T-DNA construct). Callus regeneration was done under active selection conditions using hygromycin (25 mg/L). The composition of callus and root inducing media, growth conditions and the calculation of CRE and STE were previously described (see Section 4.1.5.4. Transformation of *N. tabacum* plants with a N::LUC T-DNA construct).

5.1.6. Description of molecular techniques and assays used for analysis

5.1.6.1. Total DNA preparation

Total genomic DNA was prepared and analyzed as previously described (see Section 3.1.5.1. Total DNA, RNA and cDNA preparations).

5.1.7. Statistical treatment of data

Statistical analysis of the data was performed as previously described (see Section 3.1.6. Statistical treatment of data).

5.2. RESULTS

5.2.1. Selection of chemicals that can increase the frequency of homologous recombination while being present in growth medium

We evaluated the possibility of application of two distinct classes of chemical compounds, chlorides and rare earth elements, for the improvement of plant transformation. The first class was chosen based on our previous studies that demonstrated an increase of RR by chloride ions (Boyko and Kovalchuk, 2008b). The second class has a well-documented positive effect on plant growth and is known to enhance nitrogen metabolism (Wang, 1988; Dong *et al.*, 1992; Bai and Ma, 1993; Chen *et al.*, 1999; Fashui *et al.*, 2002; Weiping *et al.*, 2003). The first class includes potassium chloride, whereas the second class includes cerium (III) and lanthanum (III) chlorides.

5.2.1.1. High concentrations of potassium chloride increase recombination rates in *Arabidopsis*

First, we analyzed whether exposure to potassium chloride can increase HRF. Transgenic *Arabidopsis* line #11 plants were germinated and grown on solid MS media modified with various concentrations of potassium chloride. To get potassium chloride as a single source of potassium in all modified media, we omitted potassium nitrate and substituted potassium dihydrogenphosphate for ammonium dihydrogenphosphate (Table 5.1.1). Since our previous studies and published reports demonstrated that the total nitrate content in media is of great importance for plant transformation and SE (Choi *et al.*, 1998; Kothari *et al.*, 2004; Menke-Milczarek and Zimny, 2001), we adjusted the

concentration of ammonium nitrate to compensate for the total loss of nitrates in the media (Table 5.1.1). The composition of the control medium was not changed.

Our results demonstrated that the presence of potassium chloride in the growth medium significantly increased RR (Figure 5.2.1). The presence of 18.8 mM potassium chloride which corresponded to the amount of potassium present in standard MS medium resulted in a 9.3-fold increase in RR as compared to control MS medium (Student's t-test, $\alpha=0.05$) (Figure 5.2.1). Consistently, supplementation of the growth medium with 47 and 94 mM potassium chloride led to a 15.4- and 19.2-fold increase in RR, as compared to plants grown on MS medium (Student's t-test, $\alpha=0.05$) (Figure 5.2.1). Overall, a strong positive correlation between the amount of potassium chloride present in growth media and RR was observed ($r=0.93$, $P<0.05$).

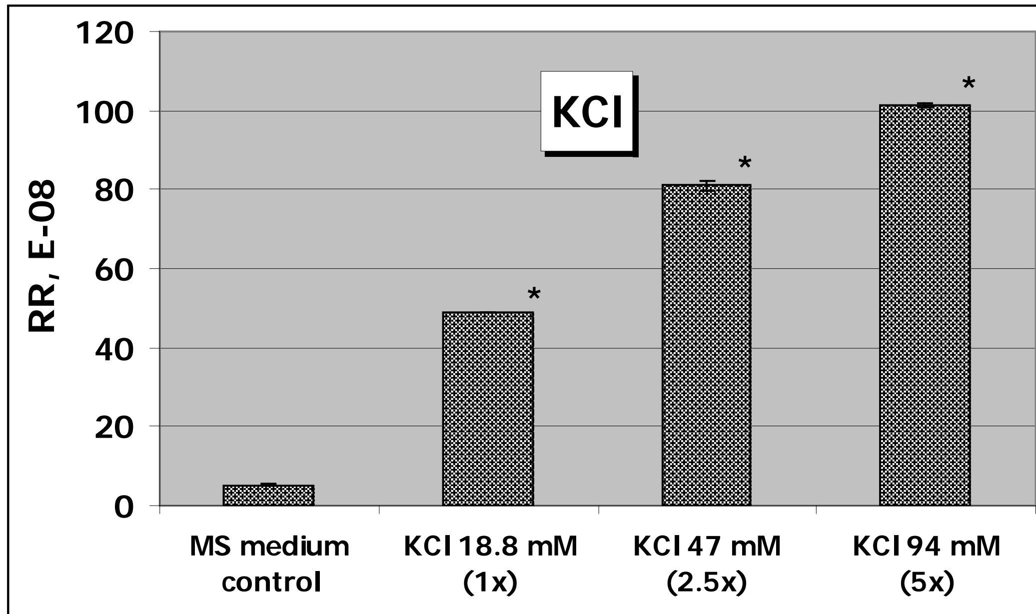

Figure 5.2.1. Recombination rates in *Arabidopsis* plants grown on solid MS medium supplemented with various amounts of potassium chloride

Recombination rates were measured in plants germinated and grown for 3 weeks on solid MS medium (control) or modified medium containing 18.8 (1x), 47 (2x) and 94 (5x) mM potassium chloride. 'x' – stands for the concentration of potassium in standard MS medium. Two independent experiments were performed. Values represent the mean ± s.d. Asterisks show a statistically significant difference as compared to control MS medium. Student's t-test, $\alpha=0.05$, $t=2.78$. ANOVA: $P<0.05$.

5.2.1.2. The presence of rare earth elements increases the frequency of homologous recombination in *N. tabacum*

Effects of rare earth elements on induction of HR was analyzed using transgenic *N. tabacum* line #LU2 plants (Ilnytskyy, *et al.*, 2004). Plants were germinated on solid MS medium supplemented with different concentrations of cerium (III) and lanthanum (III) chlorides, or a combination of both elements. Standard MS medium was used as a control.

Our results demonstrated that supplementation of growth medium with cerium (III) or lanthanum (III) chloride leads to a gradual increase of HRF (Figure 5.2.2). The highest HRF was observed in plants grown in the medium supplemented with 1.0 µM of either one of these two elements. The 2.8- and 2.7-fold differences for cerium and lanthanum, respectively, were statistically significant (Student's t-test, $\alpha=0.05$) (Figure 5.2.2). In contrast, concentrations of rare earth elements that were higher than 1.0 µM resulted in a decline of HRF (Figure 5.2.2).

Simultaneous delivery of these two elements to growth media yielded a similar response pattern. The highest HRF was found in plants grown in the presence of both 1.0 µM cerium (III) and 1.0 µM lanthanum (III) chlorides (Student's t-test, $\alpha=0.05$) (Figure 5.2.2). Interestingly, simultaneous delivery of 1.0 µM cerium and lanthanum resulted in HRF that was 1.4-fold higher, if compared to media containing 1.0 µM of either one of these two elements (Figure 5.2.2). These findings indicate that simultaneous application of various chemicals may result in the formation of an additive positive effect on HRF.

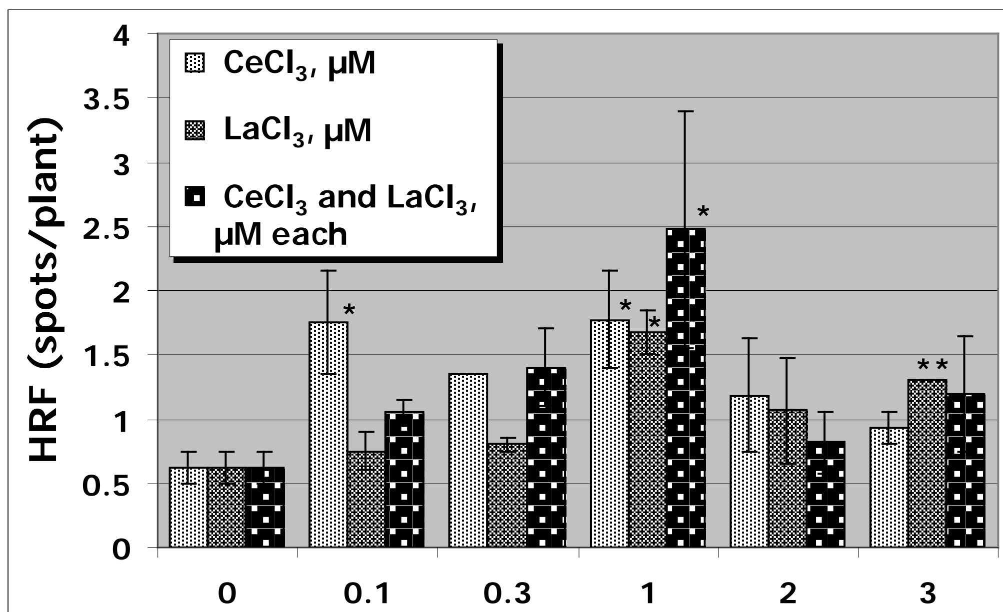

Figure 5.2.2. Homologous recombination frequency in *N. tabacum* plants grown on solid MS medium supplemented with various amounts of cerium (III) and lanthanum (III) chlorides

HRF was measured in plants germinated and grown for 4 weeks on solid MS medium (control) or MS medium supplemented with 0.1, 0.3, 1.0, 2.0 and 3.0 µM either cerium (III) or lanthanum (III) chlorides, or in the presence of both elements. Three independent experiments were performed. Values represent the mean ± s.d. Asterisks show a statistically significant difference as compared to control MS medium. One asterisk shows the Student's t-test at $\alpha=0.05$ of $t=2.45$ for 0.1 µM cerium (III) chloride and for 1 µM cerium (III), lanthanum (III) chlorides and combination of both. Two asterisks show the Student's t-test at $\alpha=0.1$ of $t=1.94$ for 3 µM lanthanum (III) chloride.

5.2.2. Preliminary assessment of the influence of potassium and cerium (III) chlorides on callus regeneration and the frequency of stable T-DNA integrations in *N. tabacum*

To provide a preliminary assessment of effects of potassium chloride and rare earth elements on stable plant transformation, we performed a pilot experiment on genetic transformation of plants grown in media supplemented with potassium or cerium (III) chloride. To compare their effects on CRE and STF with those previously described for ammonium nitrate, we also included growth media modified in their ammonium nitrate content (Table 5.1.2). The concentration of ammonium nitrate of 20.6 mM was used as a control.

Consistently with previous experiments, growing plants in 41.2 and 61.8 mM ammonium nitrate increased the total number of regenerated calli by 3.9- and 7.5-fold, respectively (Figure 5.2.3). Moreover, the presence of 50 mM potassium chloride contributed to an increase in callus regeneration by factor of 5.9; and the presence of 0.1 and 1.0 µM cerium (III) improved callus regeneration by 2.1- and 3.2-fold, respectively (Figure 5.2.3).

The positive influence of these chemicals on plant transformation was further confirmed by the analysis of stable transformants. In brief, the difference between control media and media containing 50 mM potassium or 1.0 µM cerium (III) chloride remained unchanged and amounted to 5.9- and 3.1-fold, respectively (Figure 5.2.4). The presence of 41.2 and 61.8 mM ammonium nitrate in media increased the total number of stable transformants by 3.8- and 6.7-fold, respectively (Figure 5.2.4). The difference between media containing 0.1 and 1.0 µM cerium (III) chloride remained unaffected at 1.6-fold

(Figure 5.2.4). Overall, our data support the idea that the application of potassium chloride and rare earth elements in growth media improves plant transformation efficiency.

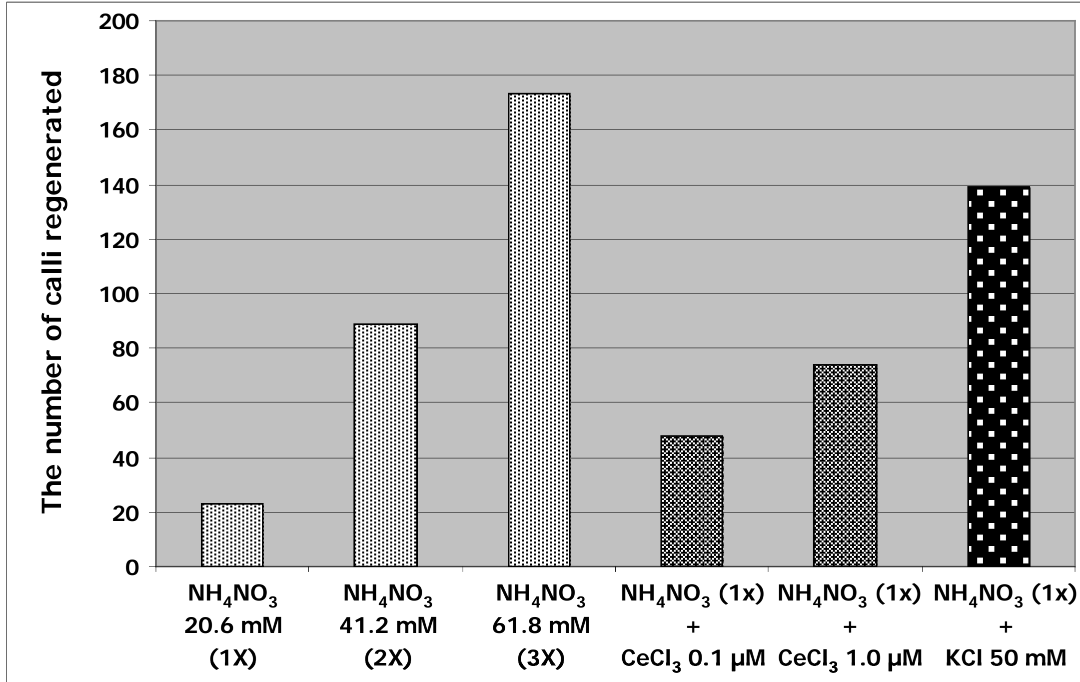

Figure 5.2.3. Callus regeneration efficiency in *N. tabacum* plants grown in liquid MS medium supplemented with various amounts of ammonium nitrate, cerium (III) and potassium chlorides

Plants grown in the presence of different amounts of ammonium nitrate (20.6 (1x), 41.2 (2x) and 61.8 (3x) mM) were used for transformation with *luciferase* gene containing a T-DNA construct. To compare the effect of cerium (III) and potassium chlorides on callus regeneration efficiency, plants were grown in 1x liquid medium containing 20.6 (1x) mM ammonium nitrate and either cerium (III) (0.1 or 1.0 µM) or potassium chloride (50 mM). 'x' – stands for the concentration of ammonium nitrate in standard MS medium. Calli were regenerated under selective conditions (hygromycin, 25 mg/L).

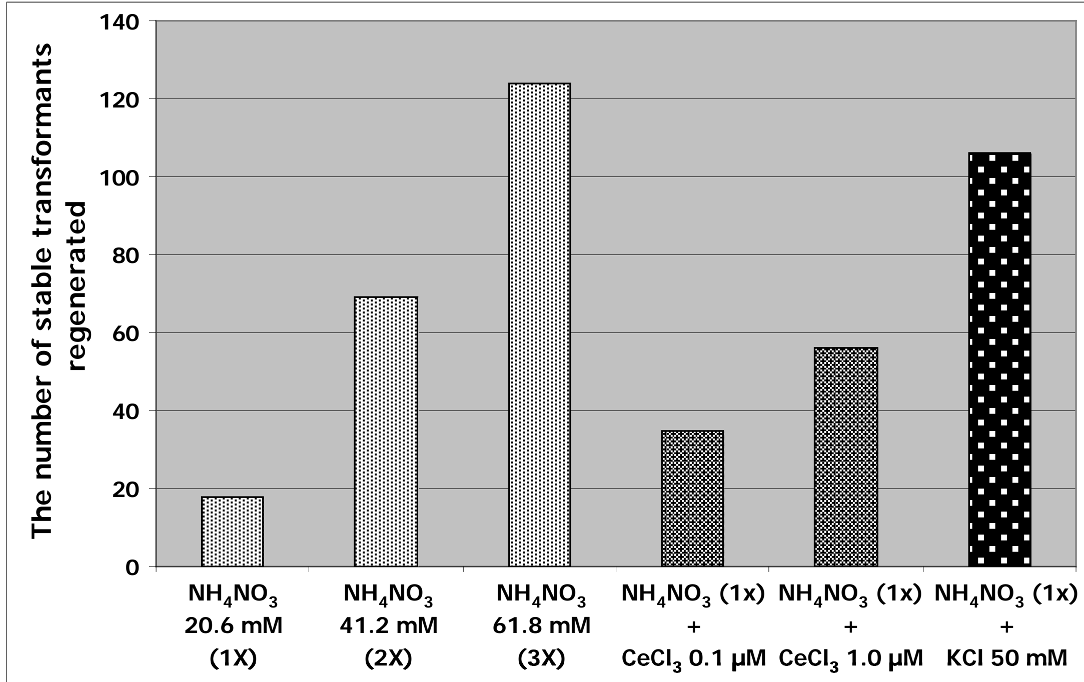

Figure 5.2.4. Efficiency of stable transformation of *N. tabacum* plants grown in liquid MS medium supplemented with various amounts of ammonium nitrate, cerium (III) and potassium chlorides

Plants grown in the presence of different amounts of ammonium nitrate (20.6, 41.2 and 61.8 mM) in liquid medium were used for transformation with *luciferase* gene containing a T-DNA construct. To compare the effect of cerium (III) and potassium chlorides on stable transformation efficiency, plants were grown in 1x liquid medium containing 20.6 mM ammonium nitrate and either cerium (III) (0.1 or 1.0 µM) or potassium chloride (50 mM). 'x' – stands for the concentration of ammonium nitrate in standard MS medium. Calli were regenerated under selective conditions (hygromycin, 25 mg/L). Stable transformation events were confirmed via *luciferase* gene expression.

5.3. DISCUSSION

In this study, we analyzed the effect of high concentrations of potassium chloride and the presence of rare earth elements in growth media on the activity of HR. A preliminary evaluation of their role in plant transformation with *Agrobacterium* was performed. We found that: a) supplementation of plant growth media with potassium chloride results in a dose-dependent increase of HRF; b) application of two rare earth elements, cerium and lanthanum, can increase HRF; c) growing plants in media supplemented with potassium or cerium (III) chloride before transformation can increase the frequency of T-DNA integration in the plant genome.

5.3.1. High concentrations of potassium chloride increase recombination rates in *Arabidopsis*

Our previous studies of the effects of salt stress on the plant genome demonstrated that the presence of sodium chloride in growth media can significantly increase RR in plants (Boyko *et al.*, 2008; Boyko and Kovalchuk, 2008b). This suggests the possibility of using this chemical for increasing HRF in plants before transformation with *Agrobacterium*. Unfortunately, high concentrations of sodium chloride result in a significant increase in the level of DSBs, therefore limiting the application of this chemical to relatively low concentrations. Furthermore, elevated levels of Na^+ ions in media are toxic to plants and result in ionic stress (Hasewaga *et al.*, 2000; Zhu, 2000, 2002). The role of Na^+ ions in HR induction is negligible. In contrast, Cl^- ions mediate an increase in RR under salt stress conditions (Boyko and Kovalchuk, 2008b).

We hypothesized that delivering Cl^- ions with other cations to growth media could increase HRF without triggering an ionic stress response. K^+ is a natural candidate to use, since it is normally present in high concentrations in growth media in the form of potassium nitrate (Murashige and Skoog, 1962).

Our data supported a hypothesis as to an important role of Cl^- ions in increasing RR. Exposure to potassium chloride resulted in a dose-dependent increase in RR. Unfortunately, the mechanism behind the effect of Cl^- ions on RR is unclear and still remains to be specified. Since both control and modified media contained the same concentration of K^+ ions, it can be suggested that the effect of potassium chloride on HR was mediated by Cl^- ions. Indeed, it was previously reported that a genotoxic aspect of salt stress is Cl^- - dependent (Boyko and Kovalchuk, 2008b).

These data strongly support the use of potassium chloride in growth media for enhancing HRF and improving plant transformation. Importantly, the effectiveness of such an approach was successfully demonstrated for ammonium nitrate, one of five MS macrosalts that are normally present in standard MS medium.

5.3.2. The presence of rare earth elements increases the frequency of homologous recombination in *N. tabacum*

Our previous studies demonstrated that enrichment of growth media with ammonium nitrate has a significant positive influence on HRF and can improve efficiency of *Agrobacterium*-mediated plant transformation. We believe that these influences can at least be partially mediated by a positive effect of enhanced nitrogen metabolism on plant growth and development (Forde, 2002a, 2002b; Ruffel *et al.*, 2008). We hypothesized that the use of microelements inducing nitrogen metabolism and increasing its assimilation could have a similar positive impact on plant transformation. Based on results reported by several independent studies, we selected two rare earth elements, cerium and lanthanum, for our experiments. The presence of these elements in growth media was shown to stimulate nitrogen metabolism, plant growth and differentiation (Wang, 1988; Dong *et al.*, 1992; Bai and Ma, 1993; Chen *et al.*, 1999; Fashui *et al.*, 2002; Weiping *et al.*, 2003).

Our data demonstrate a novel finding that the presence of rare earth elements in plant growth environment can increase HRF. Furthermore, these findings are consistent with our hypothesis and support a hypothetical link between nitrogen metabolism and HR activity. Both rare earth elements analyzed in the experiments resulted in a similar dose-dependent increase of HRF. However, in contrast to ammonium nitrate and potassium chloride analyzed before, the effect was not linear, and a slight deviation from an optimal concentration usually resulted in a substantial decline of HR activity. Interestingly, the simultaneous presence of these two elements in media led to a higher increase of HRF, as

compared to media with only one element being present. This finding suggests that the application of several rare earth elements might be beneficial for an increase of HRF.

The mechanism of a HRF increase caused by the presence of rare earth elements remains to be determined. To our knowledge, our findings represent the first report demonstrating the influence of cerium (III) and lanthanum (III) chlorides on the activity of the DNA repair pathway. We can hypothesize that this effect was mediated by enhanced nitrogen metabolism which increased the rate of cell division resulting in a higher activity of HR proteins. Importantly, correlations between nitrogen metabolism and cell division (Takei *et al.*, 2001) and increased expression of a HR protein during transition to S/G2 phase of the cell cycle (Chen *et al.*, 1997) were reported. Overall, further studies are needed to elucidate the exact mechanism of the rare earth elements' influence on HRF in plants.

Since chemicals that increased HRF were also shown to improve plant transformation, we hypothesized that cerium (III) chloride would also improve transformation.

5.3.3. Preliminary assessment of potassium and cerium (III) chlorides effects on callus regeneration and the frequency of stable T-DNA integrations in *N. tabacum*

It was previously demonstrated that the activity of host DNA repair proteins plays a critical role during T-DNA integration. In fact, it has been shown that simultaneous inactivation of HR and NHEJ pathways by mutations in *RAD52* and *KU70* genes completely inhibits T-DNA integration in yeast (van Attikum and Hooykaas, 2003). We hypothesized that an increase of HRF in plants before a transformation event should increase the frequency of stable transgene integration. Importantly, this hypothesis was consistent with our previous data demonstrating that high ammonium nitrate concentrations in growth media could increase HRF and significantly improve the transformation yield. Overall, our study provided a preliminary assessment of potassium and cerium (III) chlorides effects on *Agrobacterium*-mediated transformation efficiency in plants.

Our results confirmed the hypothesis and demonstrated a positive effect of these two chemicals on plant transformation. This effect was reflected in the higher number of calli and stable transformants obtained from plants grown in media containing potassium or cerium (III) chlorides before transformation. Using potassium chloride for the improvement of stable transformation resulted in an effect similar to that of ammonium nitrate. Unfortunately, strong genotoxicity of Cl^- ions places a limitation on the application of very high concentrations of this chemical in media (Boyko and Kovalchuk, 2008b). However, we believe that relatively low concentrations of potassium chloride can

be effectively applied, in combination with other factors, for the improvement of transformation efficiency.

Moreover, even very low concentrations of cerium (III) chloride in media were shown to be effective for the improvement of transformation. This implies that cerium can be used simultaneously with other chemicals, like ammonium nitrate, to potentiate their effect on plant transformation. Manipulation of the amount of macrosalts in growth media has its own physical and physiological limit: very high salt concentrations depress the water potential of media and are usually toxic to plants. In contrast, using cerium in combination with ammonium nitrate or potassium chloride could potentiate the effect of these macrosalts, thus making it possible to achieve maximum transformation efficiency without applying these salts in amounts that exceed plant physiological limits.

It is noteworthy that previous reports demonstrated that even very small quantities of different microelements in growth media could significantly improve SE. Supplementing standard MS medium with higher quantities of cupric sulphate promoted tissue regeneration on callus induction and plant regeneration media (Kothari, *et al.,* 2004). Similarly, zinc sulphate was shown to be essential for regeneration and proper growth of shoots (Kothari, *et al.,* 2004). In contrast, media devoid of zinc sulphate enhanced regenerative callus formation (He *et al.*, 1991; Kothari, *et al.,* 2004). Silver nitrate in media improved the total embryo yield in anther culture in *Brassica* (Dias and Martins, 1999; Sahrawat, *et al.,* 2003). Finally, supplementation of a regeneration medium with spermidine significantly improved the recovery of wheat transformants (Khanna and Daggard, 2003).

Overall, in our study we described preliminary characterization of two distinct groups of chemicals, namely chlorides and rare earth elements, and their effect on HR and plant transformation. The data obtained demonstrated that all tested chemicals have the potential for being applied during plant transformation. We also think that the simultaneous application of different transformation-enhancing chemicals in growth media could result in potentiating their effect on transformation efficiency. From this viewpoint, rare earth elements represent the most likely candidates for potentiating the influence of other macrosalts on *Agrobacterium*-mediated genetic transformation.

5.4. SUMMARY

Our study has provided a preliminary evaluation of the application of Cl^- ions and rare earth elements for the improvement of plant transformation. Supplementing plant growth media with potassium chloride, cerium and lanthanum proved to be a very efficient technique. These chemicals could significantly increase HRF, the total number of regenerated calli as well as plants containing stable T-DNA integration events.

However, genotoxicity of high Cl^- ions concentrations limits the application of high potassium chloride concentrations in media. We believe that low concentrations of this chemical can be applied to potentiate the effect of other factors that increase HRF. On the contrary, rare earth elements do not exert a negative impact on plant physiology. Moreover, they have a well-documented positive effect on plant growth and nitrogen metabolism, which, combined with a very low range of their active concentrations, makes rare earth elements an effective microsupplement for enhancing effects of other macrosalts on HRF and plant transformation. Furthermore, using these elements help achieve the maximum transformation efficiency without the application of macrosalts, such as ammonium nitrate or potassium chloride, in amounts that exceed plant physiological limits.

Overall, further studies will be needed to complete the characterisation of potassium, cerium (III) and lanthanum (III) chlorides' effects on the HR activity and plant transformation with *Agrobacterium*. A preliminary assessment of their application in growth media for the improvement of plant transformation performed in our study provides significant support for continuing research along this line.

5.5. FUTURE DIRECTIONS

Our study has demonstrated that manipulating the host growth medium for improving *Agrobacterium* genetic transformation is not restricted to chemicals normally available in standard growth medium. A number of other factors exhibiting similar influences can be suggested. A preliminary evaluation of potassium chloride and two rare earth elements performed in this study should be continued. It should include the analysis of intactness of T-DNA/plant DNA junctions, T-DNA integration sites, inheritance of integration events and stability of their expression.

Our study has revealed that a combined usage of several chemical factors in plant growth media can potentiate their effect on HRF and plant transformation. In future studies, special emphasis should be made on the application of rare earth elements for this purpose. These chemicals can be used in plant growth media as important microsupplements due to their positive effect on plant growth and nitrogen metabolism. Further research should be conducted to investigate effects of a simultaneous application of these elements in combination with high amounts of ammonium nitrate in media. The mechanisms mediating an increase of HRF by rare earth elements observed in our study should be further determined and described.

Finally, it would be important to determine the concentration range of Cl^- ions that can increase HRF without exerting a significant genotoxic influence on plants. This would allow the use of potassium chloride in growth media for enhancing HR and therefore plant transformation. It would be interesting to elucidate a combined application of potassium chloride and ammonium nitrate or cerium (III) chloride in growth media.

Overall, it is necessary to search for other chemical factors and physical conditions that will enhance HR and improve plant transformation. The transgenic plant system described in our study can be efficiently used for this purpose. Gaining knowledge of a spectrum of factors and conditions that control the efficiency of *Agrobacterium*-mediated plant transformation could help us reach a better understanding of genetic transformation mechanisms and facilitate the development of successful plant transformation protocols.

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