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Transgenic assays for the analysis of DNA repair in plants

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**TRANSGENIC ASSAYS FOR THE ANALYSIS OF
DNA REPAIR IN PLANTS**

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Abstract

In this work we studied various aspects of DNA repair in plants, focusing mainly on point mutation repair and its interconnection with double-strand break repair. We were using transgenic point mutation and recombination substrates as a primary tool in our experiments.

We have compared two transgenic homologous recombination assays (*β -glucuronidase-* and *luciferase-*based), analyzed the sensitivity of DNA repair machinery to ultraviolet radiation and assessed the involvement of AtKu80, Atm and AtXpd repair genes in point mutation repair.

Our study revealed the following: the *luciferase*-based recombination assay is more sensitive than *β -glucuronidase*-based; double-strand break repair machinery is sensitive to ultraviolet radiation, which results in increased point mutation formation; chosen DNA repair genes might be impaired in point mutation repair, however further experimentations are needed to confirm this.

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Table of Content

TITLE PAGE	i
APPROVAL	ii
ABSTRACT	iii
ACKNOWLEDGMENTS	iv
TABLE OF CONTENTS	v
LIST OF TABLES	vii
LIST OF FIGURES	viii
LIST OF ABBREVIATIONS	ix
1. INTRODUCTION	1
2. LITERATURE REVIEW	5
2.1. Types of DNA damage	5
2.1.1 Hydrolytic Damage	5
2.1.2. Alkylation damage	7
2.1.3. Oxidative damage	8
2.1.4. Damage induced by ionizing radiation	9
2.1.5. DNA damage induced by UV-irradiation	10
2.2. Mechanisms of DNA repair: general review	12
2.2.1. Base excision repair	12
2.2.2. Nucleotide excision repair	15
2.2.3 Double-strand break repair	18
2.2.4. Mismatch repair	22
2.3. DNA repair in plants	25
2.3.1. Direct reversion of DNA damage	26
2.3.2. Base excision and nucleotide excision in plants	27
2.3.3 Double-strand break repair in plants	31
2.4. Systems used in DNA damage analysis	38
2.5. Selected DNA repair related genes in Arabidopsis	44
2.5.1 Atm	44
2.5.2. AtXPD	46
2.5.3. AtKU80	48
3. MATERIALS AND METHODS	51
3.1. Plant and protoplast cultivation	51
3.1.1. Growth conditions	51
3.1.2. Sterilization of Arabidopsis seeds	52
3.1.3. Protoplast cultivation	52
3.2. Plants used in this study	53
3.2.1. GUS transgenic lines with HR substrate	53
3.2.2. LUC transgenic lines with HR substrate	53
3.2.3. GUS transgenic line with point mutation substrate	56
3.2.4 AtKu80 deficient Arabidopsis mutant	56
3.2.5. AtAtm deficient Arabidopsis mutant	59
3.2.6. UVH6 (AtXpd) Arabidopsis mutant	59
3.3. Visualization of mutation events. Histochemical staining	59
3.3.1. GUS staining procedures	59

3.3.2. LUC visualization procedures	60
3.4. DNA and RNA preparation	60
3.4.1. DNA preparation	60
3.4.2. RNA and cDNA preparation	61
3.5. Mutagenic treatments	62
3.5.1 UVC irradiation of Arabidopsis and Tobacco plants	62
3.5.2. UVC irradiation of tobacco protoplasts and leaf disks	62
3.5.3. MMS treatment	62
3.6. Generation of DNA repair deficient lines with stop codon inactivated GUS gene	62
3.6.1. Crosses	62
3.6.2. Isolation of progenies homozygous for the mutant genes	63
3.7. Real-time PCR analysis of gene expression	64
3.8. DNA digestion	66
3.9. Protoplast transfection	66
3.10. Tobacco leafes bombardment	67
3.11. N-acetyl-L-cysteine pre-treatment	68
3.12. Measurement of peroxide levels	68
3.13. Statistical treatment of the data (in study of DNA repair machinery sensitivity to UV radiation)	69
4. RESULTS	70
4.1. Transgenic plants used	70
4.2. Experiment 1: Luciferase-based transgenic recombination assay is more sensitive than β -glucuronidase-based	71
4.2.1. Spontaneous recombination frequencies as measured in GUS and LUC transgenes	71
4.2.2. Induced recombination frequency	71
4.2.3. Similarity of the level of transgene transcripts	72
4.3. Experiment 2: Double-strand break repair machinery is sensitive to UV-radiation	77
4.3.1. Generation of plasmids harboring the GUS gene inactivated by nonsense mutation	77
4.3.2. Pretreatment of the cells with UV-C increases the appearance of spontaneous reversions	77
4.3.3. Increase of reversions by UV-C is likely due to the action of free radicals	84
4.4. Experiment 3: Generation of DNA repair deficient Arabidopsis lines carrying point mutation substrate	88
4.4.1. Isolation of the homozygous Arabidopsis mutants crossed with 166 line	88
4.4.2. Contribution of mutant genes to point mutation repair	89
5. DISCUSSION	97
5.1. Experiment 1: Luciferase-based transgenic recombination assay is more sensitive than β -glucuronidase-based	97
5.2. Experiment 2: Double-strand break repair machinery is sensitive to UV radiation	99
5.3. Generation of DNA repair deficient Arabidopsis lines carrying point mutation substrate	104

5.3.1. AtAtm	104
5.3.2. AtXpb	105
5.3.3. AtKu80	105
BIBLIOGRAPHY	108

List of tables

Table 4.1. Spontaneous recombination frequency in <i>Arabidopsis</i> and tobacco as measured in <i>GUS</i> and <i>LUC</i> transgenes.	74
Table 4.2. UVC-induced recombination frequency in <i>GUS</i> and <i>LUC</i> transgenes of <i>Arabidopsis</i> and tobacco plants.	75
Table 4.3. The real-time PCR analysis of <i>GUS</i> and <i>LUC</i> transgene activity.	76
Table 4.4. Point mutation frequencies measured in AtAtm deficient plants crossed with line 166 in comparison with 166 control line upon UV-C and MMS treatments.	91
Table 4.2. Point mutation frequencies measured in Uvh6 deficient plants crossed with line 166 in comparison with 166 control line upon UV-C and MMS treatments.	92
Table 4.6. Point mutation frequencies measured in AtKU80 deficient plants crossed with line 166 in comparison with 166 control line upon UV-C and MMS treatments.	93

List of Figures.

Figure 3.1. Detection of recombination events in <i>Arabidopsis</i> .	55
Figure 3.2. Structure of recombination substrate in <i>Arabidopsis</i> and tobacco.	58
Figure 4.1. Schematic representation of GUS#166 construct used in the experiments.	80
Figure 4.2. (A) <i>N. plumbaginifolia</i> protoplasts and <i>N. tabacum</i> leaves were irradiated with UV-C of 5000 erg. At 15 minutes later the circular or linearised forms of the GUS#166 plasmids were delivered to the irradiated tissue.	81
Figure 4.3. Increased levels of GUS reactivation upon the repair of extrachromosomal DNA in UV irradiated protoplasts (A) and tobacco leaves (B) and in protoplasts (C) and detached leaves (D) pretreated with NAC and subsequently treated with UV-C.	82
Figure 4.4. The amount of organic (A) or aqueous (B) peroxides was measured in control, UV-C-pretreated, NAC-pre-treated and NAC/UV-C-pre-treated protoplast samples transformed with circular (non-cut) or linear (BclI) plasmids.	86
Figure 4.5. PCR analysis of cDNA derived from the F2 progenies of atku80 mutant and line 166 cross.	94
Figure 4.6. PCR analysis of genomic DNA isolated from the F2 progenies of atm-2 mutant and line 166 cross.	95
Figure 4.7. Picture of the plants obtained from the cross of uvh6 mutant with line 166 (F2 generation), which were grown at 37°C for 4 days.	96

List of Abbreviations

6-4PP – 6-4 pyrimidine pyrimidone
8-OHdG – 8-hydroxydeoxyguanosine
AP – apurinic/aprimidinic
BER – base excision repair
CAK – component in associated complex
CaMV – cauliflower mosaic virus
CPD – cyclobutane pyrimidine dimer
DSB – double-strand break
EMS – ethylmethane sulfonate
EYFP – yellow fluorescent protein
FYDR – fluorescent yellow direct repeat
GGR – global genomic repair
hGH – human growth hormone
HR – homologous recombination
IDL – insertion/deletion loop
IR – ionizing radiation
MMR – mismatch repair
MMS – methylmethane sulfonate
MS – Murashige and Skoog (medium)
NAC – N-acetyl-L-acetyl
NER – nucleotide excision repair
NHEJ – non-homologous end joining
PAM – polycyclic aromatic hydrocarbons
PCB – polychlorinated biphenils
PCD – programmed cell death
PCR – polymerase chain reaction
PPT – phosphinotricin
RF – recombination frequency

ROS – reactive oxygen species
RT-PCR – real-time PCR
SCID – severe combined immunodeficiency
SDSA – synthesis-dependent strand annealing
SSA – single-strand annealing
SSB – single-strand break
TCR – transcription-coupled repair
UV – ultraviolet (light)
UV-A – ultraviolet light (400 – 320 nm)
UV-B - ultraviolet light (320 – 290 nm)
UV-C - ultraviolet light (290 – 100 nm)

1. Introduction

DNA is constantly subjected to chemical modifications. This poses considerable threat for genome integrity and therefore successful survival of the organism. However, these modifications serve as a source of genetic variability, a fundamental base of natural selection and evolution. In an attempt to protect their genetic material, organisms have evolved various mechanisms to prevent and repair DNA damage. The study of mechanisms that provide genetic stability is thus an important task for modern biology.

The study of DNA damage induction and its repair in mammalian systems has been of interest largely because of the postulated role in carcinogenesis, aging and possible contribution of mutations to the genetic load (Britt, 1996). DNA damage has been studied with regard to its possible role in seed stock and perennial crop "aging". Some DNA damage products cause significant growth inhibition. Many act as blocks to the progress of both DNA and RNA polymerases. This could lead to cell cycle arrest and apoptosis (Hanawalt, 1998). Another important aspect of DNA damage and repair is the creation of genetic diversity. Certain DNA repair pathways that allow cells to bypass the DNA lesion and replicate are of special interest.

Internal and external factors classified either as chemical or physical, are capable of altering genetic material. The most common physical factors contributing to DNA damage are ultraviolet and ionizing radiation.

Ultraviolet (UV) radiation can directly damage DNA molecules resulting in the creation of cyclobutane pyrimidine dimers (CPD) and pyrimidine (6 - 4) pyrimidinone dimers (Britt, 1996). UV radiation damages protein and biomembranes (Kramer et al.,

1991; Pang and Hays, 1991). In addition, the toxicity of many environmental organic contaminants can be activated and enhanced by UV light as many have strong absorbance bands in the UV-B range (Huang et al., 1993). Another major source of DNA damage is ionizing radiation (IR). IR was shown to directly and indirectly (via production of free radicals) influence the integrity of organic molecules in the cell, including DNA (Ward, 1975). Direct absorption of radiation by the sugar-phosphate backbone likely results in double strand breaks (DSB) of the DNA molecule. Thus IR is capable of generating chromosomal breaks, inversions, duplications and translocations. Point mutations are also possible.

Known genotoxic compounds can be divided by chemical nature into two groups: inorganic and organic mutagens. Heavy metals are the main inorganic genotoxic compounds. Metal ions such as Cu^{2+} , Ni^{2+} and Cd^{2+} can cause the formation of free radicals capable of damaging genetic material (Imlay and Linn, 1988). Metal ions can interfere with DNA repair and replication machinery lowering efficiency and thus increasing the mutation rate (Hartwig, 1995). Some ions like Ni^{2+} , As^{3+} are known to alter methylation patterns of DNA and therefore change the stability of genetic material (Mass and Wang, 1997; Lee, 1998).

Genotoxic organic compounds represent an extremely variable group of organic molecules that influences the DNA stability in different ways. Good examples are alkylating agents that cause double-strand breaks (DSBs), ethylmethane sulfonate (EMS) and methylmethane sulfonate (MMS). Many chemicals containing aromatic rings like polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) are extremely genotoxic and carcinogenic; they might act as intercalating agents.

Double-strand breaks represent a major challenge for the genomic integrity. This kind of lesion usually leads to cell division arrest and apoptosis (Karanjavala et al., 2002; Rinaldo et al., 2002). DSBs can be repaired by one of two mechanisms: homologous recombination (HR) or non-homologous end-joining (NHEJ). The HR mechanism is relatively error free. NHEJ frequently cause deletions and/or insertions of various sizes.

The study of DNA repair depends strongly on the availability of relatively simple and inexpensive *in vitro* or *in vivo* systems that allow direct observation of point mutation or DSB events. Many recombination assays have recently been developed. They include for example protoplast based extrachromosomal repair of linear vectors (Gorbunova et al., 1997), the somatic eye mutation test in *Drosophila* (Rasmuson, 1985), the transgenic mouse based systems (Sacco et al., 1997, Mayer et al.1998), the transgenic-zebra-fish assay (Amanuma et. al., 2000), the chromosome aberration assay involving higher plants such as *Allium cepa* (Fiskesjo, 1988, Kovalchuk et al., 1998a), *Vicia faba* (Kanaya et al., 1994), *Tradescantia* (Ichicava, 1992) etc. and the single cell gel/comet assay (Tice et al., 2000). The most sensitive and reliable assays for the analysis of strand-break repair were constructed in plant systems (Puchta et al., 1995; Swoboda et al., 1994; Leonard et al., 2003; Li et al., 2004; Kovalchuk et al., 2000, Kovalchuk et al., 2001). A β -glucuronidase-based system, designed in middle of ninetieth allows detection of the recombination events throughout the entire life span of the plants (Swoboda et al., 1994; Puchta et al., 1995). This assay is based on the restoration of activity of the visible marker transgene - β -glucuronidase.

Point mutations can be caused by one of two types of events. Either a chemical modification can replace an amino acid or a malfunction during DNA replication can

cause the insertion of an incorrect base. Various *in vivo* transgenic systems such as mice (*lacI*) or zebra fish (*rspL*) have been developed to allow point mutation study and observation. (Kohler et al., 1991; Amanuma et al., 2000). An *Arabidopsis* based system used in this study utilizes the stop-codon inactivated β -glucuronidase (*uidA*) (Kovalchuk et al., 2000).

In this study we analyzed the efficiency of different transgenic systems for DSB detection, the influence of UV radiation on the repair of DSBs and the role of several known and putative DNA repair genes in strand processing.

2. Literature review

2.1. Types of DNA damage

Genetic material of any given organism, even under the best of circumstances, is constantly exposed to a number of mutagenic factors of an endogenous and exogenous nature. Whereas the endogenous factors are mostly associated with cell metabolic activity, the exogenous factors may be chemical (hydrolyzing, alkylating, oxidative, intercalating agents) or physical (UV and ionizing radiation) in nature. DNA damage that arises can be broadly classified into three types of lesions: mismatched bases, double-strand breaks, and chemically modified bases. Each of these is repaired via distinct pathway (Britt, 1996).

2.1.1 Hydrolytic Damage

The DNA of any living cell is a target for different hydrolytic reactions. The most common is the hydrolysis of the glycosylic bond between purine bases and the DNA backbone. The outcome of this reaction would be an apurinic/apyrimidinic (AP) site. AP sites arise spontaneously at a substantial rate (Lindahl et al., 1973). AP sites have been shown to be a threat to cellular viability and genomic integrity as they are capable of preventing normal DNA replication and transcription (Yu et al., 2003). Collision of replication fork may lead to a double-strand break. The DSB can also form as a result of the activity of endonucleases, which are normally involved in repair of the AP sites (Loeb and Preston, 1986; Gentil et al., 1990; Gentil et al., 2000; Yu et al., 2003). Recent studies show that DSBs themselves can influence the point mutation rate. For example

misincorporation of nucleotides during the gap filling steps that take place both in DSB and SSB repair are likely due to DNA end processing during the course of microhomology search (Kovalchuk et al., 2003).

There is evidence that AP sites, arising as a result of an occasional lesion bypass events during DNA replication and are potentially mutagenic (Gentil et al., 1984). Generally AP sites are easily recognized and repaired and therefore do not play a significant role in mutagenesis under normal conditions.

The second known type of hydrolysis reaction is the hydrolytic deamination of cytosine and methyl-cytosine that leads to formation of uracil and thymine, respectively. This reaction is highly mutagenic due to the pairing with adenine which is the cause of the transition of C:G to T:A during replication. However, uracil and thymine differ significantly in their mutagenic abilities. Uracil, being an inappropriate base for the DNA, is easily recognized and repaired. A transition to thymine (a normal DNA component) is less likely to be detected as an error and therefore is not recognized by proofreading enzymes. It is suggested therefore that 5-methylcytosine deamination is one of the most important causes of point mutations in mammalian cells (Rideout et al., 1990).

The fact that the plant genome contains 10 times more 5-methylcytosine than the human genome (Shapiro, 1976) suggests a greater role for this source of point mutations in plants. Another interesting aspect of DNA hydrolysis is the accumulation of hydrolytic damage in dried seeds. It is known that DNA in desiccated seeds is less prone to hydrolysis than in fully hydrated cells (Dandoy et al., 1987). Seeds stored for long period display a delay in replication, synthesis of low molecular weight untranslated RNAs and

unscheduled repair (Osborne, 1983). This is consistent with a period of genomic repair required for cell division and points to a beneficial effect of “osmopriming” for uniform germination (Ashraf and Bray, 1993).

2.1.2. Alkylation damage

Simple methylating agents such as methyl methane sulfonate (MMS) and dimethyl sulfate methylate DNA *in vivo* and *in vitro* mainly at 7-deoxyguanine and 3-deoxyadenine. The resulting 3-methyladenine blocks DNA synthesis and is therefore considered as a major lethal lesion (Evensen and Seeberg, 1982). MMS is known also to indirectly induce DSBs (Hryciv et al., 2002). Even in the absence of artificially applied alkylating agents, however, any genome experiences spontaneous DNA methylation at a significant level (Rebeck and Samson, 1991). The most frequently generated alkylation product is 7-methylguanine, that basepairs in a normal way and is considered neither toxic nor mutagenic. Another frequent alkylation lesion is O⁶-methylguanine. It has the ability to pair efficiently with thymine and therefore is a potent source of G:C to A:T transitions. Consistent with this survey, *Arabidopsis* seeds treated with ethyl methane sulfonate have revealed that all acquired mutations represented G:C to A:T transitions (Oroszo et al., 1993). We can say that alkylating agents produce various lesions that can be either neutral, lead to point mutations or DSBs.

2.1.3. Oxidative damage

The living cell is constantly exposed to potentially damaging reactive oxygen species (ROS) that can modify various biomolecules including DNA. The main source of free radicals is the activity of chloroplasts (Bowler et al., 1992) and mitochondria (Ward, 1975). The extracellular ROS inducing factors include ozone (Kanofsky and Sima, 1991), high levels of UV-B radiation (Hariharan and Cerutti, 1977), ionizing radiation (Evans et al, 2003) and heavy metal ions (Kovalchuk et al., 2001). These species cause oxidative damage to DNA resulting in the formation of modified bases and sugars, DNA-protein cross-links, strand breaks, abasic sites, tandem lesions such as 8,5'-cyclopurine-2'-deoxyribonucleosides and clustered damaged sites (Friedberg et al., 1995;). Like alkylating agents, ROS can cause point mutations via the chemical modification of bases. For example O⁶-methylguanine can basepair efficiently with thymine and therefore create G:C to A:T transitions (Dolferus et al., 1990; Niyogi et al., 1993; Oroszo et al., 1993).

The most prevalent base damage to purines is 7,8-dihydro-8-oxoguanine more commonly named 8-oxoguanine. The most common damage to pyrimidines is the formation of thymine glycol (Slupphaug et al., 2003). 8-oxoguanine has been shown to basepair with equal capability to A and C and represents an important source of point mutations (Maki and Sekiguchi, 1992). 5-methylcytosine can be effectively converted to thymine glycol by ionizing radiation under aerobic conditions or peroxide. Thymine glycol can act as a block for replication and also be an additional source of mutations in organisms that utilize 5-methylcytosine to regulate gene expression (Burdzy et al., 2002). Several uracil analogues are also generated from cytosine after exposure to ionizing radiation and ROS from different sources. In addition to base damage, ROS are capable

of reacting directly with the sugars of the sugar-phosphate backbone. This leads to a single-strand and, eventually, to double-strand breaks upon replication (Dizdaroglu, 1998; Pfeiffer et al, 2002). Another important outcome of ROS-induced DNA damage are DNA-protein cross-links. For example the formation of thymine-tyrosine cross-link has been observed in mammalian chromatin *in vitro* and in living cells when exposed to ionizing radiation, peroxide, metal ions and carcinogenic compounds (Olinski et al; 1992). Thus oxidative damage of DNA is a powerful source of both point mutations, strand-breaks and also has certain cytotoxic effects.

2.1.4. Damage induced by ionizing radiation

Ionizing radiation produces a wide spectrum of DNA lesions such as base damage, sugar damage, single-strand breaks, DNA-DNA and DNA-protein crosslinks (Belli et al., 2002). Its distinctive feature is the complete lack of target specificity. The probability of any cell component to interact with ionizing radiation depends only on the mass fraction it makes up of the cell. For this reason the most frequent primary target is water that comprises the biggest part of the cell. It is believed that the majority of the DNA damage is produced due to the ROS generated by ionizing radiation (Ward, 1975). Another cause of DNA damage is the direct absorption of radiation by the sugar-phosphate backbone. This leads to the generation of nicks and DSB and then, most probably converts to a DSB during replication. Numerous authors have related cell survival to the initial level of induced DSB (Kelland et al., 1988; Schwartz et al., 1991). There is a a more convincing correlation with residual DSBs (Blocher, 1988). Among DNA lesions induced by ionizing radiation, DSBs are the least efficiently repaired and

their frequency is correlated with that of cell death (Friedberg et al., 1995). It has been shown that *Escherichia coli* and most other organisms cannot survive if more than three DSBs are introduced per chromosome independently of their physiological state (Krasin and Hutchinson, 1977; Resnick, 1978). Some organisms are extremely resistant to ionizing radiation. For example *Deinococcus radiodurans* has the capability to repair more than 100 induced DSBs without the loss of viability (Moseley, 1983).

Single-strand breaks (SSB) are another important type of mutagenic lesion introduced by ionizing radiation (Povirk, 1996). Ionizing radiation induced SSBs are chemically defined and consist of two equally distributed forms. The lesion is composed of a one nucleotide gap containing a 5'-phosphate and either a 3'-phosphoglycolate or a 3'-prime phosphate (Henner et al., 1983). Between them the SSB that contains 3'-phosphoglycolate has been shown to be more cytotoxic (Buchko and Weinfeld, 1993).

We can see that ionizing radiation is both genotoxic and cytotoxic due to its ability to cause DNA lesions indirectly via the production of free radicals and directly affecting the DNA strand with induction of single-strand and double-strand breaks.

2.1.5. DNA damage induced by UV-irradiation

UV radiation is one of the most prominent natural mutagens. UV is cytotoxic even in the low dose range. It leads to the inhibition of DNA replication and the cell undergoes transient arrest of the cell cycle. A high dose of UV damage leads to an initial replicative arrest followed by death of the cell by apoptosis (Latonen et al., 2001).

In plants it penetrates tissues and damages DNA and other cellular targets, for example photosystem II and plasma membrane ATPase (Stapleton, 1992). The DNA

integrity may be influenced either by direct damage or by the influence of UV on proteins involved in DNA repair. Longer UV wavelengths (UV-B and UV-A) have been proven to induce oxidative stress and protein denaturation (Ravanat et al, 2001). Exposure of cells to UV-C (shorter wavelength) primarily results in the formation of the two most common lesions, the cyclobutane pyrimidine dimer (CPD) and the 6-4 pyrimidine pyrimidone photoproduct (6-4PP) at adjacent pyrimidines (Friedberg et al., 1995). CPDs and 6-4PPs make up approximately 70-80% and 20-30% respectively of the total UV photoproducts (Mitchell and Nairn, 1989). If not removed, these lesions block both DNA replication (Painter, 1985) and transcription (Protic-Sabljis and Kraemer, 1985). They are normally removed by nucleotide excision repair (NER) in most organisms including humans (Hoeijmakers, 2001). CPD has been shown to have only a modest influence on DNA structure. By contrast, 6-4PP induces a large structural distortion and is repaired more rapidly by NER (Mitchell, 1988). Due to incomplete repair damaged bases may be misinterpreted during replication (de Gruijl et al., 2001). Transitions been reported at dipyrimidine sites at high frequency (Witkin, 1976). Formation of pyrimidine dimers or (6-4) photoproducts could explain the base changes found in mutated sequences like GGC→AGC (Nishigori, 2000). Mutations at CC sites can be due to the indirect influence of UV light mediated by ROS (Ried and Loeb, 1993). One of the most important types of base damage caused by UV induced ROS is 8-hydroxydeoxyguanosine (8-OHdG) produced at the 5'-site of 5'-GG-3' sequence in double-stranded DNA by treatment with UV radiation and riboflavin. 8-OhdG is believed to cause G:C to T:A transversions by pairing with adenine at 50% probability. (Ito et al., 1993).

2.2. Mechanisms of DNA repair: general review

2.2.1. Base excision repair

Base excision repair (BER) was discovered by Thomas Lindahl in 1974 as a result of a search for enzymatic activity that would act on deaminated cytosine (Lindahl, 1974). Most damaged or inappropriate bases in DNA are removed by excision repair, while a minority are repaired by direct damage reversal (Lindahl and Wood, 1999). BER is the multistep process used to repair damaged bases that do not distort the DNA helix (e.g. uracil 3-methyladenine). BER is initiated by a damage specific DNA glycosylase that releases the damaged base. This leaves an abasic site that is subsequently processed in a pathway that restores the correct DNA sequence (Mol et al., 1999).

For instance, hydrolytic deamination of cytosine leads to the formation of a uracil. This lesion produces C:G to T:A transitions and therefore is highly mutagenic. In humans, four of eight identified glycosylases can remove uracil from DNA. Each of them has a specialized function. The UNG, which is homologous to *E.coli* Ung enzyme is associated with DNA replication forks and correct uracil misincorporated opposite adenine; SMUG1, which is unique to higher eucaryotes removes uracil that arises from cytosine deamination. MBD4 removes uracil only from deaminated CpG and 5-methyl-CpG sequences. TGD slowly removes uracil and thymine from G:U and G:T base pairs (Lindahl and Wood, 1999).

All mammalian glycosylases have N-terminal extensions that are not present in bacterial counterparts and serve for targeting the enzymes to nuclei or mitochondria and interactions with other proteins that might be involved in BER (Otterlei et al., 1999).

DNA glycosylase can cleave the glycosylic bond between the base and deoxyribose releasing the damaged base and leaving an abasic site that is cytotoxic and mutagenic and therefore must be repaired. Glycosylases can be divided into two classes: monofunctional and bifunctional. For monofunctional glycosylases hydrolysis of the glycosylic bond involves nucleophilic attack by a water molecule, activated by the carboxyl side chain of an Asparagine (Asp) residue. For bifunctional DNA glycosylases the nucleophile is an activated amino group of a Lysine (Lys) residue activated by an Asp residue (Lindahl et al., 1977). Removal of the damaged base is the only function of monofunctional DNA glycosylases, such as uracil DNA glycosylases (UNG), mismatch specific thymine/uracil DNA glycosylase (TDG) and the methylpurine DNA glycosylase (MPG). Whereas UNG and TDG have narrow substrate specificities, MPG removes a large array of damaged bases which have a weakened glycosylic bond as their only common feature (Krokan et al., 1999). Several DNA glycosylases have associated lyase activities that cleave the 3' side of the abasic site. These are the enzymes that remove oxidized purines such as 8-oxoG DNA glycosylase (OGG1) and the human endo III homologue hNTH1 that removes oxidized pyrimidines (Matsumoto et al., 1999). Important factor in BER is XPG protein, which enhances DNA activity of glycosylases, for example NTH1 (Klungland., 1999).

In vitro repair in HeLa cell extracts revealed the removal of two substrates by the monofunctional MPG takes place via both short and long patch repair, while repair of 8-oxoG initiated by the bifunctional OGG1 takes place mainly via short patch repair (Fortini et al., 1999).

In case of the short patch pathway DNA polymerase β (Pol β) is recruited along with major AP endonuclease (HAP1) to insert 1 nt (Bennet et al., 1997; Fortini et al., 1998). The 5'-deoxyribose 5-phosphate (dRP) moiety is removed by dRPase activity of Pol β (Deterding et al., 2000). Evidence suggests that the Pol β - dependent short patch pathway is responsible for 75 – 90% of BER in human cells (Sobol et al., 1996). The single strand nick is then ligated by ligase III in complex with the scaffolding protein XRCC1 (Nash et al., 1997).

DNA damage processing initiated by monofunctional glycosylases is performed by long patch BER. In this case HAP1 leaves a nick with 3'-OH and dRP end (Parikh et al., 1999). Long patch BER involves the synthesis of 2-8 nt stretches beginning at the damaged site (Deterding et al., 2000). Strand displacement synthesis creates the flap structure from which the damaged base is removed as part of an oligonucleotide by structure-specific flap endonuclease (FEN1) (Lieber, 1997) or by AP lyase activity associated with Pol β (Matsumoto and Kim, 1995). In long patch BER the DNA synthesis is performed mainly by DNA polymerases δ and ϵ (Stucki et al., 1998), however it can be done also by Pol β (Dianov et al., 1998). Pol δ and Pol ϵ are stimulated and strongly dependent in their activity on proliferating cell nuclear antigen (PCNA) and replication factor C (RFC) (Stucki et al., 1998) suggesting the involvement of long patch BER primarily during replication. On the final step of this process created nick is sealed by ligase I or III.

2.2.2. Nucleotide excision repair

Nucleotide excision repair (NER) is one of the most versatile and flexible DNA repair pathways that deals with a wide range of lesions, which common feature is DNA double helix distortion. The NER mechanism includes several distinct steps: DNA lesion recognition, opening of the double helix by the sequential action of helicases and endonucleases, excision of the DNA segment containing the lesion, gap polymerization using the intact strand as a template and ligation (Costa et al., 2003).

All 25 polypeptides, the key factors in NER required for the successful repair by NER *in vitro* have been cloned and identified (Abboussekhra et al., 1995). The protein assembly required for NER action is likely to be formed in sequential manner rather than through the interaction of damaged DNA with an already pre-assembled complex (Volker et al., 2001).

Two distinct subpathways that differ in the mechanism of DNA damage recognition represent NER: transcription-coupled repair (TCR) and global genome repair (GGR). In the case of TCR the lesion is detected by RNA polymerase II in the transcribed region. This pathway allows faster recognition and repair than GGR that deals with non-transcribed segments (Hanawalt, 2002). The repair rate also depends on the nature of the lesion. For example 6-4 PPs that create considerable distortion of the DNA helix are repaired 5 times faster than another UV photoproduct, CPDs that has less influence on DNA structure (Tornaletti and Hanawalt, 1999). In addition genome condensation in chromatin may also interfere with the rate of lesion recognition and removal (Moggs and Almouzni, 1999).

The first factor involved in the recognition of a damaged base in case of GGR is the XPC-hHR23B (Volker et al., 2001; Yokoi et al., 2000). It has been shown that besides the recognition of a damaged base, XPC-hHR23B can distort the DNA double-helix (Volker et al., 2001). This complex has affinity for a wide range of lesions, however in some cases (e.g. CPDs), this affinity is reduced (Kusumoto et al., 2001). It has been suggested that in some cases other factors like XPE can be recruited to cooperate in the detection of a lesion (Kusumoto et al., 2001). XPE has been shown to correspond to a damaged DNA binding factor (Hwang and Chu, 1993). The XPC-hHR23B complex is not required in TCR. Rather, the block of DNA polymerase II serves as signal for detecting DNA damage. Two other proteins, CSB and CSA, are also required for this process (Henning et al., 1995). CSB protein has been shown to alter DNA conformation and remodel chromatin. This activity is ATP dependent (Citterio et al., 2000).

Following DNA damage recognition by the XPC-hHR23B complex or TCR machinery, other factors required for subsequent step of NER are recruited. The most important among them are TFIIH, XPD XPA, XPB, RPA, XPF and XPG (Evans et al., 1997; Evans et al., 1997). TFIIH is a transcription initiation factor that consists of 9 proteins that are also required for NER (Winkler et al., 1998; Coin et al., 1999). XPD and XPB proteins display ATP dependent DNA helicase activity (Weeda et al., 1990; Weber et al., 1990). XPB helicase unwinds the double-helix in 3'-5' direction and XPD in opposite (Schaeffer et al., 1994; Roy et al., 1994). The replication protein A (RPA) possesses a DNA binding activity. It has been shown to bind to the undamaged strand providing full double-helix opening around the lesion (Christians and Hanawalt, 1993). Another known NER protein, XPA binds in the form of a homodimer to RPA creating

XPA2-RPA complex (Yang et al., 2002). It is suggested that this complex is able to provide the correct three-dimensional assembly of NER components required for DNA damage excision (Missura et al., 2001).

The next step, which involves the endonucleolytic cleavage of DNA in the vicinity of the lesion, is performed by the XPG factor. XPG possesses structure specific 3' endonucleolytic activity (Mudgett and MacInnes, 1990). Endonucleolytic activity of XPG is likely to be induced by its interaction with the XPA2-RPA complex (Laat et al., 1998). Another endonuclease required for this process is XPF in complex with the ERCC1 protein (Laat et al., 1998). XPA2-RPA plays a crucial role in the recruitment of the XPF-ERCC1 complex to the site of NER (Volker, 2001). This way, the XPA2-RPA complex helps to build the correct assembly of the endonucleolytic complex, providing appropriate positioning of endonucleases on the damaged strand (Saijo et al., 1996). Incisions occur when the assembly of endonucleolytic complex is completed, 3' incision is believed to precede the 5' incision (Mu et al., 1996). The XPG protein cleaves the DNA strand 3' to the lesion 2-8 nt away and XPF-ERCC1 – 15-24 nt 5' (Evans et al., 1997). Then the segment of excised DNA containing the lesion dissociates (Mu et al., 1996). The 3' end left by XPF-ERCC1 may act as primer for DNA polymerase activity in the last step of NER (Sijbers et al., 1996). As in the case of the long patch BER the gap refilling is provided by DNA polymerases ϵ and δ (Hunting et al., 1991; Coverley et al., 1992). Both require two additional protein factors. PCNA and RFC (Wood et al., 1997). The last step of NER – ligation of the 5'-end of the newly synthesized patch is performed by DNA ligase I (Tomkinson and Levin, 1997).

2.2.3 Double-strand break repair

Double-strand breaks are usually regarded as the most dangerous kind of DNA damage. If left unrepaired, even in non-essential chromosomes, DSBs can cause cell-cycle-checkpoint arrest or cell death in yeast and mammals (Weaver, 1995; Bennet, 1996). There are three distinct pathways of DSB repair: non-homologous end-joining (NHEJ), single-strand annealing (SSA) and homologous recombination (HR) (van Gent et al., 2001; Khanna and Jacckon, 2003; Valerie and Povirk, 2003). Both NHEJ and SSA require first exonucleolytic processing of the DNA ends to allow the homology search and annealing. Microhomology, of several basepairs is necessary for NHEJ (Chen et al., 2001), while up to several hundred basepairs are necessary for SSA (Sugawara et al., 2000).

In somatic mammalian cells NHEJ is a common pathway for repairing broken chromosomes (Weaver, 1995; Lehman et al., 1993). The NHEJ mechanism is highly conserved as yeast and mammals require the same core set of proteins: the DNA end binding proteins KU70 (Yku70) and KU80 (Yku80), as well as DNA ligase IV (Dnl4) and associated XRCC4 (Lif1) protein. Homologues of these proteins were identified in yeast using a gene knock-out approach (Critchlow and Jackson, 1998; Lewis and Resnick, 2000). A homologue of the DNA-dependent protein kinase (DNA-PK_{cs}) in yeast was found (Chen and Kolodner, 1999). In the initial step of NHEJ KU proteins bind to the DNA ends, align them, prepare them for ligation and protect them from degradation. Two heterodimers must come together to bridge matching ends (Feldman et al., 2000). The Ku heterodimer also recruits DNA-PK_{cs} to the DSB site and activates its kinase function. Then it must phosphorylate target proteins to dissociate and allow NHEJ to

proceed (Smith and Jackson, 1999). DNA-PK_{cs} can also bind and be activated by DNA ends in the absence of KU (Yaneva et al., 1997; Hammersten and Chu, 1998), but KU is likely to be required for stabilization of DNA binding by DNA-PK_{cs} *in vivo* (Hammersten and Chu, 1998). According to the atomic-force microscopy assay, DNA-bound KU self-associates and can juxtapose two DNA ends (Cary et al., 1997). KU is likely to be the first protein complex that interacts with a damaged site. However it is also possible that yeast transcriptional silencing factors (Sir2p, Sir3p and Sir4p) are recruited first to create heterochromatin like regions around the break (Boulton and Jackson, 1998). It has been proposed that DNA-PK_{cs} can phosphorylate XRCC4 and therefore remove or relocate the Ligase IV-XRCC4 complex from the KU-bound DNA ends. It also can regulate the accessibility of DNA via autophosphorylation or/and phosphorylation of KU proteins and prevent transcription at the damaged site (Calsou et al., 1999; Chan et al., 1999). The next step of NHEJ includes terminal processing of the DNA ends via nucleolysis and polymerization.

It has been suggested that nucleases remove several nucleotides at the broken ends especially when microhomology occurs and then DNA polymerases fill in the gaps. The protein complex involved in this process might include Mre11, RAD50 and Nbs1 proteins, which also participate in DNA damage signaling and protection of the ends from degradation (Khanna et al., 2001). Other proteins involved in this process could be exonuclease FEN1, the unwinding enzymes WRN (Werner syndrome helicase) and BLM (Bloom syndrome helicase) (Wu et al., 1999; Wilson., 2003). On the final step of NHEJ the Ligase IV-XRCC4 is recruited tetramer in order to ligate the DSB (Junop et al., 2000; Lee et al., 2000; Sibanda et al., 2001). Although the function of XRCC4 in DSB repair is

not clear it has been shown to activate Ligase IV *in vitro* (Grawunder et al., 1997). It may also work as an adapter providing appropriate targeting of Ligase IV to DNA-PK_{sc} (Critchlow et al., 1997).

Number of discovered factors impaired in DSB is growing continuously. Recent experiments performed on human SCID (severe combined immunodeficiency) cells revealed a novel DNA DSB repair protein Artemis. Artemis is required for lymphoid differentiation in bone marrow (Moshous et al., 2001; Lieber et al., 2003). Artemis has been shown to possess 5' exonuclease activity (Ma et al., 2002). Recently it was discovered that another novel NHEJ protein polynucleotide kinase (PNK) was capable of modifying the DNA termini (Chappel et al., 2002). The non-protein factor inositol phosphate (IP₆) can specifically interact with human KU70/KU80 and stimulate end joining *in vitro* (Hanakahi et al., 2002).

Another important pathway used to repair double-strand breaks is homologous recombination (HR). Unlike NHEJ, HR involves site-specific interaction between a broken DNA molecule and a specific homologous template. HR is preferentially used in yeast throughout the cell cycle except for the G1 phase of haploid cells. Cells deficient in HR, such as rad52 mutants, are extremely sensitive to ionizing radiation (Bressan et al., 1999). Mammals however, appear to be less dependent on HR. For instance, HR deficient rad54 adult mice do not exhibit any IR sensitivity (Essers et al., 2000) whereas mice carrying deleterious mutations in DNA-PK_{sc} are hypersensitive to IR (Gao et al., 1998). Studies performed on a yeast model revealed a number of genes involved in HR called the Rad52 epistatic group. They include: Rad50; Rad54; Rad55; Rad57; XRS2 and Mre11 (Game and Mortimer, 1974). Some of these proteins are highly conserved.

Rad51p from *Saccharomyces cerevisiae* for example, is 55% identical to human and mouse proteins.

The early step of HR includes the processing of the damaged DNA ends into extended 3' overhangs which are subsequently loaded with proteins that help to invade the sister chromatid and recognize the region of homology (Thompson and Shield, 2001). According to recent studies, RAD proteins are assembled in a step-wise fashion (Sugawara et al., 2003; Wolner et al., 2003). As in NHEJ, the Rad50/Mre11/Nbs1 (MRN) complex is believed to be responsible for the initial clean up of DNA ends and for 5' to 3' exonuclease activity (Tsuchi et al., 2002). Rad51p has been shown to catalyze *in vitro* strand exchange between ssDNA and homologous dsDNA (Sung, 1994). Rad51p forms a nucleoprotein filament on ssDNA regions (Ogawa et al., 1993) and catalyzes search for homologous sequence strand pairing and strand exchange (Baumann et al., 1996, Baumann and West, 1998). The usual target for the Rad51p mediated homology search is the sister chromatid which is used in late S or G2 stages at least 100-fold more frequently than the homologous chromosome (Richardson et al., 1998; Johnson and Jasin, 2000). Several other proteins including Rad51B, Rad51C, Rad51D and XRCC2 might also facilitate the formation of Rad51 filaments on the gapped DNA sequence (Masson et al., 2001). The BRCA2 protein has been shown to bind directly to Rad51p (Wong et al., 1997) and regulate the Rad51-DNA interaction (Davies et al., 2001). Additionally, Rad52p is able to interact with the N-terminal domain of Rad51p stimulating binding to ssDNA during the pre-synaptic stage of strand exchange (Krejci et al., 2002). Strand invasion is further stimulated by Rad54p (Petukhova et al., 1998). This protein forms negative supercoils in duplex DNA increasing accessibility for strand invasion (Tan et al.,

2003). As soon as the homologous sequence is found, Rad51p is removed from the recipient strand (Sugawara et al., 2003).

Upon invasion of complementary DNA strands, 3'-ends can be used as primers. Polymerization is driven by the replication polymerases δ and ϵ (Holmes and Haber, 1999; Halas et al., 1999). After invasion and DNA polymerization, ligation immediately rejoins the broken arms (Aylon et al., 2003).

Single-strand annealing (SSA) can be considered a type of HR frequently employed in the presence of repeated sequences in the region of resected 3' overhangs. Rad52p and the replication protein A (RPA) may initiate SSA repair between repeated sequences (van Dyck, 1999). The Rad52p binds to ssDNA 3' ends (Stasiak et al., 2000), whereas RPA is bound to 3' ssDNA overhang (Wold, 1997). The region between annealed repeats is flipped out on either side. It serves as a substrate for ERCC1/XPF endonuclease (Sargent et al., 1997; Sargent et al., 2000). The product formed by SSA is a deletion. The pathway is therefore highly error-prone yet it has been shown to be frequently used in the repair of repetitive sequences (Liang et al., 1998).

2.2.4. Mismatch repair

DNA mismatch repair is one of the most important repair pathways that target base-base mismatches and insertion/deletion loops (IDLs). Mismatches arise frequently during DNA replication and homologous recombination (Evans and Alani, 2000; Harfe and Jinks-Robertson, 2000) and can be the result of DNA damage (Harfe and Jinks-Robertson, 2000).

MMR-defective human tumor lines display elevated spontaneous mutation rates (Eshleman and Markowitz, 1996). Mutations in MMR pathway were also linked with the cancer predisposition syndrome, hereditary non-polyposis colorectal cancer, HNPCC (Harfe and Jinks-Robertson, 2000; Buermeyer et al., 1999). The importance of MMR is also highlighted by the fact that most of the human tumor lines (SW48; DU145; HEC1A) are defective in MMR genes.

To date the best-characterized MMR pathway is the methyl-directed MutHLS system of *E. coli*. This pathway is able to recognize and repair all base-base mismatches except C:C and small IDLs. MutS proteins act as a homodimer and recognize structurally altered sites (Lamers et al., 2000; Obmolova et al., 2000). MutS has been shown to possess ATPase activity (Obmolova et al., 2000; Junop et al., 2001). In the presence of ATP and a mismatch it recruits the MutL protein. Together they activate MutH, a latent endonuclease that preferentially cleaves the unmethylated strand at the hemimethylated dGATS site (Modrich and Lahue, 1996). Nicking of the newly synthesized unmethylated strand ensures that the repair activity will take place in the strand actually containing the error. MutL was also shown to contain an ATP processing site and is able to undergo a conformational change when ATP binding and hydrolysis occurs (Ban and Yang, 1998; Ban et al., 1999). It was shown that MutL, but not MutS, interacts with MutH and this interaction stimulates endonuclease activity of MutH (Hall and Matson, 1999). A conformational change of MutS upon ATP binding may serve to activate MutL, triggering downstream repair events (Obmolova et al., 2000). MutL may also recruit the next MMR protein, helicase II (UvrD) to the site of a nick. Unwinding DNA (Mechanic et al., 2000). The strand containing a nick and unwound by helicase II is degraded by

exonucleases of different polarities. If the nick is located 5' to the mismatch, degradation is initiated by ExoVII or RecJ (Cooper et al., 1993). If it is located 3' it is processed by ExoI or ExoX in 3'-5' direction (Viswanathan and Lovet, 1999). Finally, the gap is specifically filled by the DNA polymerase III holoenzyme in a step which requires a single-strand binding protein SSB (Modrich and Lahue, 1996). The remaining nick is then ligated by DNA ligase.

In eukaryotes general features of MMR are conserved (Kolodner, 1996), such as 7 MutS homologs (MSH1-7) and 4 MutL homologs (MLH1-3 and PMS1) (Mansour et al., 2001; Tornier et al., 2001). Yeast MSH1 has a function in the protection of mitochondrial DNA (Chi and Kolodner, 1994; Sia et al., 2000). As in yeast MutS homologues have been identified in mammals. They comprise 4 genes, MSH2-5; the MSH1 orthologue is missing (Drummond et al., 1995; Palombo et al., 1995). MutL homologues in humans are represented by four genes – PMS1, PMS2, MLH1 and MLH3. Human PMS2 corresponds to yeast PMS1 and human PMS1 does not have a clear orthologue in *S. cerevisiae* (Raschle et al., 1999; Lipkin et al., 2000).

In yeast and mammals, MMR is initiated by the heterodimeric complexes MSH2-MSH6 (MutS α) and MSH2-MSH3 (MutS β). MutS α recognizes base-base mismatches whereas MutS β recognizes IDLs with about eight unpaired nucleotides. IDLs with one unpaired nucleotide can be redundantly recognized by both heterodimers (Acharya et al., 1996; Macpherson et al., 1998). Coimmunoprecipitation has shown that four MutL homologues form three heterodimers with Mlh1p as a common component (Wang et al., 1999). Similarly, in yeast and mammals, MLH1-PMS1 and MLH1-PMS2 play major roles in postreplication repair (Harfe and Jinks-Robertson, 2000). MLH1-MLH2 and

MLH1-MLH3 have more specialized roles in yeast MMR (Flores-Rozas and Kolodner, 1998; Harfe et al., 2000). Mammalian MLH1-MLH3 and MLH1-PMS1 have also been shown to play minor roles in MMR (Kato et al., 1998; Raschle et al., 1999).

Unlike *E.coli*, initial steps of MMR in yeast and human involve proliferating cell nuclear antigen (PCNA) that interacts both with MSH and MLH heterodimers (Johnson et al., 1996; Gu et al., 1998; Clark et al., 2000). It has been proposed that PCNA enables specific binding of MutS α to mismatched DNA and subsequently recruits the MutL heterodimer (Bowers et al., 2001).

The downstream steps of MMR in mammals and yeast are performed by the only identified, candidate exonuclease EXO1. EXO1 shows interactions with MSH2, MSH3 and MLH1 (Schnutte et al., 2001; Tran et al., 2001). Exo1 is a 5'-3' exonuclease, *S. cerevisiae* strains defective for this gene show only a mild mutator phenotype, suggesting a possible redundancy with other exonucleases (Sokolsky and Alani, 2000). Finally the DNA gap is filled by DNA polymerases δ and ϵ , that also are implicated in 3'-5' exonuclease activity of MMR (Tran et al., 1999).

2.3. DNA repair in plants

Being sedentary organisms, plants, unlike animals, cannot avoid the influence of harmful genotoxic agents (Ries et al., 2000). It is expected that plants would evolve extremely robust molecular mechanisms of DNA damage repair. To date our knowledge about DNA repair in plants lags far behind data collected about animal systems.

2.3.1. Direct reversion of DNA damage

There are two major strategies of DNA repair in plants: direct reversion and excision of DNA damage. Both are present in plant organisms. Direct reversion is primarily applied to UV induced photoproducts – CPDs and 6-4 PPs. It has been shown that these types of DNA damage can be reversed after subsequent exposure to radiation in the 360-420 nm range (UV-A to blue). This phenomenon was termed “photoreactivation” and is due to the action of proteins named photolyases. These enzymes specifically recognize and bind to pyrimidine dimers. They contain two chromophores. The first is flavin cofactor (FADH) which acts as a transient electron donor to reverse the crosslink between the bases. The second acts as an antenna pigment to excite the electron donor (Sancar, 1994). In general photoreactivation results in reversal of several UV induced effects in plants including mutagenesis, chromosome rearrangements (Ikenaga and Mabuchi, 1966), inhibition of growth, induction of flavonoid pigments (Beggs et al., 1985) and unscheduled synthesis of DNA (Jackson et al., 1979). *Arabidopsis* has been shown to contain two specific photolyases corresponding to genes UVR2 and UVR3, for both types of UV photoproducts (Jiang et al., 1997).

CPD repair by photolyases is regulated by visible light. For instance, the CPD repair activity in the common bean is enhanced two fold by subsequent exposure to red light. This effect however, is reversed by exposure to far red light suggesting that the induction is phytochrome mediated (Langer and Wellman, 1990). In *Arabidopsis* the intensity of CPD reversion depends on exposure to visible light prior and after UV irradiation (Chen et al., 1994). Similarly, *Arabidopsis* possesses a light dependent pathway for 6-4 PPs repair (Chen et al., 1994). In contrast to the CPD-specific photolyase

activity, it doesn't require prior induction by visible light and is not dependent on the UVR1 gene product essential for dark repair of 6-4 photoproducts (Britt et al., 1993) and possesses the ability to photoreactivate both of the major UV photoproducts. This photolyases activity probably extends to other plant species as it is also characterized in wheat seedlings (Taylor et al., 1995).

2.3.2. Base excision and nucleotide excision in plants

DNA damage that cannot be reversed directly is processed in pathways that include base excision and nucleotide excision repair. As mentioned above base excision repair involves different types of damage specific glycosylases that remove single damaged bases leaving the an AP site. The intact DNA molecule is then restored via the combined action of exonucleases, gap filling DNA polymerase and ligase.

In plants, uracil incorporated opposite to adenine is likely removed by an ortholog of the Ung protein. Although the corresponding protein has not been identified, its activity has been purified from several plant sources (Bones, 1993; Talpaert-Borle, 1987). Plants, however, appear to lack the so called Mug glycosylases, found in bacteria and mammals but not in yeast, so they may also use Ung activity to remove uracil produced via cytosine deamination. Plants don't have orthologs to human MBD4 and TGD, required to remove uracil and thymine opposite to guanine. However, turnip plants are able to remove thymine from G:T mismatches (Riederer et al., 1992).

The *Arabidopsis* genome encodes a surprisingly large number of alkyl-purine glycosylases similar to Tag glycosylase of *E. coli*. Some of these glycosylase genes are also seen in sugarcane and rice (Costa et al., 2001; Yu et al., 2002). The product of the

Tag gene is highly specific for 3-methyladenine (Evensen and Seeberg, 1982; Karran et al., 1982). Another *E. coli* alkyl-purine glycosylase – AlkA has a broad substrate specificity that includes 7-methylguanine, 3-methylguanine, O2-methylthymine and O2-methylcytosine (Karran et al., 1982). *Arabidopsis* encodes orthologs for AlkA, its yeast ortholog and mammalian Aag. That points to an interesting phenomenon of coexistence of several dissimilar glycosylases with similar substrate ranges.

8-oxoguanine is a highly mutagenic lesion due to its ability to basepair with adenine (Maki and Sekiguchi, 1992). Plants possess a gene functionally orthologous to both human Ogg1 and bacterial MutM (FPG) proteins (Garcia-Ortiz et al., 2001; Dany and Tissier, 2001). Bacterial MutM removes 8-oxoguanine from 8oxoG:C base pairs and recognizes the ring-opened formamidopyrimidine products of adenine and guanine (FaPy-A and FaPy-G) (Boiteux et al., 1990; Tchou et al., 1991). The human and yeast OGG1 gene have been cloned via complementation of bacterial mutM mutator phenotype, suggesting that they play similar roles in 8-oxoguanine removal (Radicella et al., 1997; Rosenquist et al., 1997). AtFPG is encoded by two splice-variant cDNAs (Gao and Murphy, 2001). The longer protein AtFPG-1 is targeted to the nucleus, while AtFPG-2 is suggested to enter both organelles. The two forms also differ in substrate specificity. Both cleave depurinated DNA and remove FaPy-G sites, however AtFPG-1 but not AtFPG-2 cleaves at 8-oxoguanine sites in short oligoduplexes (Gao and Murphy, 2001). MutM activity is complemented in bacterial systems by other glycosylases. MutY recognizes 8-oxoG:A pairs and removes adenine providing the second opportunity to avoid mutation (MacGoldrick, 1995). MutT acts as a triphosphatase hydrolyzing 8-oxodGTPs to 8-oxodGMPs an inorganic phosphate therefore depleting the available

nucleotide precursor pool (Mo et al., 1992). *Arabidopsis* also encodes the MutY ortholog and protein similar to human and bacterial MutT enzymes. Plants therefore possess various glycosylases with a similar substrate range, displaying a high level of redundancy.

Bulky helix-distorting lesions, including major UV photoproducts and other types of DNA damage are recognized and removed by NER proteins. Light independent repair is much slower than direct reversion (Britt et al., 1993). Most NER proteins that mediate recognition, initiation of repair, incision and removal are highly conserved among species including yeast and humans (Sancar, 1996). A GenBank search including a nearly complete *Arabidopsis* genomic sequence revealed that higher plants possess obvious homologs to many of the eucaryotic genes required for NER (Britt, 1999). The presence of NER pathways in plants was confirmed by the discovery of light-independent CPD repair in several plant species (MacLennan, 1987). The rate of dark repair of CPDs was found to vary widely between plant species, with a high rate of repair demonstrated in the case of a carrot suspension culture (Howland, 1975), protoplasts of carrot, petunia and tobacco (Howland, 1977), whereas it was undetectable in cultured soybean cells (Reilly, 1980). Some evidence suggests also that NER is a primary repair pathway in proliferating cells, while in non-proliferating UV photoproducts are repaired via photoreactivation (Kimura et al., 2004). NER seems to be especially recruited in the presence of high dose UV (Costa et al., 2003).

A complex of XPC (RAD4) and HR23B (RAD23) has been shown to initiate global genomic repair (GGR) via the recognition of the DNA damage (Volker et al., 2001; Yokoi et al., 2000). Neither the single nor the four apparent *Arabidopsis* RAD23

homologues has been associated yet with UV sensitive mutants (Hays, 2002). This could be caused by redundancy and/or lesion specialization of the latter proteins. The process of damage recognition in transcription coupled repair (TCR) requires two proteins: CSA and CSB. *Arabidopsis* encode several CSB and two CSA like proteins. The role of latter proteins is yet unknown (Hays, 2002). There is no direct evidence, however, indicating the presence of TCR in plants. An important player recruited for NER is the multi-subunit transcription complex TFIIN including XPB and XPD helicases required for the opening of the double-helix around a lesion. Recent findings confirmed that the *Arabidopsis* UV sensitive mutant *uvh6-1* carries the mutation in XPD/RAD3 homologue (3'-5' helicase) (Liu et al., 2003). The search for a homologue of the NER 5'-3' helicase XPB/RAD25 in plants surprisingly revealed that this gene has undergone a duplication event in *Arabidopsis*. Complementation assays in yeast *rad25* mutant strains suggested the involvement of AtXPB2 in DNA repair as already was shown for AtXPB1 (Costa et al., 2001), indicating that these proteins may be functionally redundant in the removal of DNA lesions in *A. thaliana* (Morgante et al., 2005). The duplicated genes were named AtXPB1 and AtXPB2 (Ribeiro et al., 1998; Costa et al., 2001). In *Arabidopsis thaliana*, disruption of AtXPB1 was not lethal possibly due to the duplication. However, AtXPB1 deficient plants display developmental delay, lower seed viability, loss of germination synchrony, and an increased sensitivity to alkylating agents (Costa et al., 2001).

Another key player in the NER pathway – XPG 3' endonuclease has been related an *Arabidopsis* mutant that exhibited hypersensitivity to UV light, peroxide and ionizing radiation (Liu et al., 2001). In *S. cerevisiae* and humans the incision 5' to the DNA lesion is carried out by the RAD10/RAD1 (human ERCC1/XPF) complex (Davies et al., 1995).

Arabidopsis encodes single orthologs of each and the mutation of AtXPF causes UV sensitivity, decreased removal 6-4 PPs in the dark and surprisingly high sensitivity to ionizing radiation (Liu et al., 2000; Fidantsef et al., 2000; Gallego et al., 2000). The *Arabidopsis* ERCC1 mutant has displayed increase of IR sensitivity as well (Hefner et al., 2002). *Arabidopsis* doesn't encode *E. coli* NER recognition/incision proteins UvrA, UvrB and UvrC, but surprisingly there are homologs of Mfd protein, which mediates transcription-coupled NER in *E. coli*, and of the UvrD/MutU/RecL protein. The latter protein plays essential roles in post-recognition and incision steps in bacterial NER and MMR, and appears to be involved in some recombinational processes (Hays, 2002). As in BER, plants seem to display a certain redundancy in the NER pathway. Interestingly some NER proteins appeared to be involved also in double-strand break repair and recombination.

2.3.3 Double-strand break repair in plants

Our knowledge of proteins involved in DSB repair in plants lags far behind our knowledge of these pathways in bacteria, yeast and mammals (Gobunova and Levy, 1999). A number of UV sensitive *Arabidopsis* mutants have been isolated. Further analysis has shown that some display sensitivity to IR (Jenkins et al, 1995; Jiang et al., 1997). This suggests their possible involvement both in excision and DSB repair. Another group of mutants only displayed increased sensitivity to gamma-radiation (Davies et al., 1994). Similarly X-ray sensitive mutants were simultaneously sensitive to either mitomycin C or methylmethane sulfonate (MMS) but not to UV-C light (Masson and Paszkowski, 1997).

As organisms with a large and highly repetitive genomes, plants appear to share with mammals a primary dependence on NHEJ instead of HR (Vergunst and Hooykaas, 1999; Puchta et al., 1996) NHEJ however, has been proven to be a highly error-prone pathway (Mengiste and Paszkowski, 1999; Gorbunova and Levy, 1997). NHEJ proteins in plants are similar to those in yeast and mammals with some interesting exceptions and redundancies. The first step of NHEJ is recognition and binding of the exposed DNA ends by a heterodimer of KU70 and KU80 proteins (KU) (Dyran and Yoo, 1998). *Arabidopsis* homologues of KU70 and KU80 have been recently isolated (Tamura et al., 2002, West et al., 2002). The importance of these proteins in yeast and mammals is manifested by the extreme IR sensitivity of mammalian cell lines containing mutations in any of these genes (Li et al., 2002). Cells from KU70 and KU80 mutant mice display high levels of spontaneous chromosomal abnormalities underscoring their critical role in genomic stability (Difilippantonio et al., 2000). In contrast to mammalian mutants, *Arabidopsis* plants defective in KU70 or KU80 genes are viable, fertile and developmentally normal in the absence of damaging factors (Tamura et al., 2002; West et al., 2002). Both AtKU80 and AtKU70 mutants are, however sensitive to IR and MMS but not to UV-C light (West et al., 2002; Bundock et al., 2002). KU70 mutants in *Arabidopsis* display enhanced telomere length (Bundock et al., 2002) while animal cell lines lacking KU70 have shortened telomeres (Bertuch and Lundblad, 1998). It is not yet known if the KU80 protein is absolutely required for the NHEJ repair pathway in plants. There is data indicating that AtKU80 is not required for DNA end-binding activity *in vitro* (West 2002). In vertebrates a third protein, DNA dependent protein kinase catalytic subunit (DNA-PK_{cs}), binds to the KU dimer and has a role in intracellular DNA damage

signaling (Smith and Jackson, 1999). Like yeast, *Arabidopsis* doesn't encode an obvious putative homologue of DNA-PK_{cs} suggesting that DNA damage signaling pathways may differ significantly in yeast, plants and vertebrates. The *S. cerevisiae* cell-cycle checkpoint proteins Rad9 and Mec1, which have homologues in plants and mammals may play a role in DSB signaling in yeast (Lustig, 1999; Mills et al., 1999). A similar role for these proteins in *Arabidopsis* is possible.

Mammalian KU also interacts with MRE11. This is a component of RAD50/Mre11/Mbs1 responsible for DNA end processing in NHEJ and HR (Goedecke et al., 1999). *Arabidopsis* encodes both RAD50 and Mre11 orthologs. The third component is also an ortholog of human Nbs1 but not yeast Xrs2 contributing to the similarities between human and yeast NHEJ (Gallego et al., 2001; Bundock and Hooykaas, 2002). In yeast cells mutations of RAD50, Mre11 and Xrs2 give rise to shortened telomeres (Boulton and Jackson, 1996; Nugent et al., 1998; Boulton and Jackson., 1998). RAD50 deficient *Arabidopsis* plants also display shortened telomeres (Gallego et al., 2000), suggesting that *Arabidopsis* RAD50/Mre11/Nbs1 complex influence telomere length maintenance .

The KU70 dimer also recruits the DNA ligaseVI/XRCC4 complex to the site of the lesion and stimulates its activity (Ramsden and Gellert, 1998). Homologues of Ligase IV and XRCC4 have been recently identified in *Arabidopsis* (Taylor et al., 1998; West et al., 2000). DNA ligase IV deficient plants are sensitive to IR and undergo developmental arrest after treatment with doses of gamma-irradiation that do not affect wild-type plants. However in contrast to animal models, in which mouse knockouts of Ligase IV (LigIV) are lethal, *Arabidopsis* mutants are viable and fully fertile (Frank et al., 1998; Friesner

and Britt, 2003). High IR sensitivity of LigIV *Arabidopsis* mutants suggests its role in DSB repair. However, their viability in comparison to animal models suggests that plants either can tolerate much higher rate of DSBs or LigIV activity can be partially substituted. The ERCC1 *Arabidopsis* mutant also displays increased sensitivity to gamma-irradiation, however, it is viable in contrast to ERCC1 deficient mutants in mammals (Hefner et al., 2003; Selfridge et al., 1995).

It has been shown that ionizing radiation could induce crossing over between homologous chromosomes. This suggests that not only NHEJ but HR may play a role in DSB repair (Carlson, 1974). Recent studies have discovered that HR in plants plays a role in enhancing T-DNA integration at homologous loci (Puchta, 2002; Reiss et al., 2000), repairing a DSB with homologous sequences close to the break (Siebert and Puchta, 2002; Xiao and Peterson, 2000) or at an ectopic position in the genome (Puchta, 1999). It has been demonstrated that homologous recombination is a significant DSB repair pathway in plants and that both ends of the break are processed independently in a step-wise fashion (Puchta, 1999). HR can take place between sister chromatids as well as between homologous chromosomes. A recent study has shown that DSB induced allelic recombination. In this case the break is repaired by a faithful copying process from the homologue, however it occurs at the same order of magnitude as between ectopic sites, and therefore is too infrequent to act as a major repair pathway. These point to the prevalence of intrachromatid rearrangements over allelic recombination (Gisler et al., 2002).

The RecA protein is a key player involved in HR repair pathways in bacteria. It catalyzes exchange between two homologous DNA strands and is capable of providing

homologous DNA pairing at double-strand breaks (Cox, 2000). RecA homologues have been identified in all bacterial species as well as in many eucaryotes, including plants (Angulo et al., 1985; Ogawa et al., 1993; Sato et al., 1995). The *Arabidopsis* homologue of RecA targeted to mitochondria also has been found (Khazi et al., 2003). In yeast the homology to RecA protein is shared by Rad51 and another gene – DMC1 (Thaker, 1999). Similar to bacterial RecA, the yeast Rad51 acts in homology search, DNA pairing and strand exchange (Haber, 2000). The loss of RAD51 function is lethal in both chicken DT40 and mouse cells but is not lethal in yeast (Thaker, 1999). RAD51 has been shown to interact directly and colocalize with nuclear loci formed after DNA damage. (Pellegrini et al., 2002; Yu et al., 2003). Yeast RAD51 mutations also cause meiotic defects including an accumulation of meiosis-specific DSBs and reduced formation of physical recombinants (Shinohara et al., 1992). The meiotic function of RAD51 seems to be conserved among species as it was shown to localize to recombination foci along early meiotic chromosomes in yeast, maize and mouse (Ashley et al., 1995; Anderson et al., 1997; Franklin et al., 1999). Detailed analysis of RAD51 foci localization during normal and mutant maize meiosis has shown that Rad51 is important for homologous chromosomes pairing in addition to its role in recombination (Pawlowski et al., 2003; Franklin et al., 2003). In contrast to a RAD51 deficient vertebrates, loss-of-function *Arabidopsis* exhibit normal vegetative growth and flower development and has no detectable abnormality in mitosis, however they are completely male and female sterile. AtRad51 likely plays a critical role in the repair of double-stranded breaks generated by AtSpo11-1. This suggests that the Rad51 function is essential for chromosome pairing and synapsis formation at early stages of meiosis in *Arabidopsis* (Li et al., 2004). Most

accessory proteins associated with the RecA-dependent RecBCD and RecFOR pathways in *E. coli*, including the “title-role” proteins are absent in *Arabidopsis* (Hays, 2002).

The *Arabidopsis* DMC1 protein, which is a structural homologue of RecA, has been implicated in meiosis. Inactivation of DMC1 in *Arabidopsis* severely disturbs chromosome behavior during both male and female meiosis and reduces fertility to 1.5% of normal rates (Couteau et al., 1999). It is interesting that *Arabidopsis*, but not yeast, encodes proteins similar to human BRCA1 and BRCA2. Their function in *Arabidopsis* is yet to be elucidated. Recently BRCA2 protein was shown to interact with DMC1 protein and its function is crucial for meiotic DSBs repair (Siaud et al., 2004).

The number of the genes involved in NER pathway has been related also to HR in plants. The Rad1p/Rad10p heterodimer plays a crucial role in NER as a 5' structure specific endonuclease (Davies et al., 1995). In plants it was shown to be involved in the removal of UV induced photoproducts (Liu et al., 2000). Unlike Rad10 and Rad1 mutants in mammals and yeast, plants deficient in AtRad1 are also hypersensitive to IR (Harlow et al., 1994; Jenkins et al., 1995; Jiang et al., 1997). Consistent with this, recent findings have shown that AtRad1 plays a role in the removal of non-homologous tails in HR (Dubest et al., 2002). It is not surprising that a recently characterized *Arabidopsis* homologue of Rad10 – AtErcc1 displays 12-fold reduced recombination between plasmid DNA substrates containing non-homologous tails. It is also required for bleomycin induction of mitotic recombination in the chromosomal context (Dubest et al., 2004).

The function of Rad50/Mre11/Nbs1 is also conserved in plants. *Arabidopsis* homologues of Rad50 (AtRad50) and Mre11 (AtMre11) have been identified and shown to form a complex (Daoudal-Cotterel et al., 2002). Mutants defective for AtRad50 and

AtMre11 are sterile, suggesting a role for this complex in plant meiosis (Bundock and Hooykaas, 2002; Gallego et al., 2001). A recent study has shown that both male and female gametogenesis are defective in the *Arabidopsis* AtRad50 mutant and cytological observation of male meiosis indicates that in the absence of the AtRad50 protein, homologous chromosomes are unable to synapse. This suggests a role for the *Arabidopsis* Rad50/Mre11/Nbs1 complex in the early stages of meiotic recombination (Bleuyard et al., 2004).

Arabidopsis also encodes six homologues of RecQ helicase (Hartung et al., 2000). RecQ helicase is an initiator of homologous recombination in an *E. coli* recBCsbcBC mutant (Nakayama et al., 1985). It is functional not only in the initiation step for producing the single-strand substrates, but also in the latter steps for moving the Holliday junction and resolving the recombined molecules (Harmon and Kowalczykowski, 1998). Recent findings have shown that transient expression of the bacterial RecQ gene in rice embryogenic cells increases the homologous recombination efficiency as much as 4-fold, and stable expression even 20-40-fold in leaf tissue from different transgenic lines (Li and Li, 2004). One of the *Arabidopsis* RecQ homologues RecQsim has been shown to suppress the MMS hypersensitivity of the yeast Sgs1 mutant (Bagherich-Najjar et al., 2003). *Arabidopsis* homologues of RecQ helicases include both proteins similar to human helicases, which defects are associated with Werner's syndrome and Bloom's syndrome, as well as more direct homologues of bacterial RecQ (Hays, 2002).

2.4. Systems used in DNA damage analysis

Various systems have been developed to help researchers in the evaluation of DNA damage in different organisms. Such systems serve a number of purposes, like estimation of the DNA damage caused by various genotoxic agents, biomonitoring of environmental pollutants, functional study of DNA repair related genes. These assays can be based on either *in vivo* or *in vitro* systems. *In vivo* systems exist in a number of eucaryotic and prokaryotic organisms and often utilize specially constructed transgenes.

Among the tests suitable for toxicity monitoring the *Allium* test (*Allium cepa*, common onion) is well known and commonly used (Fiskesjo, 1988). The *Allium* test is a useful tool for quantitative measurements of changes in the onion genome and thereby to evaluate cyto- and genotoxic effects of various substrate pollutants. This test provides a rapid and reliable screen of toxic effects caused by mutagenic chemicals and heavy metal ions (Grant, 1994; Grant, 1982; Fiskesjo, 1988). Also it was shown to be effective in the evaluation of cyto- and genotoxicity of chronic irradiation (Kovalchuk et al., 1998). The test has been used extensively for wastewater monitoring and it is the first of nine plant assays in the Gene-Tox Program of the US Environmental Protection Agency (Grant, 1994; Constantine and Owens, 1982; Rank and Nielsen, 1994; Smaka-Kincl et al., 1996). Results obtained from this assay support other test systems such as V79 Chinese hamster fibroblast and human lymphocytes (Pavlica et al., 1991; Fiskesjo, 1995). Mutagenic environmental effects can be analyzed by microscopic parameters such as root shape and root growth and cytological parameters such as the types and frequencies of chromosomal aberrations and cell divisions (Nielsen and Rank, 1994; Fiskesjo, 1997; Grinikh and Shevchenko, 1992). The *Allium* test is a relatively non-expensive and

simple system to monitor the cyto- and genotoxicity of environmental chemicals and radioactive pollutants. The onions are easy to store and to handle, and the root tip cells constitute a convenient system for macroscopic as well as for microscopic parameters (c-mitosis; stickiness; chromosome breaks) (Fiskesjo, 1988). The system has number of limitations. It requires a rather time-consuming microscopy step, and also cannot be used in genetical studies.

Additional plant assays along with the *Allium* test are the *Tradescantia* stamen-hair system and the *Vicia faba* chromosomal aberration tests. The stamen-hair system of *Tradescantia* is an excellent botanical tester of mutagenicity. It can be used for the detection of the genetic effects of both ionizing radiation and chemical mutagens at low levels of exposure. The system exhibits high accuracy in laboratory experiments, and is also applicable for *in situ* monitoring of an environment. The use of this system is inexpensive and requires limited training time (Ichikawa, 1992). The *Vicia faba* chromosomal aberration assay was also shown to be an efficient and reliable short-term bioassay for the rapid screening of chemicals for clastogenicity (Kanaya et al., 1994).

A useful approach in study of recombination is the engineering of non-functional overlapping truncated cassettes carrying a marker gene (Paques and Haber, 1999; Hellgren, 1992; Lambert et al., 1999). Overexpression of the construct in cultured cells allows the activity of a restored gene to be visualized. Direct-repeat substrates provide evidence that HR contributes between 30-50% of DSBs repair in mammalian cells (Liang et al., 1998). Direct repeats are also useful in genetic studies of DNA repair. The application of this system revealed that many tumor-suppressor genes including BRCA1, BRCA2, ATM, WRN, MSH2 and NBS1 modulate spontaneous mitotic homologous

recombination in mammalian cells (Moynahan et al., 2001; Bishop et al., 2000; Moynahan et al., 2001; Elliot and Jasin, 2001; Saintigni et al., 2001; Tauchi et al., 2002). There are also animal based models available (Murli et al., 1992; Moynahan et al., 1996; Mathis et al., 1997; Carrie et al., 2003; Burkhart, 2000). For instance, the fluorescent yellow direct repeat (FYDR) was used in a mouse system (Carrie et al., 2003). It consists of two different copies of expression cassettes for truncated coding sequences of enhanced yellow fluorescent protein (EYFP), arranged in tandem. HR between these repeated elements could restore a full-length EYFP coding region and yield fluorescent phenotype that can be detected by flow cytometry. Analyses have shown that this system detects gene conversions based on the arrangement of the integrated recombination substrate, unequal sister-chromatid exchanges and repair of collapsed replication forks (Carrie et al., 2003). This system although expensive and requiring complicated and time-consuming techniques, was shown to be very effective.

Mice systems allow analysis of mutagenic and cytotoxic effects produced by certain compounds. One system is based on transgenic mice carrying human growth hormone (hGH) gene under the control of the human hsp70 promoter. HGH response can be elicited by heat shock and also by toxic compounds injected intraperitoneally (Sacco et al., 1997).

Another animal model useful for mutation studies is based on zebra fish (*Brachidanio rerio*) (Amanuma et al., 2000). This system uses a shuttle plasmid that can be moved from zebra fish to bacteria that will detect mutagens based on the acquisition of mutation induced streptomycin resistance. One of the advantages of such species as zebra fish is their transparent eggs, which allow the study of mutagenesis to be easily combined

with rapid evaluation of development (Burkhart, 2000). Fish provide one of the best opportunities in vertebrates to study chronic low-dose exposures to mutagenic factors with accuracy and control using flow-through or renewal designs that can easily span generations (Burkhart, 2000). Unlike rodent systems, transgenic fish can be effectively used for environmental monitoring.

An interesting transgene system was used in plants to study double-strand breaks and genome evolution (Kirik et al., 2000). It was based on a transgene harboring an 18mer recognition site for I-SceI within the negative selectable marker gene cytosine deaminase (Stougaard, 1993). When DSB is induced via *Agrobacterium*-mediated transient expression of I-SceI (Puchta et al., 1996), its repair results in genomic alterations associated with the loss of marker-gene function the cells become selectable by their resistance to 5-fluorocytosine (Salomon and Puchta, 1998).

An extrachromosomal *in planta* system has been applied recently to study possible contribution of NHEJ to DSB repair (Kovalchuk et al., 2004). It was based on a stop codon inactivated reporter gene. DSBs have been introduced in different places of the inactivated reporter (GUS). Point mutations that arise during the course of repair lead to the reversion of stop codon and activation of the GUS gene. This system, also used in this study, allows detection of point mutations as an outcome of DSB repair.

In plants transgenic recombination systems have been used to study DNA repair since early the 90^s. The recombination assays were based on the restoration of two non-functional partially overlapping parts of the marker gene (Lebel et al., 1993; Swoboda et al., 1994). The first assay included kanamycin as a marker gene for the detection of recombination events. This assay could be used only for screening of meiotic

recombination or inherited somatic events. The frequency of somatic recombination could be analyzed only following the generation of callus material and this restricted use of this assay. The second assay appeared to be much more useful. Based on the recombinational restoration of the β -glucuronidase (GUS) gene, it provided relatively simple and inexpensive method to evaluate HR frequency throughout the plant life span. The recombination substrate in this case contains two non-functional deletion mutants of chimeric GUS gene with 566 bp of overlap in inverted orientation. Following the recombination, as proven by Southern blot analysis, a functional gene is restored and its product can be detected via histochemical staining that results in blue spots and sectors on the leaves. Cells in which recombination events occurred, and their progeny, can be precisely localized in the whole plants using a dissecting microscope. This system is currently used in a variety of DNA repair studies. These include the use of transgenic plants as biomonitors (Kovalchuk et al., 1998; Kovalchuk et al., 2001), to study various DNA damaging agents (Ries et al., 2000; Ries et al., 2000; Kovalchuk et al., 2003) and the induction of HR in various plant organs at different stages of the life cycle (Swoboda et al., 1994). The most notable disadvantage of this system is that plants are destroyed during the staining procedure and therefore a dynamic study of HR is impossible.

This drawback was recently overcome by the creation of a new luciferase-based (LUC) assay. The system is constructed similar to the GUS system but allows HR event observation without killing the plants (Gorbunova et al., 2000; Kovalchuk et al., 2003; Filkowski et al., 2004). This was a significant breakthrough, as the system allowed the analysis of recombination and influencing factors within the intact plant. The LUC-based system allowed the analysis of the dynamics of the appearance of recombination

events during the application of biotic stress (Kovalchuk et al., 2003). Moreover this assay appeared to be more sensitive than the GUS based system and therefore allows the average HR frequency in plants to be reevaluated (Ilnytskyy et al., 2004).

The transgenic system based on the restoration of marker gene activity is also available for point mutation studies in plants (Kovalchuk et al., 2000). To analyze point mutations the transgenic lines carry a GUS construct with single-nucleotide substitutions that lead to the formation of a stop codon. It was shown that only a reversion to the original nucleotide can restore enzyme activity (Kovalchuk et al., 2000) and therefore point mutations could be detected by histochemical staining. This system was effectively used for biomonitoring of genotoxic environmental factors (Kovalchuk et al., 2001) and to study genome-wide variation of somatic mutation frequency in *Arabidopsis* (Kovalchuk et al., 2000). The transgenic construct used in the current study contains a substitution (GGA → TGA) creating a stop codon at position 166 (Kovalchuk et al., 2000). The stop codon was generated in a triplet followed by adenine or guanine, which had been shown to be essential for efficient termination of translation (Atkinson and Martin, 1994; Betzner et al., 1997). Point mutations that restore the original triplet lead to the activation of the GUS gene driven by 35S promoter (Kovalchuk et al., 2000).

2.5. Selected DNA repair related genes in *Arabidopsis*

2.5.1 Atm

The deficiency of the ATM gene is responsible in humans for Ataxia-telangiectasia (AT), an autosomal recessive disorder characterized by progressive neurodegeneration, immunodeficiency and a predisposition to cancer (Sedgwick et al., 1991). Mammalian ATM has been found to play a critical role in cellular responses to ionizing radiation and in normal cell-cycle progression and meiosis (Xu and Baltimore, 1996; Rotman and Shilon, 1998). It is believed to function in response to DSBs that occur as a result of IR and due to normal cellular processes (Rotman and Shilon, 1998). ATM deficiency in mammals also results in sterility and severe meiotic disorders, possibly due to the incapability of the cells to repair DNA breaks that occur normally in meiosis (Barlow et al., 1998; Rotman and Shilon, 1998). This gene is a member of the phosphoinositide 3-kinase (P13-kinase) family that includes the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) and the RAD3 related protein (ATR). All are involved in the recognition of damage in DNA (Abraham, 2001). The ATM protein is involved not only in DNA damage cell cycle checkpoints but also in the regulation of DNA repair activities and apoptosis (Garcia et al., 2003). It is believed that ATM responds specifically to double-strand breaks, as judged by the sensitivity of cells from AT patients to ionizing but not to UV-B irradiation (Xu and Baltimore, 1996). The mechanism of DNA damage recognition is yet unknown. ATM deficient mutants are defective in repair only a small component (10%) radiation-induced breaks (Foray et al., 1997).

ATM is rapidly activated following radiation by a mechanism involving autophosphorylation on ser1981 followed by dissociation into an active monomer (Bakkenist and Kastan, 2003; Kozlov et al., 2003). The activation of ATM triggers a phosphorylation cascade which leads to cell-cycle arrest at various check points (Zhou and Elledge, 2000). It also may signal DNA repair complexes, for example via the phosphorylation of proteins such as BRCA1 (Cortez et al., 1999), NBS1 (Gatei et al., 2000), and c-ABL (Baskaran et al., 1997), which in turn phosphorylates RAD 51. NBS1 might play a key role in this process. It acts as an adapter protein for ATM-dependent phosphorylation of Chk2, FANCD2 and SMC1 (Buscemi et al., 2001; Nakanashi et al., 2002; Yadzi et al., 2002). Mre11 also becomes phosphorylated in response to IR damage in ATM-dependent fashion, although no specific sites for this have been identified (Yuan et al., 2002; Yuan et al., 2002; Dong et al., 1999). Therefore ATM is positioned at the top of an intricate regulatory network, which allows the cell to sense DNA damage and choose responses ranging from the activation of DNA repair to cell cycle arrest and apoptosis.

The *Arabidopsis* homologue of ATM – AtATM spans over 30 kb and contains 79 exons. It is a single copy gene . Transcription into 12 kb mRNA is ubiquitous and not induced by ionizing radiation (Garcia et al., 2000).

AtATM mutants are partially sterile and hypersensitive to ionizing radiation (IR), methylmethane sulfonate but not to UV-B. Partial sterility of ATM mutants in *Arabidopsis* is caused by a major meiotic disorder, which results generally in chromosome fragmentation in anaphase I and II (Garcia et al., 2002). This information confirms the role of ATM in mammals where the ATM deficiency leads to defects in

meiosis and cell cycle arrest (Cato and Ogawa., 1994). This might be the result of a p53-mediated apoptotic response to DSBs. Meiosis has been shown to progress in *atm/p53* double mutants (Barlow et al., 1997). In contrast ATM deficiency in *Arabidopsis* doesn't lead to complete meiotic arrest. This suggests that *Arabidopsis* lacks strong meiotic checkpoints altogether. *Arabidopsis* appears to lack a p53 homologue that is the ATM target in mammals.

AtATM is also known to regulate the transcription of several DNA repair genes AtRAD51, AtPARP1, ATGR1 and AtLIG4 (Garcia et al., 2002). The absence of a transcriptional induction of these genes can provide an explanation for the higher sensitivity of AtATM mutants to IR.

2.5.2. AtXPD

The *Arabidopsis* gene Uvh6 (AtXPD) is a homolog of the human XPD and yeast RAD3 repair genes. AtXPD deficient plants exhibit higher UV sensitivity shown as yellow-green coloration of leaves and mild growth defects (Liu et al., 2003). In humans XPD belongs to one of the Xeroderma pigmentosum complementation groups. This disease results in higher UV sensitivity and usually increased risk of skin cancer. It is the consequence of nucleotide excision repair (NER) disorders. NER is the major pathway that provides protection from UV photoproducts that consist primarily of cyclobutane pyrimidine dimers (Mitchell and Nairn, 1989). It involves recognition of the lesion, incision of the damaged strand on both sides of the lesion, removal of the damaged fragment, repair by gap filling and ligation (Batty and Wood, 2000).

The conservative nature of NER and high structural similarities between AtXPB, XPB and RAD3 and high sensitivity of Uvh6 mutant to UV irradiation suggest that this gene provides similar function in plants (Britt et al., 1993; Fidantsef et al., 2000; Gallego et al., 2000; Liu et al., 2000). Human XPD and yeast RAD3 proteins function as helicases during the DNA repair, presumably unwinding DNA surrounding target lesions to facilitate incision (Winkler et al., 2000). The *Arabidopsis* homologue of XPD is likely to act the same way. The latter one is supported by the structural analysis that confirmed seven highly conserved motifs required for helicase activity (Gorbalenya et al., 1989).

In humans, XPD protein acts not only as a DNA helicase but also as a part of the transcription initiation factor TFIIH (de Boer and Hoeijmakers, 2000; Prakash and Prakash, 2000; Lehmann, 2001). Considerable evidence suggests that human XPD mutations can cause developmental abnormalities due to the defects in transcription. For example trichothiodystrophy is associated with brittle hair and Cockayne syndrome with skeletal deformation (de Boer and Hoeijmaker, 2000; Lehmann, 2001; Viprakasit et al., 2001; Keriél et al., 2002). Interestingly, human TFIIH contains six core proteins and three additional components in an associated kinase (CAK) complex. In contrast only three core proteins and one CAK components appear to be conserved in *Arabidopsis* (Liu et al., 2003). The existence of two expressed AtXPB genes and the low conservation of TFIIH core components suggest that multiple forms of this complex may exist in plants (Costa et al., 2001).

The T-DNA knockout of this Uvh6 gene results in 100% lethality suggesting that this gene is involved in plant development. Uvh6/AtXPD deficient mutant plants derived from EMS treatment are smaller in size, contain subnormal amount of chlorophyll and

have poorly organized grana stacks within the thylakoid membrane. They are also more sensitive to high temperatures. Four days of exposure to 37 °C is lethal for mutant plants but not for wild type (Jenkins et al., 1997). Some clue to the role of AtXPD in plant growth and stress response might be given by the fact that human XPD and yeast RAD3 play a significant role in transcription initiation as part of the TFIIF complex (Lehmann, 2001).

This mutant demonstrates a slightly reduced efficiency in the removal of UV photoproducts (Liu et al., 2003). This finding contrasts with the observation of Jenkins who pointed out that the AtXPD mutant is not DNA repair mutant, but rather exhibits additional phenotypes including failure to grow at elevated temperatures with lower chlorophyll content (Jenkins et al., 1997). Leaves of 14 day old AtXPD (-/-) plants were extremely sensitive to UV-B and UV-C irradiation, however their sensitivity to IR doesn't differ from wild-type plants. This mutant also exhibits significant sensitivity to increased temperatures. Apparently the AtXPD protein plays an important role in the heat shock/UV stress response that is somehow interconnected in plants. In AtXPD(-/-) plants the response to UV and heat shock occurs at a much lower threshold than in the wild-type. This stress response was proposed to lead to a programmed cell death pathway (PCD) (Jenkins et al., 1997).

2.5.3. AtKU80

The Ku70/Ku80 dimer has been shown to bind DNA ends at the site of a DSB and initiate NHEJ pathway (Dynan and Yoo, 1998; Koike et al., 1998; Wang et al., 1998). Structural studies suggest that mammalian KU binds to an exposed DNA ends as a

heterodimer, and the interaction between the two heterodimers brings DNA ends together (Cary et al., 1997; Walker et al., 2001). When bound to DNA, the KU dimers protect the ends from large-scale exonuclease degradation and modification by DNA ligases (Lieber, 1999). In vertebrates, a third protein – DNA-PK binds to KU dimer forming DNA-PK_{cs} that has a role in intracellular DNA damage signaling (Smith and Jackson, 1999). KU may also recruit other components of the NHEJ pathway into the DSB repair complex and may interact with other proteins involved in DNA repair and damage signaling (Lewis and Resnick, 2000; Nick-McElhinny et al., 2000). Ku has also been shown to stimulate DNA ligase activity *in vitro* (Rasmusen and Gellert, 1998). Neither yeast nor *Arabidopsis* contain putative homologues of DNA-PK, suggesting that DSB signaling pathways may differ significantly in this organism from those in vertebrates. In *S. cerevisiae* RAD9 and Mec1 (cell-cycle checkpoint proteins) may play a role in DNA damage signaling, a similar role of their homologues in *Arabidopsis* is possible (Lustig, 1999; Mills et al., 1999). The *Arabidopsis* Ku dimer has been shown to also have ATPase dependent DNA helicase activity, however the mutations in this domain have no effect on its function (Dyran and Yoo, 1998; Tamura et al., 2002; Singleton et al., 1997).

The Ku dimer was also shown to have a role in telomere length maintenance. KU deficient yeast cells display shorter but stable telomeres (Boulton and Jackson, 1996; Porter et al., 1996; Kironmai and Muniyappa, 1997; Nugent et al., 1998). Telomere length is deregulated in Ku80 mutant mouse cells. Contradictory results showing both shortening and lengthening of telomeres have been reported, however. In both cases, a high frequency of end-to-end chromosome fusions was found suggesting a role for the

Ku80 protein in protecting telomeres from fusion (Bailey et al., 1999; Samper et al., 2000; d'Adda di Faganga et al., 2001; Espejel et al., 2002).

The availability of KU proteins might regulate the relative involvement of NHEJ and HR in DSB repair (Goedecke et al., 1999; Van Dyck et al., 1999). Consistent with this hypothesis, NHEJ-mutant mammalian cells show up-regulation of HR-mediated DSB repair with the greatest increase in HR found in Ku70 mutants (Pierce et al., 2001). Normally NHEJ is the prevalent DSB repair pathway in plants (Gelvin, 2000; Gorbunova and Levi, 1999; Zupan et al., 2000).

Recently *Arabidopsis* homologues of Ku70 and Ku80 have been cloned. Both display constitutive low-level expression in all tissues and more than a three fold induction of expression when exposed to DNA damaging agents (Tamura et al., 2002). Later studies have revealed that AtKu70/AtKu80 heterodimer and AtKu70, but not AtKu80 could bind DNA ends *in vitro* (West et al., 2002), although AtKu80 is required for DNA repair *in vivo* (West et al., 2002). AtKu80 deficient plants do not exhibit any developmental defects under normal growth conditions. They do display increased sensitivity to DNA damaging agents however, including menadione (quinone that causes oxidative damage to the cells), bleomycin (DSB inducing agent) and methyl-methane sulfonate (West et al., 2002; Riha et al., 2002). Hypersensitivity was not observed in the case of UV-C (West et al., 2002). AtKu80 deficient cells are also deficient in end joining of a linear plasmid DNA demonstrating the Ku80 protein is involved in NHEJ in plant. No change in the HR rate was observed in AtKu80 mutants (Gallego et al., 2003). But most surprisingly, AtKu80 deficiency doesn't show any defects in T-DNA integration, suggesting the existence of an alternative mechanism of end-joining for T-DNA

integration in plant cells (Gallego et al., 2003). Recent studies also have proven the role of AtKu80 in telomere length maintenance in *Arabidopsis*. The Ku protein was shown to control telomere length in *Arabidopsis* by direct or indirect inhibition of telomerase at the telomere ends (Gallego et al., 2003).

3. MATERIALS AND METHODS

3.1. Plant and protoplast cultivation

3.1.1. Growth conditions

Arabidopsis plants were grown for three weeks in sterile conditions at 16h day/8h night. The high light intensity ($32.8 \mu\text{Em}^{-2}\text{s}^{-1}$) was provided by Cool White Halogen bulbs (Sylvania; Mississauga, ON, Canada) and Longlife Incandescent bulbs (Sylvania). Seeds were kept for five days incubated at 4°C prior to germination.

Plants were grown on sterile Murashige and Skoog (MS) basic medium (Sigma; Oakville, ON, Canada), with the addition of Murashige and Skoog vitamins (Sigma), 20 g/liter sucrose and 8 g/liter agar) aliquoted into 85 mm Petri dishes.

Tobacco plants were grown on sterile all purpose potting soil (Plants Etc; Lethbridge, AB, Canada). They were germinated and grown in a Conviron growth chamber (Winnipeg, MB, Canada) with constant 85% humidity. Tobacco plants were grown in high light conditions ($32.8 \mu\text{Em}^{-2}\text{s}^{-1}$) provided by Cool white Fluorescent bulbs (Sylvania). The day/night regime was 16/8 hours with temperatures at $22/18^{\circ}\text{C}$.

3.1.2. Sterilization of *Arabidopsis* seeds

Arabidopsis seeds were incubated in 70% ethanol for two minutes with constant shaking. Seeds were then sterilized with 0.5% sodium hypochloride and 0.05% Tween-80 solution for 3 minutes and washed two times 5 minutes with large volumes of sterile distilled water.

3.1.3. Protoplast cultivation

Protoplasts were prepared as described by Goodal et al. (1990). Cell subculture suspension (1:10, 50 ml total) was prepared two days before preparing chloroplasts. Prepared cells were centrifuged in 50 ml falcon tubes (2 minutes, 800 rpm). Supernatant and fluid in pellet were aspirated. Cells were mixed with enzyme solution (Cellulose Onozuka R-10(1%); Macerozym R-10 (0.25%); Mannitol (0.5%); CaCl₂; pH 5.6). Cell walls were digested overnight (16-18 hours) in the dark at 26 – 28 °C with gentle agitation. Cells were filtered through 100 µM pore size sieve. Cells were mixed with W5 solution (NaCl₂ (9g); CaCl₂ (18.3g); Glucose (0.99g) in 1L; pH 5.6) by inversion and centrifuged for 5 min at 1000 rpm. Supernatant was aspirated and pellet resuspended in 0.6 M Sucrose solution (Sucrose (204.5g), MES (1g) in 1L; pH 5.6). Cells were placed in sterile 14 ml tubes and carefully overlaid with 1 ml of W5 solution. Protoplasts were then centrifuged at 800 rpm for 10 min with low acceleration and low brake. Good protoplasts are collected in interphase. Protoplasts were transferred to new 14 ml tube, ten milliliter of W5 solution added. The suspension was then centrifuged for three minutes at 800 rpm. Ten milliliters of W5 solution was added after the aspiration of the supernatant. Centrifugation was repeated. Pellet was resuspended in 5ml of MMM

solution (MgCl₂ (15mM); MES (0.1%); Mannitol (0.5 M); pH 5.6). Protoplasts were counted and diluted to 2X10⁶ protoplasts per milliliter.

3.2. Plants used in this study

3.2.1. GUS transgenic lines with HR substrate

The structure of β -glucuronidase HR substrates for the different lines is described by Puchta et al. (1995) and Swoboda et al. (1994; Figure 3.1.).

The following transgenic *Arabidopsis* lines were used: lines A11, A87, A94 and A211 (one copy in direct orientation), A231 (two copies in direct orientation), A651 (one copy in indirect orientation). Two transgenic tobacco lines were used to estimate the average recombination frequency: N9, carries four transgene copies in inverted orientation; N29, has a single copy in direct orientation (Figure 3.2).

3.2.2. LUC transgenic lines with HR substrate

The construction of the luciferase recombination substrate was described previously (Gorbunova et al., 2000). It consisted of two non-functional overlapping copies of luciferase transgene cloned in direct orientation (Figure 3.2). These constructs contain deletion derivatives of the luciferase gene sharing 1146 bp of overlap, which are positioned within the T-DNA in a direct orientation. Intrachromosomal recombination within the common luciferase sequence restores a functional gene. Recombination LUC systems based on direct repeats has been shown to be more effective than those based on inverted repeats (Gorbunova et al., 2000).

The recombination events were visualized as bright sectors on a dark background using a luciferase CCD camera (Figure 3.2). LUC based *Arabidopsis* lines are: LU# 2B.2; 2G.5; 2H.2; 2H.7; 2H.9; 15D.8; 15D.10; 15E.8. Tobacco lines used in the current study are: LU2; 5; 9; 12; 14; 15; 17; 22; 23; 29. All the luciferase lines used in the experiments carried single transgene copies.

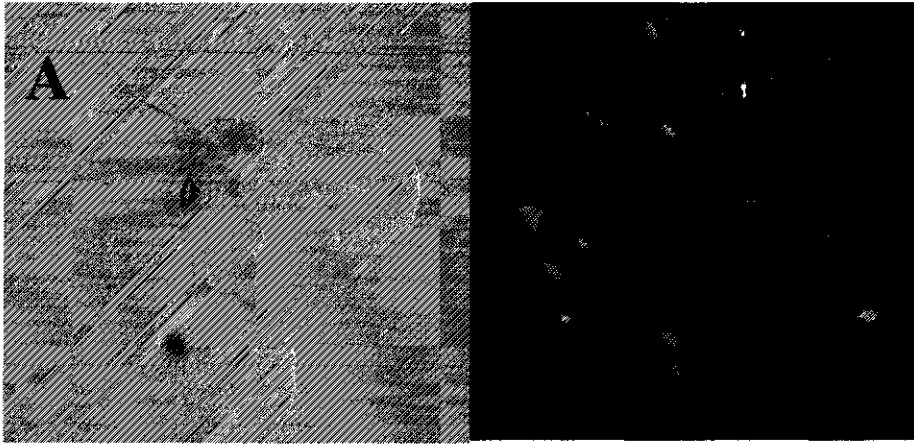


Figure 3.1. Detection of recombination events in *Arabidopsis*. (A) GUS-based recombination assay in *Arabidopsis* and (B) LUC-based recombination assay in *Arabidopsis*.

3.2.3. GUS transgenic line with point mutation substrate

The construction of this line is described in Kovalchuk et al. (2000). *Arabidopsis* plants carry β -glucuronidase gene driven by the 35S promoter. *Stop codons* were introduced into the marker gene by site directed mutagenesis. In order to increase the efficiency of the newly generated *stop codons*, they were followed by adenine or guanine. The PGUS23 vector used for the transformation of *Arabidopsis* (Columbia ecotype) plants contains the BASTA resistance (bar gene) as the selection marker. Lines used in the current work contained the marker gene with the *stop codon* (GGA \rightarrow TGA) introduced at position 166 (line 166).

3.2.4 AtKu80 deficient *Arabidopsis* mutant

Identification of the T-DNA insertion mutant of AtKu80 is described in West et al. (2002). A T-DNA insert was found in the ninth exon (of 12) of AtKu80 gene. Southern analysis confirmed that the T-DNA insert was in the AtKu80 gene, and only one T-DNA insert was present in the genome of the mutant. The left border region showed a 3 bp deletion of the T-DNA and a 33 bp insertion of filler DNA, including 21 bp duplicated from the AtKu80 gene at the insertion site and 12 bp of unknown origin. RT-PCR (reverse transcription) using RNA from above ground tissues could not detect any wild-type AtKu80 transcript in the mutant plant. However, mutant transcript was detected by RT-PCR and this included the 5' end of the mutated gene; however no 3' end was detected. This suggested that transcription termination occurs within 17 kb T-DNA insertion. The mutant transcript if translated could encode 490 of the 621 amino acids of

the wild type protein. This truncated form however would likely not be functional. The mutant was derived from the Wassilewskija background (West et al., 2002).

Mutant plants did not have developmental defects under normal growth conditions (West et al., 2002).

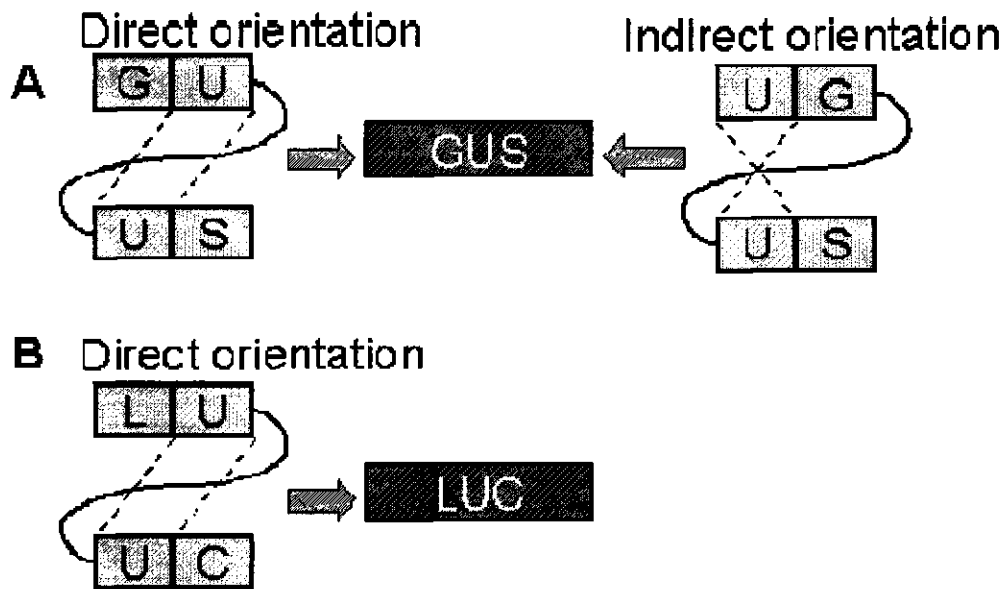


Figure 3.2. Structure of recombination substrate in *Arabidopsis* and tobacco. (A) The structure of GUS recombination substrates and (B) the structure of LUC recombination substrate.

3.2.5. AtAtm deficient *Arabidopsis* mutant

The atm-2 mutant was originally described by Garcia et al. (2003). For this work we have used the atm-2 line derived in the Columbia background. In this mutant, a T-DNA was inserted into intron 64, with an intact left border and a truncated right border. The insertion was complexed with the loss of four basepairs of genomic DNA and the insertion of filler DNA from other regions of the genome on both sides of the T-DNA. DNA gel blot analysis showed that a single insertion was present in this line.

All of the homozygous mutant plants were partially sterile with normal vegetative development.

3.2.6. UVH6 (AtXpd) *Arabidopsis* mutant

The mutant was described by Liu et al. (2003). The UVH6 mutant was derived from EMS treatment. The mutated transcript contains a single-nucleotide change at codon 521. This mutation results in a Gly to Glu missense substitution. Plants displayed slightly slower development and lower chlorophyll content that resulted in a yellow-green phenotype. Plants were sensitive to heat shock and did not survive four days of incubation at 37 °C. This would not be a lethal condition for normal plants.

3.3. Visualization of mutation events. Histochemical staining

3.3.1. GUS staining procedures

Histochemical staining, as described by Jefferson (1987), was applied to plants at the full rosette stage (3 weeks) for *Arabidopsis* and three weeks for tobacco leaves. Plants were vacuum infiltrated for 10 min in sterile staining buffer containing 100 mg of 5-

bromo-4-chloro-3-indolyl glucoronide (X-glu: Jersey labs Inc.,USA) in 200 ml of 100 mM phosphate buffer pH 7.0, 0,05% sodium azide, 0,1% Triton X-100. Plants were then incubated at 37 °C for 48 hours and bleached using 70% ethanol. Cells that contained the restored GUS gene were able to degrade X-glu, resulting in blue coloration on bleached leaves. Recombination or point mutation events were thus visualized as blue spots on the leaves using a dissecting microscope.

3.3.2. LUC visualization procedures

The transgenic plants harboring a luciferase based recombination substrate were examined using a CCD camera (Gloor instruments; Basel, Switzerland). The surface of the leaves was treated with a 0.5 mM beetle luciferine (Promega; Madison, WI, USA), 0,05% Tween-80 solution. Samples were incubated without light 30 minutes then photographed with the CCD camera. Cells that contained a restored LUC gene were able to cleave the luciferine resulting in ATP-dependent light production. Superimposing a light-exposed image (5 – 10 seconds under white light) on a dark-exposed image (20-minute exposure with no background light to expose auto fluorescent, luciferase expressing cells) through analiSIS program (Soft Imaging System; Munster, Germany) allowed for the quantification of recombination events.

3.4. DNA and RNA preparation

3.4.1. DNA preparation

Plant tissues were ground in liquid nitrogen and homogenized in *Arabidopsis* or Tobacco extraction buffer. Upon addition of β -mercaptoethanol the mixture was

incubated at 65 °C with occasional vortexing. Solid particles were precipitated after short centrifugation in a table top centrifuge and the liquid phase was retained. The DNA was precipitated using equal volumes of cold isopropanol followed by centrifugation. The pellet was washed with cold 70% ethanol and then resuspended in sterile distilled water. Organic contamination was removed by phenol and chlorophorm. DNA was precipitated from the aqueous phase retained after the phenol/chloroform step. The DNA pellet was washed with 70% ethanol and resuspended in sterile distilled water.

3.4.2. RNA and cDNA preparation.

RNA was extracted using Trizol™ and following the manufacturer's instructions (Invitrogene; Burlington, ON, Canada). Briefly, tissue samples were homogenized in Trizol. In a second step chloroform was added to separate DNA and proteins from RNA, which remained in aqueous phase. After centrifugation, the organic phase was discarded and the RNA was precipitated from the aqueous phase with isopropyl alcohol. The RNA pellet was washed with 75% ethanol. The RNA pellet was dried (10 minutes at room temperature) then resuspended in sterile distilled water. ReverAid™ Minus First Strand cDNA Synthesis Kit (Fermentas) was used for cDNA synthesis. For each reaction, up to 5 µg of total RNA preparation, 2 µl oligos were used. The mixture was briefly incubated at 70 °C and vortexed. 4 µl of 10x reaction buffer, 1 µl Ribolock™ RNAase inhibitor, 2 µl of dNTPs were added during the second step. The reaction mixture was vortexed and briefly incubated at 37 °C. Finally, the reaction was supplemented with 1 µl of ReverAid™ H Minus M-MuLVRT enzyme and incubated at 42 °C for one hour. The enzyme was inactivated by heating at 70 °C during for 10 min.

3.5. Mutagenic treatments

3.5.1 UVC irradiation of *Arabidopsis* and Tobacco plants

Arabidopsis plants were germinated on soil or sterile plates and were grown using the conditions of 16 h light 8 h dark. Two weeks after germination, plants were UVC irradiated. Plants were given a dose of 5000 ergs (200 ergs/s) and mutations were analyzed 10 days after irradiation.

3.5.2. UVC irradiation of tobacco protoplasts and leaf disks

N. plumbaginifolia protoplasts and tobacco leaf disks were UV-C irradiated under sterile conditions. Samples were treated with a dose of 5000 ergs (200 ergs/s) 15 minutes prior to protoplast transfection or leaf bombardment. To provide efficient irradiation, protoplasts were spread on the surface of a 10 cm×10 cm Petri dish.

3.5.3. MMS treatment

Arabidopsis plants grown in sterile conditions were treated with methylmetane sulfonate (MMS). The MMS (Sigma, Germany) was added to MS medium after autoclaving at a concentration of 30 ppm.

3.6. Generation of DNA repair deficient lines with *stop codon* inactivated GUS gene.

3.6.1. Crosses

To generate DNA repair deficient lines carrying point mutation substrates, we crossed DNA repair deficient *Arabidopsis* mutants (*atm-2*, *uvh6* and *atku80*) to the line with stop codon inactivated GUS substrate (166 line). F₂ progenies harboring at least one

copy of the GUS marker gene were selected using phosphinotricin (PPT) added to MS medium (10 µg/L). Progeny of selected plants were grown on soil. Each plant was labeled and some of their leaves were collected for DNA and RNA extraction. Using PCR on gDNA and cDNA, we were able to isolate homozygous plants. Seeds from plants homozygous for both mutated genes were subsequently grown on PPT-containing medium to determine plants homozygous for the point mutation substrate. Seeds collected from plants homozygous for the DNA repair mutant and the GUS reporter were used for further experiments.

3.6.2. Isolation of progenies homozygous for the mutant genes

PCR methods were used to isolate homozygous progeny derived from the cross of AtATM and AtKu80 deficient lines. To localize homozygous deficient AtKu80 plants we designed primers (forward – TGATCTTACTCCTAACCCGG; reverse – AATCTCTTAGAAGCCTTCTTC), that annealed to the 9th and 10th exons, upstream and downstream of the insertion respectively. In homozygous T-DNA plants, product could not be observed in either DNA or cDNA templates. If present, the intact gene yielded 250 bp and 170 bp products respectively. Using a PCR on genomic DNA and RT-PCR one homozygous plant atku80 was isolated (atku80#11). Subsequent propagation of atku80#11 seeds on phosphinotricin (PPT) media showed that this line was heterozygous for the GUS gene.

In the case of AtATM deficient plants we designed primers (forward – TCCATACCATAACAATCTTGCAG; reverse – TTGATACGGTCACCGTTTGCC) flanking intron 64 that carries the large T-DNA insertions. PCR on genomic DNA

didn't show any trace of this product suggesting that the T-DNA present in the gene was too long to be amplified. In the control PCR samples with genomic template from wild type plants we observed a 250 bp product. RT-PCR (reverse transcriptase) performed with the same primer pair proved that the transcript was not produced in the AtATM deficient plants.

Using both RT-PCR and PCR on genomic DNA, eight plants were isolated homozygous for the T-DNA insertion. Results obtained by PCR confirmed the morphology of the mutated plants. Plants were partially sterile and had significantly shorter siliques than the wild type plants (Garcia et al., 2003).

AtXPD (Uvh6) mutant was used for crosses derived from EMS treatment. The mutated transcript contained a single nucleotide change at codon 521. This mutation resulted in Gly to Glu missense substitution (Liu et al., 2003). Due to the nature of mutation we had to use alternative to PCR in order to determine if plants were homozygous. F2 plants that displayed a yellow-green phenotype under normal conditions were checked by exposing the progeny to heat shock (4 days; 37 °C). Plant progeny that did not show any segregation during this trial were considered homozygous. Presence of the GUS construct in the genomic DNA template was confirmed by PCR. Plants homozygous for both the mutation in Uvh6 and the stop codon inactivated GUS transgene were named atxpd#21.

3.7. Real-time PCR analysis of gene expression

For real-time PCR expression, all plant lines were plated as previously mentioned. Three-week-old plants were harvested and frozen in liquid nitrogen. RNA was extracted

from plant tissues using TrizolTM reagent. Reverse transcriptase PCR (You-Prime-First-Strand, ready to go PCR beads; Amersham) was carried out on all samples providing a transcriptome copy for each of the mutant lines. After quantification, 1 µg of RNA was taken for cDNA preparation (You-Prime-First-Strand, ready to go PCR beads, Amersham). The transgene activity was detected by amplification of 150 base pair (bp) 5' region of the *uidA* or *LUC* transgene. The following primers were used for amplification:

LUC, forward: 5'-ACTTCGAAATGTCCGTTCCGG-3'; reverse: 5'-GCAACTCCGATAAATAACGCG-3';

GUS, forward: 5'-ATGGATCCCCGGGATCATCT-3'; reverse: 5'-ACAGTTTTTCGCGATCCAGAG-3'.

Real-time PCR was performed in a total volume of 25 µl using 1 µl of the first strand cDNA synthesis mixture as a template, 300 nM forward primer, 300 nM reverse primer and 12.5 µl of 2×SYBRGreen PCR Master Mix (Applied Biosystems). The duplicate reactions were carried out with the 1:3 and 1:15 dilutions of the first strand cDNA synthesis mixture. A SmartCycler (Cepheid; Sunnyvale, CA) was used to perform the PCR cycles and fluorescence was quantified against standards. The cDNAs were amplified under the following conditions: (i) 95 °C for 5 min for one cycle; (ii) 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min for 30 cycles; and (iii) 72 °C for 10 min for one cycle. The melting temperatures were estimated for every gene product. The standards for the expression of each gene were amplified from the cDNA of following dilutions: 1:1, 1:4, 1:20, 1:100. Equal loading of each amplified sample was determined by the control actin gene *Act2* PCR product (forward primer: 5'-TGGACAAGTCATAACCATCGGAGC-3'; reverse primer: 5'-TGTGAACAATCGATGGACCTGAC-3').

3.8. DNA digestion

DNA (5 µg) from GUS#166 construct was digested overnight with *BclI*, *BsmI* or *SnaBI*. The digestion with *BclI* leaves 5' overhangs, with *BsmI*, 3' overhangs and *SnaBI*, blunt ends. The completion of the digestion was confirmed by agarose gel electrophoresis and PCR amplification across the region of the generated DSB (data not shown).

3.9. Protoplast transfection

Protoplasts prepared from leaves of *N. plumbaginifolia* and transfected with equal amounts of DNA from various linear and circular constructs were used for evaluating the activity of the *uidA* gene. Subsequently, 1 µg of pM1-Luc plasmid (Roche) that carries the kanamycin resistance marker was included in all protoplast transfections as a control for equal transfection. For the positive control protoplasts were transformed with 5 µg of the plasmid carrying an active GUS gene. Four protoplast samples were transformed per construct. The experiments were repeated three times. Materials were added to 15 ml Falcon tubes in the following order: 5 µg DNA, 0.3 ml of a suspension containing 2×10^6 protoplasts/ml, 0.3 ml of 40% (w/v) PEG 6000. After careful mixing the tubes were allowed to stand at room temperature for five minutes, then 4 ml of K3 medium (Bilang, 1994) was added and the tubes were incubated in horizontal position at 27 °C overnight in the dark. For harvesting the transfected protoplasts, 10 ml of W5 buffer (150 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 6 mM glucose) was added to each tube to reduce the high osmolarity of the K3 medium and thereby facilitate sedimentation of the protoplasts (ten minutes at 1000 rpm). Protein extracts were prepared by re-suspending protoplasts in 200 µl of GUS extraction buffer (50 mM NaH₂PO₄ (pH 7.0), 10 mM EDTA, 0.1% Triton

X-100, 0.1% Sarkosyl). Aliquots of 20 μ l were immediately taken for the fluorometric reporter gene assay. The fluorometric readings for the GUS#166 and the firefly luciferase (LUC) construct as well as water were related to the fluorometric reading of the protoplasts transfected with the wild-type GUS-expressing construct (taken as 100% protein activity). The activity of the reporter gene was measured by MUG assay and standardized to protein contents in the Bradford assay.

3.10. Tobacco leafes bombardment

N. tabacum plants (cultivar Havana) were grown under sterile conditions at 24 °C and 16 hours light regime. Leaves of six week-old plants were removed and placed in the center of the Petri dish. Gold particles (20 μ g) (1 μ M diameter, BioRad, CA) were incubated in absolute 100% ethanol for two days. After washing in sterile water, the particles were re-suspended in 500 μ l of 50% glycerol. A bombardment mixture was prepared by mixing 25 μ l of gold particle suspension with 5 μ l (0.1 μ g/ μ l) of plasmid DNA and 25 μ l of 1 M $\text{Ca}(\text{NO}_2)_3$. Plasmid pM1-Luc (100 ng) (Roche) carrying luciferase gene was used as bombardment control. The mixture was incubated for 20 minutes at room temperature with occasional mixing. After a brief centrifugation, the supernatant was discarded and the gold-coated DNA was re-suspended in 100 μ l of 100% ethanol. The plasmid/gold suspension was transferred to a macro carrier. Biolistic transformation was performed after complete evaporation of the ethanol from the disk, using the Biolistic PDS 1000-He Particle Delivery System (BioRad) with a pressure of 1.0 kpsi and a gap size of 0.2. On average, four leaves per construct were bombarded. Each experiment was performed three times. The similarity of the bombardment efficiency

between different constructs was analyzed by detecting the luciferase fluorescence (stemming from pM1-LUC plasmid) in the CCD camera (Gloor Instruments, Switzerland) after spraying the luciferine, the cleavage substrate, on the leaves. Importantly, bombardment of leaves with plasmid carrying an active GUS gene resulted in totally blue leaves after histochemical staining.

3.11. N-acetyl-L-cysteine pre-treatment

N. plumbaginifolia protoplasts (1 ml; 2×10^6) were distributed on the surface of a 10 cm×10 cm Petri dish. The protoplast suspension was sprayed with 0.05 mM of N-acetyl-L-cysteine (NAC) (ca 100 µl of NAC was delivered). Detached tobacco leaves were sprayed with similar concentration of NAC containing 0.05% of Tween80 (for better penetration through the waxy cuticle). Both tissues were irradiated with UV-C 15 minutes after the NAC application.

3.12. Measurement of peroxide levels

Four protoplast samples per each treatment were harvested for analysis five minutes after the DNA delivery. Samples were ground thoroughly in liquid nitrogen and 10 ml of a 2 chloroform:1 methanol solution was added. The extract was shaken in the fume hood for one hour and then transferred to a 50 ml conical tubes and spun down at 10,000 rpm for five minutes. The supernatant was filtered through a cotton-stuffed Pasteur pipette into another 50 ml conical tube. Two ml of a 0.9% (w/v) NaCl solution was added to the filtrate and mixed well. The content was centrifuged again at 10,000 rpm for five minutes. Top (aqueous) and bottom (organic) phases were transferred

to two Rotovap vials. Each solution was allowed to evaporate from Rotovaps. Dried extracts were re-dissolved in 6 ml of 90% methanol for the organic half of the extract, and in 1 ml of water for the aqueous half of the extract. Peroxide content was determined using the Sigma PeroxiDetect KIT (PD-1) as described in the manufacturer's protocol.

3.13. Statistical treatment of the data (in study of DNA repair machinery sensitivity to UV radiation)

All of the peroxide level measurements were performed three times with four samples per each construct per each experiment. In all cases the mean, the maximum and the minimum, the 99% confidence interval and SD were calculated using Microcal Origin 6.0. The statistical significance of the experiments was confirmed by performing single factor ANOVA for all the experimental groups.

4. Results

4.1. Transgenic plants used

Transgenic *Arabidopsis* and tobacco plants that carried either luciferase- or β -glucuronidase-based recombination substrates in their genomes were used. Both transgenic recombination markers were driven by the 35S promoter cauliflower mosaic virus (CaMV). This promoter has previously been shown to be active in virtually all plant tissue (Jefferson, 1987).

Arabidopsis and tobacco plants carrying the HR substrate were generated by Swoboda et al. (2000) and have been used intensively over the last decade. The *GUS* recombination substrate is based on two overlapping (500–1200 bp) truncated non-functional parts of the *uidA* gene cloned in either inverted or direct orientation. Several transgenic *Arabidopsis* and tobacco lines carrying this transgene have been generated (Puchta et al., 1995; Ries et al., 2000). Previously, we generated a luciferase-based HR substrate. Similarly, two overlapping (ca 900 bp) truncated non-functional parts of the luciferase gene were cloned in direct orientation (Kovalchuk et al., 2003). Many independent single copy transgenic *Arabidopsis* and tobacco lines have been generated (Filkowski et al., 2004).

T-DNA insertional mutants *atku80*, *atm-2* and EMS derived mutant *uvh6* (*atxpd*) have been crossed with an *Arabidopsis* line harboring point mutation substrate (166 line) in order to study the possible involvement of mutated genes in the DNA repair (See Materials and Methods).

4.2. Experiment 1: Luciferase-based transgenic recombination assay is more sensitive than β -glucuronidase-based

4.2.1. Spontaneous recombination frequencies as measured in GUS and LUC transgenes

To compare the sensitivity of the *GUS*-based and *LUC*-based recombination assays, *Arabidopsis* and tobacco plants were germinated on soil and assayed for the frequency of HR at the age of 3 weeks.

In *Arabidopsis*, the average recombination frequency in the *GUS* transgene was 0.43 spots per plant, whereas recombination frequency measured with the *LUC* recombination substrate gave an average (calculated from nine lines) of 3.9 spots per plant (Table 4.1.). The recombination frequency measured in *LUC* substrate was on average 9.0-fold higher. Similarly, the recombination frequency of the *GUS* transgene in tobacco averaged 0.9 spots, whereas the *LUC* transgene averaged 11.2 spots per plant (calculated from 10 lines; Table 4.1.). A difference of more than 12-fold between *LUC* and *GUS* recombination substrates was observed.

The data suggests that the difference in HR frequency observed between *GUS* and *LUC* recombination substrate is probably due to the different sensitivity of the detection of the transgene's activity.

4.2.2. Induced recombination frequency

In order to understand whether the difference in HR frequency between *GUS* and *LUC* recombination substrates is indeed due to the difference in sensitivity, we analyzed the HR frequency upon UVC treatment. If the difference in recombination frequency

observed in *GUS* and *LUC* transgenes is indeed due to the sensitivity of the detection of the transgene activity, then the number of recombination events for UVC-treated plants will be similarly high for *LUC*-transgene-containing plants and similarly low for *GUS*-transgene-containing plants. In this case, a similar increase of recombination in both *GUS* and *LUC* transgenes should be expected. Recombination frequencies were measured in plants grown under normal conditions and plants that were exposed to 5 kJ m^{-2} of UVC. The average fold induction of HR frequency in *Arabidopsis* was similar in both *GUS* and *LUC* transgenes, 4.9- and 4.6-fold, respectively (Table 4.2.). The increase of HR frequency was also in close range for both *GUS* and *LUC* transgenes in tobacco plants, 3.9- and 3.4-fold, respectively (Table 4.2.).

As expected the frequencies of UVC-induced HR in *LUC*-containing transgenic *Arabidopsis* and tobacco plants were much higher than in *GUS*-containing plants (Table 4.2.). Again, this is apparently due to the different sensitivity of the detection of the activity of recombination substrates.

4.2.3. Similarity of the level of transgene transcripts

Although higher spontaneous and induced frequencies of HR were observed in *LUC*-transgene-containing plants as compared to those containing the *GUS*-transgene-, it was possible that these differences were due to the different levels of expression of the transgenes. Since both transgenes were driven by the same promoter, their expression pattern was supposed to be similar.

To analyze the expression of *GUS* and *LUC* transgenes, the steady state RNA levels in *Arabidopsis* and tobacco plants were compared. We used primers to amplify the

first 150 bp of the 5'-end of the truncated *GUS* or *LUC* transgenes. Real-time PCR analysis was performed. Although the expression of *GUS* and *LUC* transgenes was different in different transgenic lines, the average expression levels were comparable between transgenes (Table 4.3.). Similar expression of transgenes suggests that the differences observed in the frequency of HR visualized with β -glucuronidase- or luciferase-based marker could only be attributed to the difference in the detection procedure.

Table 4.1. Spontaneous recombination frequency in *Arabidopsis* and tobacco as measured in *GUS* and *LUC* transgenes LU#2B.2-LU15E.8 are *A. thaliana* single copy homologous recombination lines; LU2-LU29 are *N. tabacum* single copy homologous recombination lines. Recombination frequency (RF) was estimated by counting the recombination events in the population of 20–100 plants and relating it to the total number of plants. The average RF is shown in bold. The data for lines #231 and N9 were standardized to a frequency per single transgene copy by relating the average number of events to the number of transgene copies in these lines.

<i>Arabidopsis</i>				Tobacco			
<i>GUS</i>		<i>LUC</i>		<i>GUS</i>		<i>LUC</i>	
Line	RF	Line	RF	Line	RF	Line	RF
#651	0.21	<i>LU#2B.2</i>	2.4	N9	0.6	LU2	7.5
#11	1.90	<i>LU#2G.5</i>	1.4	N29	1.1	LU5	10.1
#211	0.12	<i>LU#2H.2</i>	4.7			LU9	19.8
#231	0.02	<i>LU#2H.5</i>	2.9			LU12	9.3
#87	0.11	<i>LU#2H.7</i>	2.6			LU14	16.1
#94	0.20	<i>LU#2H.9</i>	1.9			LU15	13.2
		<i>LU#15D.8</i>	6.7			LU17	14.8
		<i>LU#15D.10</i>	7.8			LU22	10.5
		<i>LU#15E.8</i>	4.5			LU23	8.9
						LU29	5.8
Average	0.43		3.9		0.9		11.2

Table 4.2. UVC-induced recombination frequency in *GUS* and *LUC* transgenes of *Arabidopsis* and tobacco plants. LU#2B.2-LU15E.8 are *A. thaliana* single copy homologous recombination lines; LU2-LU29 are *N. tabacum* single copy homologous recombination lines. "+" shows the number of recombination events per plant in UVC-treated plants. Fold ("F") induction of RF was estimated by relating UVC-induced RF to spontaneous RF. The data for lines #231 and N9 were standardized to a frequency per single transgene copy by relating the average number of events to the number of transgene copies in these lines. The average is shown in bold.

<i>Arabidopsis</i>						Tobacco					
<i>GUS</i>			<i>LUC</i>			<i>GUS</i>			<i>LUC</i>		
Line	+	F	Line	+	F	Line	+	F	Line	+	F
#651	0.9	4.4	#2B.2	12.5	5.2	N9	3.0	5.0	LU2	25.5	3.4
#11	4.0	2.1	#2G.5	8.7	6.2	N29	3.1	2.8	LU5	37.4	3.7
#211	1.0	8.3	#2H.2	23.0	4.9				LU9	37.6	1.9
#231	0.1	5.0	#2H.5	7.3	2.5				LU12	25.1	2.7
#87	0.3	2.4	#2H.7	11.2	4.3				LU14	38.6	2.4
#94	1.4	6.9	#2H.9	15.6	8.2				LU15	51.5	3.9
			#15D.8	20.8	3.1				LU17	60.7	4.1
			#15D.10	19.5	2.5				LU22	33.6	3.2
			#15E.8	20.3	4.5				LU23	31.2	3.5
									LU29	27.3	4.7
Average	1.3	4.9		15.4	4.6		3.1	3.9		36.8	3.4

Table 4.3. The real-time PCR analysis of *GUS* and *LUC* transgene activity. LU#2B.2-LU15E.8 are *A. thaliana* single copy homologous recombination lines; LU2-LU29 are *N. tabacum* single copy homologous recombination lines. The real-time PCR data for each line was calculated from two independent real-time PCR runs and presented as an average±S.D. Numbers show arbitrary units of fluorescence. Averages for *GUS*- and *LUC*-containing *Arabidopsis* and tobacco lines were calculated and compared and they are shown in bold.

<i>Arabidopsis</i>				Tobacco			
<i>GUS</i>		<i>LUC</i>		<i>GUS</i>		<i>LUC</i>	
Line	A	Line	A	Line	A	Line	A
#651	212 ± 34	#2B.2	183 ± 23	N9	317 ± 43	LU2	312 ± 58
#11	315 ± 56	#2G.5	112 ± 34	N29	266 ± 37	LU5	249 ± 35
#211	182 ± 26	#2H.2	362 ± 92			LU9	147 ± 32
#231	160 ± 31	#2H.5	337 ± 76			LU12	213 ± 43
#87	229 ± 46	#2H.7	263 ± 56			LU14	167 ± 13
#94	336 ± 89	#2H.9	218 ± 35			LU15	236 ± 65
		#15D.8	413 ± 97			LU17	442 ± 102
		#15D.10	315 ± 64			LU22	216 ± 54
		#15E.8	365 ± 43			LU23	298 ± 62
						LU29	156 ± 32
Average	239 ± 71		285 ± 99		292 ± 36		244 ± 89

4.3. Experiment 2: Double-strand break repair machinery is sensitive to UV-radiation

4.3.1. Generation of plasmids harboring the GUS gene inactivated by nonsense mutation

Using site-directed mutagenesis, *stop codons* were introduced into the 5' proximal part of the *uidA* gene, changing the first nucleotide (166th from the ATG) in the triplet. The sequence context around the mutated nucleotide was taken into consideration and the *stop codon* was followed by an adenine. This has been previously shown to be important for efficient translation termination (Angenon et al., 1990). This change was sufficient to completely abolish the protein's enzymatic activity (Kovalchuk et al., 2000). A plasmid was generated carrying an ampicillin resistance marker and reporter genes inactivated by the stop codon at position 166 (G:C/T:A change, construct GUS#166) (Figure 4.1.). Only reversions of the original nucleotide were expected to restore the activity of the gene, since we have previously shown that none of the other mutations that would lead to the amino acid change could restore the activity of the protein (Kovalchuk et al., 2000).

4.3.2. Pretreatment of the cells with UV-C increases the appearance of spontaneous reversions

We have shown previously that transfection of *Nicotiana plumbaginifolia* protoplasts and bombardment of detached tobacco leaves with the GUS#166 plasmid resulted in restoration of GUS activity via true spontaneous reversions (Kovalchuk et al., 2004). Moreover, we showed that DSBs positioned close to the *stop codon* significantly increased the reversion frequency (Kovalchuk et al., 2004). We concluded that the

processing of extrachromosomal strand breaks is a highly error-prone repair event that results in a significant number of point mutations. It was not clear, however, what contributed to the high incidence of reversions.

To determine if external DNA-damaging factors influence the precision of strand break repair, *N. plumbaginifolia* protoplasts and detached *Nicotiana tabacum* leaves were UV-C-irradiated 15 minutes prior to the delivery of the GUS#166 construct (Figure 4.2.(A)). Irradiated tissue was transformed with either circular plasmid or plasmids that were linearized with BclI (5 nt downstream of mutated nt), BsmI (26 nt upstream) or SnaBI (300 nt downstream), respectively (Figure 4.1.). Protoplasts and leaves were also transformed with the intact version of the pM1-Luc plasmid (either with the circular or linear GUS#166 construct) carrying the luciferase gene and serving as a transformation control.

Significant differences were observed in the GUS activity of non-irradiated protoplast samples transfected with plasmids cut with BclI ($\alpha=0.01$, $p<0.001$) or BsmI ($\alpha=0.01$, $p<0.001$) but not with SnaBI ($\alpha=0.01$, $p=0.79$) as compared to the samples transfected with circular plasmids (Figure 4.3.(A)). Interestingly, the activity of the GUS protein due to the *stop codon* reversions in the *uidA* gene was increased in irradiated protoplasts transfected with both circular and linear forms of GUS#166 (Figure 4.3(A)). The GUS protein was increased by greater than twofold when comparing the activity in UV-C-treated protoplasts transfected with circular vector to the protein activity in non-treated protoplasts (Figure 4.3(A); $\alpha=0.01$, $p<0.001$).

There was a similar increase in protein activity between irradiated and non-irradiated protoplasts transformed with plasmids linearized at distances of 26 nt and

300 nt from the mutated nucleotide (Figure 4.3(A), BsmI and SnaBI; $\alpha=0.01$, both $p<0.001$). In contrast, the activity of *uidA* observed in irradiated protoplasts transfected with BclI digested plasmids was significantly higher; a fourfold increase was observed (Figure 4.3(A), BclI; $\alpha=0.01$, $p<0.001$). The luciferase activity measured in transfected protoplasts was similar in both UV-C-pretreated and control samples (data not shown).

The percent of GUS protein activity in protoplasts transformed with circular or linear forms of GUS#166 plasmid was calculated as a ratio to the protein activity in protoplasts transformed with active GUS form (set as 100%). The detection limit of the fluorimetric assay is set over the noise level of 0.1% of the β -glucuronidase activity stemming from the plasmid carrying the active GUS gene (Kovalchuk et al., 2000). The rare spontaneous reversions in the circular GUS#166 plasmid delivered to protoplasts accounted for 0.8–1.0% of the activity stemming from the circular form of plasmid carrying the active GUS gene. This does not mean that the stop codon in the GUS#166 plasmid reverts with the frequency of 1%. The increase of the protein activity is most likely due to increase of the reversions in protoplasts pre-treated with UV-C.

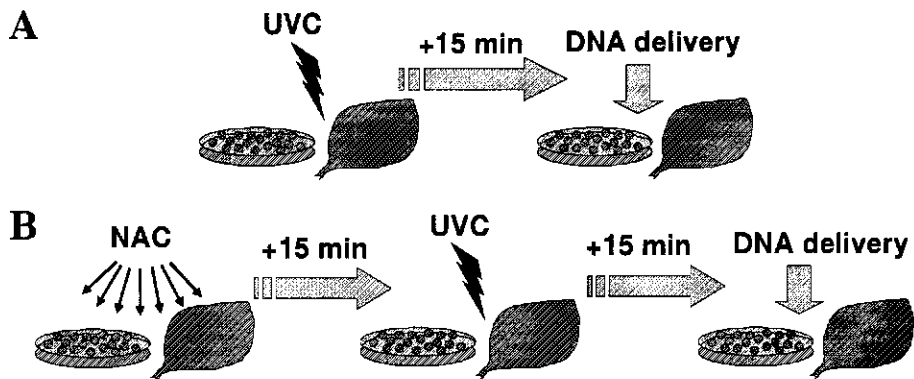


Figure 4.2. (A) *N. plumbaginifolia* protoplasts and *N. tabacum* leaves were irradiated with UV-C of 5000 erg. At 15 minutes later the circular or linearised forms of the GUS#166 plasmids were delivered to the irradiated tissue (either by PEG-mediated transfection or by biolistic bombardment). (B) *N. plumbaginifolia* protoplasts and *N. tabacum* leaves were sprayed with 0.05 mM NAC. At 15 minutes later both types of tissue were irradiated with UV-C of 5000 erg. In another 15 minutes the circular or linear forms of the plasmid were delivered to the irradiated tissue.

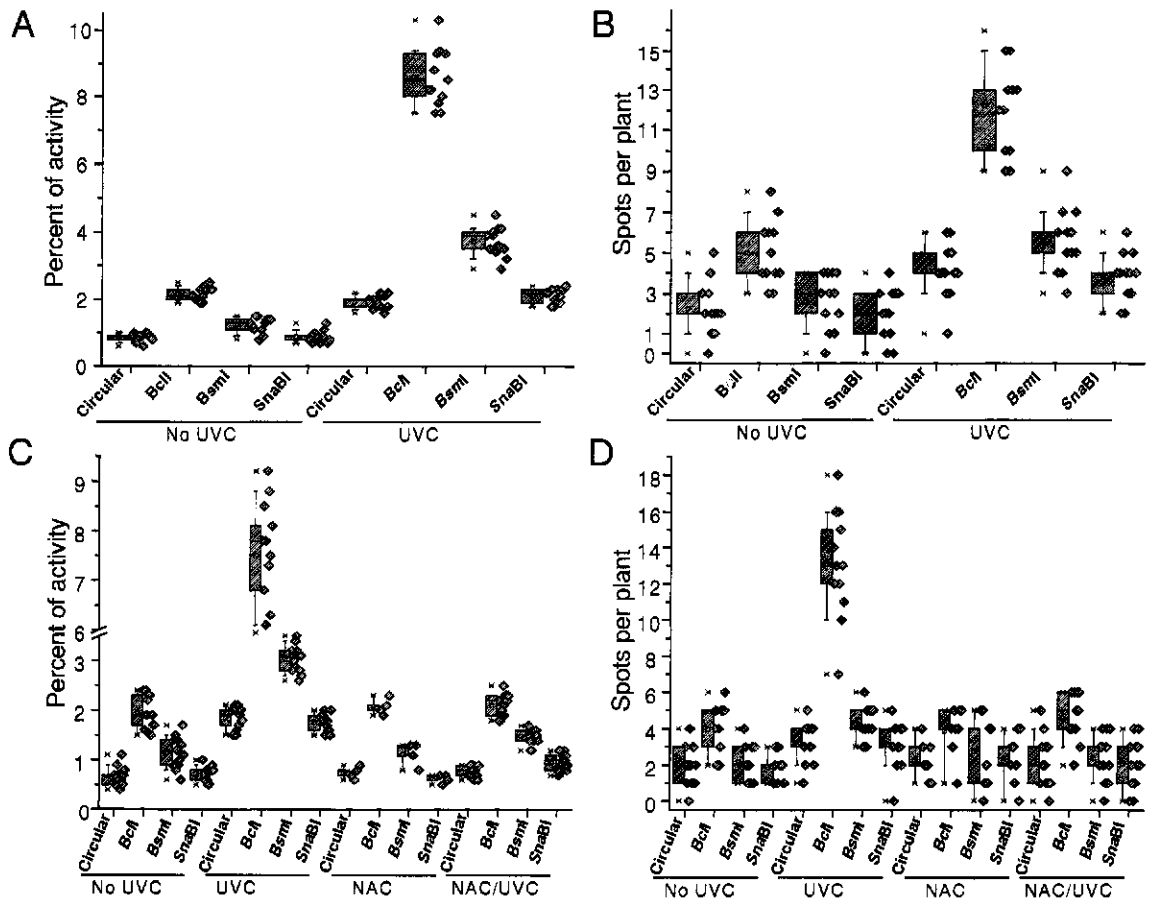


Figure 4.3. Increased levels of GUS reactivation upon the repair of extrachromosomal DNA in UV irradiated protoplasts (A) and tobacco leaves (B) and in protoplasts (C) and detached leaves (D) pretreated with NAC and subsequently treated with UV-C. Each graphic shows all the data points, the mean, the maximum and the minimum, the 1–99% confidence interval as well as SD calculated from 8–12 protoplast samples or leaves from three independent experiments. (A) Relative protein activity of the GUS gene in the plasmid GUS#166 was measured by MUG assay and related to the activity of the wild-type GUS gene (taken as 100% protein activity). The ordinate shows the activity in the control and irradiated protoplasts. (B) The average GUS reactivation (reversion) frequency was calculated as a number of blue spots on the transformed control and irradiated tobacco leaves and related to the number of leaves scored. The ordinate shows the number of reversion events in the control and irradiated leaves. (C) Relative protein activity of the GUS gene in the plasmid GUS#166 was measured by MUG assay and related to the activity of the wild-type GUS gene (100% protein activity). The ordinate shows the activity in the control and irradiated protoplasts. (D) The average GUS reactivation (reversion) frequency was calculated as number of blue spots on the transformed control and irradiated tobacco leaves and related to the number of leaves scored. The ordinate shows the number of reversion events in the control and irradiated leaves.

These data suggests that UV-C has a deleterious effect on the DNA repair machinery but not on DNA itself, as tissue was irradiated prior to DNA delivery. It is possible that UV-C stimulated the production of various toxic intermediates such as radicals that damage DNA after transfection. It has been previously shown that UV-C irradiation results in an increase of the activity of viral promoters (Francis and Rainbow, 2003). Although in our experimental system there was no direct irradiation of the marker gene driven by 35 S promoter, we wanted to ensure that the pretreatment of tissue with UV-C did not result in the change of the 35 S promoter activity. We delivered the active GUS gene into UV-C-pre-irradiated and non-treated protoplasts and found no significant difference in the marker gene activity (data not shown).

There was a significant increase in the number of reversions in non-irradiated tobacco leaf samples bombarded with plasmids cut with BclI ($\alpha=0.01$, $p<0.001$) but not with BsmI ($\alpha=0.01$, $p=0.56$) or SnaBI ($\alpha=0.01$, $p=0.65$) as compared to the samples bombarded with circular plasmids (Figure 4.3(B)). Pretreatment of leaves with UV-C by contrast, resulted in a significant (70–80%) increase of reversions in bombarded circular plasmid (Figure 4.3(B); $\alpha=0.01$, $p<0.01$) or plasmid cut at a distance from the mutated nucleotide (Figure 4.3(B); BsmI and SnaBI, $\alpha=0.01$, $p<0.001$, $p<0.01$, respectively) as compared to the plasmids delivered to non-treated leaves. Plasmids linearized at a proximal distance (BclI) exhibited a 2.6-fold increase (Figure 4.3.(B); $\alpha=0.01$, $p<0.001$) in reversion frequency. There was a significant difference in the number of reversions between circular plasmids and plasmids cut with BclI ($\alpha=0.01$, $p<0.001$), BsmI ($\alpha=0.01$, $p<0.05$) but not SnaBI ($\alpha=0.01$, $p=0.64$) when delivered to irradiated leaves (Figure 4.3(B)). We observed individual variations in the number of blue spots per transformed

leaf from 0 to 18, although the luciferase activity measured in bombarded leaves prior to the histochemical staining was comparable between different constructs (data not shown).

4.3.3. Increase of reversions by UV-C is likely due to the action of free radicals

The reason for the increase of the mutation frequency is unclear but could be due to the influence of the free radicals created in the irradiated tissue; pretreatment of protoplasts and tobacco leaves with UV-C prior to the DNA delivery may have resulted in the generation of free radicals that directly or indirectly damaged DNA. If true, pretreatment of plant tissue with a radical scavenging compound should prevent the increase of reversion frequency. *N*-acetyl-L-cysteine (NAC) is a compound that efficiently removes excessive radicals (He and Hader, 2002; Kovalchuk et al., 2003).

To test whether the NAC pretreatment itself had any effect on the GUS protein activity we exposed protoplasts to NAC without subsequent UV-C treatment. Protoplasts and leaves were exposed to 0.05 mM of NAC 15 minutes prior to UV-C irradiation (Figure 4.2(B)). NAC-treated and non-treated protoplasts were transformed with either active GUS gene or GUS#166. No significant differences were found in the protein activity between NAC-treated and non-treated protoplasts transformed with either circular or linear forms of GUS#166 (Figure 4.3.; ct, BclI, BsmI, SnaBI, $\alpha=0.01$, $p=0.45$, $p=0.51$, $p=0.89$, $p=0.25$, respectively). A similar result was obtained using tobacco leaves (Figure 4.3(D); ct, BclI, BsmI, SnaBI, $\alpha=0.01$, $p=0.62$, $p=0.85$, $p=0.40$, $p=0.36$, respectively).

In this set of experiments we found that pretreatment of plant tissue with NAC almost completely abolished the effect of UV-C irradiation (Figure 4.3(C) and (D)).

There was no statistically significant difference observed between the activity of the GUS gene in circular plasmid or plasmid cut with BclI delivered to non-treated or NAC+UV-C treated protoplasts (Figure 4.3(C); ct, BclI, $\alpha=0.01$, $p=0.14$, $p=0.20$, respectively) or detached leaves (Figure 4.3.(D); ct, BclI, $\alpha=0.01$, $p=0.76$, $p=0.31$, respectively).

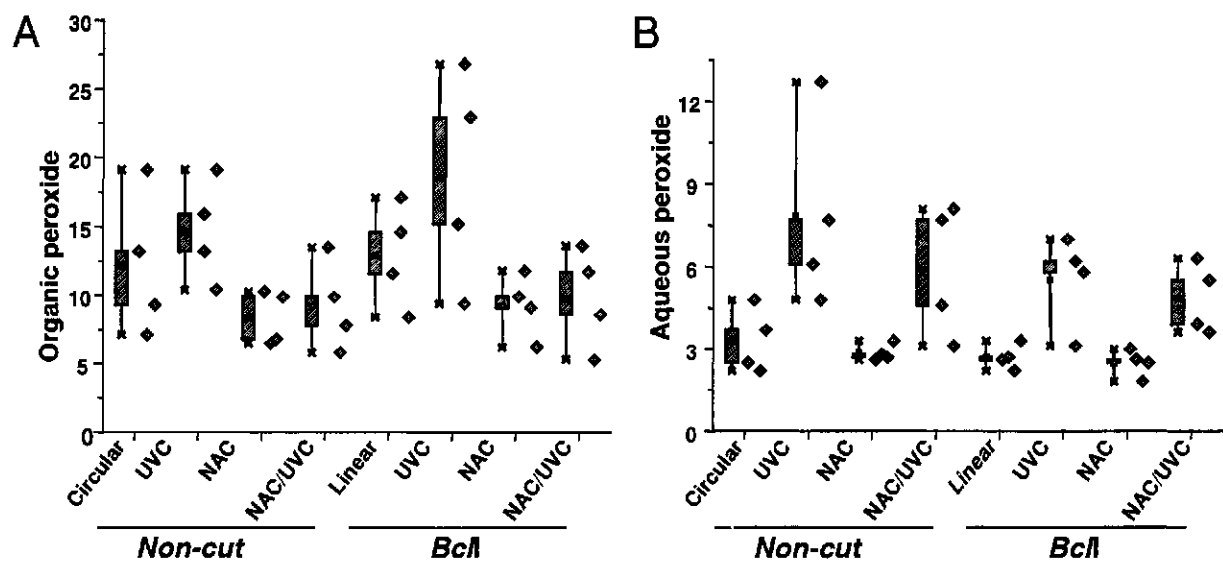


Figure 4.4. The amount of organic (A) or aqueous (B) peroxides was measured in control, UV-C-pretreated, NAC-pre-treated and NAC/UV-C-pre-treated protoplast samples transformed with circular (non-cut) or linear (BclI) plasmids. Each graphic shows all the data points, the mean, the maximum and the minimum, the 1–99% confidence interval as well as SD calculated from four protoplast samples. The ordinate shows relative peroxide units.

Similar data were observed for BsmI and SnaBI cut plasmids delivered to leaves (Figure 4.3(D); $\alpha=0.01$, $p=0.61$, $p=0.72$, respectively). Minor but significant differences were observed between non-treated and NAC+UV-C treated protoplasts transformed with BsmI and SnaBI cut plasmids (Figure 4.3.(C); $\alpha=0.01$, $p<0.01$, $p<0.05$, respectively). This set of experiments supports the hypothesis that the increase of reversion in the transgene delivered to UV-C pretreated tissue is due to the production of free radicals.

To analyze whether treatment with NAC led to a decrease in the amount of free radicals present we measured the peroxide activity in protoplasts (Figure 4.4.). The radicals were measured five minutes after the DNA delivery. Treatment with UV-C resulted in the production of a significantly higher amount of aqueous H₂O₂ in protoplasts transfected with non-cut (Figure 4.4; $\alpha=0.01$, $p<0.05$) or linear plasmid (data shown for BclI; $\alpha=0.01$, $p<0.05$). Pretreatment with NAC prior to irradiation with UV-C resulted in significant decrease of the lipid organic form of peroxide (Figure 4.4; $\alpha=0.01$, $p<0.1$) in protoplasts transfected with BclI or circular plasmids as compared to UV-C irradiated protoplasts. This was not observed for the aqueous peroxide form (Figure 4.4; $\alpha=0.01$, $p=0.39$, $p=0.53$ for circular and BclI cut, respectively). This suggests that NAC may have little or no influence on the level of peroxide in cells but rather prevented its deleterious oxidizing function. Similar data were observed for radicals measured in the bombarded leaves; there was an increase of radical production upon UV-C treatment and significant decrease of the organic form of peroxide when UV-C irradiation was preceded by NAC treatment (data not shown).

Current experiments suggest that irradiation with UV-C increases the level of free radicals and this leads to the oxidation of certain organic molecules, resulting perhaps

either in direct damage of DNA repair enzymes or in up-regulation of various signalling molecules that lead to the higher level of point mutations in extrachromosomal DNA.

4.4. Experiment 3: Generation of DNA repair deficient *Arabidopsis* lines carrying point mutation substrate

4.4.1. Isolation of the homozygous *Arabidopsis* mutants crossed with 166 line

As we mentioned in Materials and Methods, several DNA deficient *Arabidopsis* mutants (AtATM; AtXPD; AtKu80) have been crossed with an *Arabidopsis* line harboring point mutation substrate (166 line).

Atm-2 plants homozygous for the mutated gene and marker GUS transgene were selected from F2 progenies using PCR on gDNA and cDNA template (Figure 4.6) and via determination of distinguishing phenotypic features (see Materials and methods for details). Plants homozygous for mutated genes were subsequently propagated and checked for the segregation on PPT. ATM deficient lines with 100% survival on PPT assumed to be homozygous for the GUS construct. Eight plants have been proven to be homozygous for both mutant DNA repair and marker gene. Their seeds were pooled and used for experiments.

Similarly PCR on gDNA and cDNA was used to identify atku80 homozygous plants (Figure 4.5). In this case we found one plant homozygous for the mutated gene, however selection on PPT has shown that this line is heterozygous for the GUS marker gene. Plants used in these experiments were propagated on PPT to eliminate the progenies without the marker gene.

A PCR method was not applicable in the case of the EMS derived atxpd mutant. We selected a number of plants that displayed a yellow-green phenotype and a certain growth reduction. These plants have been propagated as separate lines. To confirm that these lines were homozygous their seedlings were grown under heat shock conditions (37 C° for 4 days). Wild type plants were used as control. One mutant line (#21) displayed 100% lethality in these conditions and was considered homozygous for the unh6 mutation (Figure 4.7). It was also proven to be homozygous for the marker gene via selection on PPT.

4.4.2. Contribution of mutant genes to point mutation repair

Seeds from GUS-carrying mutant lines were plated on sterile medium and subjected to UVC and MMS treatments (see Materials and Methods) and compared to plants from line 166 grown the same time at the same conditions. Plants from MS medium were used as non-treated control. UVC is considered as a mutagenic factor that mainly contributes to point mutation frequency and MMS to double-strand breaks.

Experimental plants were collected after three weeks on full rosette stage and GUS stained. Point mutations visualized as blue spots were counted under dissecting microscope.

Results are shown in tables 4.4, 4.5 and 4.6. These data suggest that UVC and MMS treatments are capable of point mutation induction, which confirms previous results (Kovalchuk et al, 2000; Kovalchuk et al., 2003). Moreover all of the analyzed DNA repair genes may contribute to the point mutation rate in *Arabidopsis*. AtKu80 deficiency lead to a two-fold increase of point mutation frequency upon UV-C treatment

and an almost 1.5 increase in case of MMS. Surprisingly, both deficiency in AtXPD and AtATM led to significant decrease of the point mutation frequency. In case of AtXPD it was a 12-fold decrease for UV-C, 7.5-fold decrease for MMS and approximately a 6-fold decrease in for control samples grown in of MS medium. Unfortunately it was impossible to estimate the difference in the point mutation rate between mutated line and control as AtATM mutant displayed zero level of point mutation events in all treatments while this line 166 showed the results consistent with those in other experiments. However we still may state that AtATM deficiency leads to significant decrease of point mutation rate in *Arabidopsis*.

Table 4.4. Point mutation frequencies measured in AtAtm deficient plants crossed with line 166 in comparison with 166 control line upon UV-C and MMS treatments.

Type of the plants Used	Point mutation frequency (spot/plant)		
	UV-C radiation	MMS	MS medium (negative control)
atm (uidA)	0 (0/271)	0 (0/159)	0 (0/169)
166 (control)	0.04 (5/126)	0.03 (8/290)	0.01 (2/173)

Table 4.2. Point mutation frequencies measured in Uvh6 deficient plants crossed with line 166 in comparison with 166 control line upon UV-C and MMS treatments.

Type of the plants Used	Point mutation frequency (spot/plant)		
	UV-C radiation	MMS	MS medium (negative control)
Uvh6 (uidA)	0.005 (1/146)	0.008 (1/188)	0.007 (1/126)
166 (control)	0.06 (11/181)	0.06 (10/175)	0.04 (7/178)

Table 4.6. Point mutation frequencies measured in AtKU80 deficient plants crossed with line 166 in comparison with 166 control line upon UV-C and MMS treatments.

Type of the plants Used	Point mutation frequency (spot/plant)		
	UV-C radiation	MMS	MS medium (negative control)
Atku80 (uidA)	0.06	0.07	0 (30 plants)
166 (control)	0.03	0.05	0.01

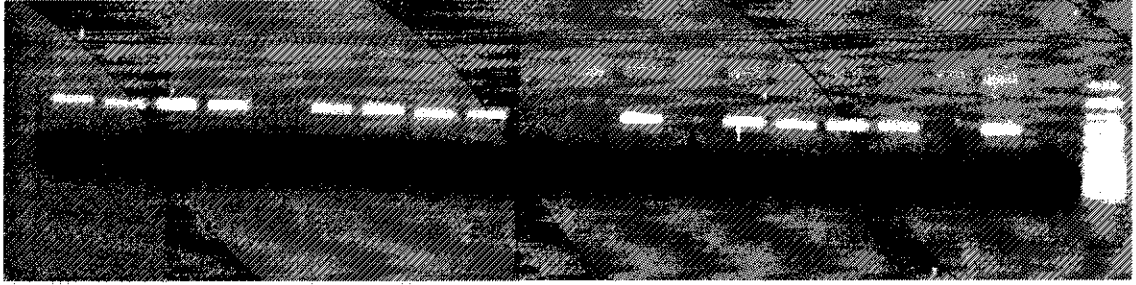


Figure 4.5. PCR analysis of cDNA derived from the F2 progenies of atku80 mutant and line 166 cross. Arrows point at the homozygous ATKU80 deficient lines with no production of wild type ATKU80 transcript (WT – wild type; - - negative control).

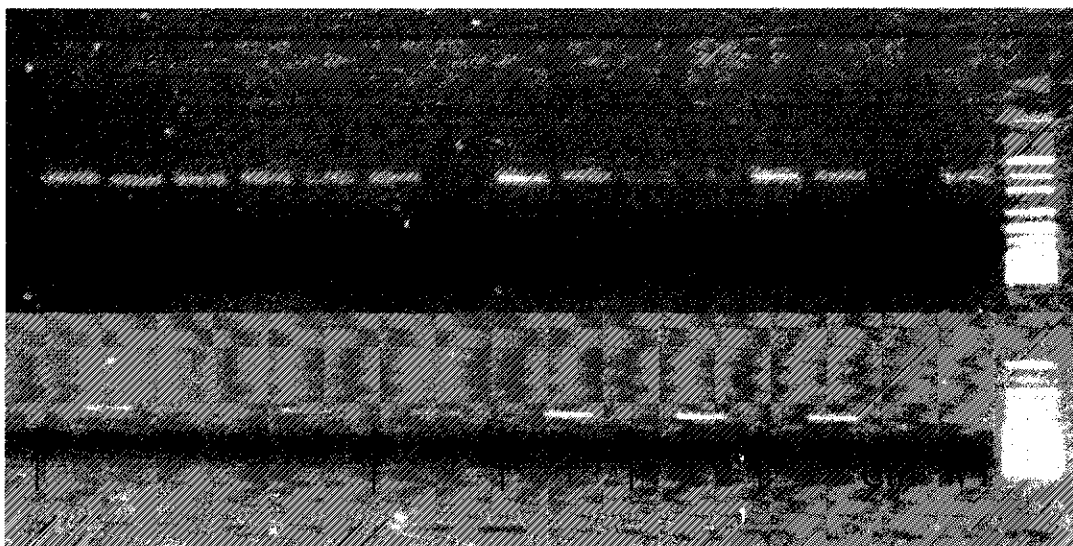


Figure 4.6. PCR analysis of genomic DNA isolated from the F2 progenies of *atm-2* mutant and line 166 cross. Arrows point to the lines homozygous for the T-DNA inserted into the ATM gene (WT – wild type, Ctrl – negative control).

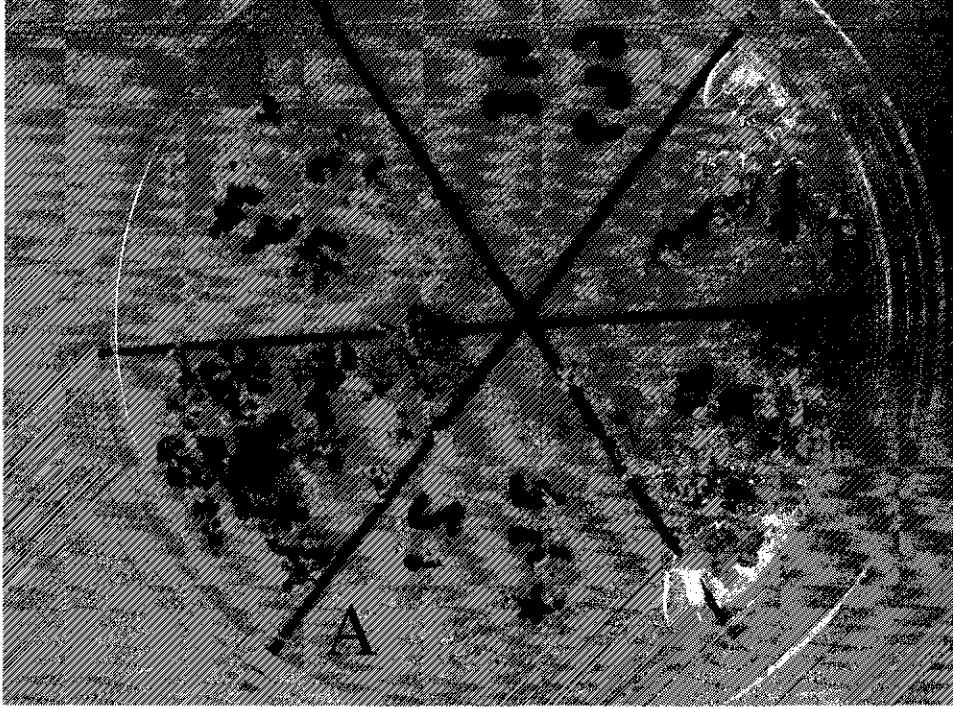


Figure 4.7. Picture of the plants obtained from the cross of *uvh6* mutant with line 166 (F2 generation), which were grown at 37°C for 4 days. Line that displayed 100% lethality was considered homozygous by *Uvh6* mutation (A – line 21; B – wild type).

5. Discussion

In this work we have compared the efficiency of various plant transgenic systems applied in DNA repair studies. These systems were further used to investigate the possible influence of DNA damaging agents on the DNA repair machinery itself and the way it is connected to the formation of point mutations. Finally we assayed several DNA repair mutants to investigate the possible contribution of these genes to the point mutation rate.

5.1. Experiment 1: Luciferase-based transgenic recombination assay is more sensitive than β -glucuronidase-based.

The sensitivity of two visible marker transgenes GUS and LUC as substrates for the detection of HR frequency was analyzed. Comparison of the average frequencies of spontaneous and UVC-induced recombination in two dicots, *Arabidopsis* and tobacco, showed approximately 10-fold higher sensitivity of the luciferase-based substrate.

To detect the activity of β -glucuronidase, plants have to be provided with the substrate X-glu. Plants are normally submerged into X-glu solution containing 0.05% Tween-80 for better penetration. Additionally, the staining process is done under vacuum condition to provide even distribution of the substrate. The cleavage of the substrate by the active enzyme results in the formation of a blue precipitate. For better detection of the precipitate, chlorophyll has to be washed away with ethanol. The detection process is therefore fully dependent on the ability of the substrate to penetrate the waxy cuticle and to reach the cells where the recombination process has taken place. It is not clear whether

the substrate penetrates all cell layers with equal efficiency. Previous experiments revealed that most recombination events take place in mesophyll cells (Ries et al., 2000).

Detection of luciferase events is also dependent on penetration of the substrate luciferine into the cell that produces an active luciferase protein. Cleavage of the substrate provides the luminescence that is detected by the CCD camera. It is possible that detection of the luciferase activity allows visualization of more recombination events than detection of the β -glucuronidase activity. This could be due to either better penetration of luciferine substrate as compared to X-glu substrate or due to better "visibility" of cells with active luciferase protein.

It is also possible that the activity of the promoters that were driving *LUC* and *GUS* transgenes were significantly different and resulted in a 10-fold difference in the detection of recombination events. Both transgenes are driven by the same double 35S promoter (Jefferson, 1987). This promoter was previously shown to be active in virtually all plant cells although its activity was significantly lower in flowers (data not published). The activity of the 35S promoter was shown to be influenced by light and temperature as well as the stage of plant development (data not published).

To analyze whether expression of the *LUC* and *GUS* transgenes resulted in similar activity we measured the steady state levels of mRNA by performing real-time PCR. Although transgene activities varied 2–3-fold between different lines, the average transcription level was comparable (Table 3). Therefore the 10-fold difference between the frequencies of recombination events analyzed with *LUC* transgene versus *GUS* transgene is likely attributed to the difference in the detection procedure. The luciferase-based recombination system should therefore be the preferred assay for the detection of

recombination events. Moreover, it would be a better marker gene for promoter analysis, especially for those promoters that are weakly expressed.

Our data show that HR rates per single cell genome calculated for the *LUC* transgene are 10-fold higher than the previously calculated 10^{-6} for *GUS* transgene (Swoboda et al., 1994) and therefore constitutes 10^{-5} events per single cell genome. HR is an important mechanism involved in gene targeting—a process that to date, has had little success (Hanin and Paszkowski, 2003). Any contribution to elucidating its function would therefore be an important step toward the success of gene targeting as well as the general knowledge of plant DNA repair.

5.2. Experiment 2: Double-strand break repair machinery is sensitive to UV radiation

We used a marker gene-based extrachromosomal repair assay to analyze DNA strand processing in plants. The delivery of circular and linear stop codon inactivated plasmids into plant tissue allowed us to analyze spontaneous reversions. Specifically, we wanted to analyze whether ultraviolet radiation influences the precision of the repair of DSBs. Here we show that the pretreatment of plant tissue (protoplasts or leaves) with UV-C results in an increased number of reversions at the stop codon. The increase of reversion frequency is greater when plasmids are linearized in close proximity to the stop codon. By contrast, there were no differences in the increase of reversion frequency between circular plasmid or plasmids cut at a distance from the stop codon. The pretreatment of irradiated tissue with the radical scavenging enzyme NAC blocked the UV-C effect.

In our previous work we discussed several possible explanations for the reversion increase in plasmids that were linearized in close proximity to the stop codon (Kovalchuk et al., 2004). We suggested that the primary reason for this phenomenon were mistakes that accumulated during the processing of strand breaks by the non-homologous end-joining machinery. The model that fitted best is a synthesis-dependent strand annealing (SDSA) system where the 3' end of one of the strands invades the unwound intact DSB template and primes DNA synthesis (Gorbunova and Levy, 1997; Gorbunova and Levy, 1999). Because SSA-like and SDSA types of DSB repair are the main mechanisms of end-joining repair in plants, it is possible that SDSA is a major mechanism that generates point mutations. The DSB repair (especially in plants) is dependent on a long stretch of ssDNA for invasion (Gorbunova and Levy, 1997; Gorbunova and Levy, 1999) and therefore will produce a significant portion of “to be filled-in” DNA. According to the SDSA model, each of the strands in plants can independently invade the template and prime DNA synthesis. This results in the formation of long stretches of single-stranded DNA. It is not clear how long this invading ssDNA “arm” is or how long this re-synthesis (fill-in) part could be. One of the current models suggests that it is either Ku70/86 or Rad51 that is involved in the invasion process. Both proteins require space for DNA binding. For example, RPA (the protein that protects ends for Rad51 to bind) is most efficient when ssDNA of 26 nt is present (Kantake et al., 2003). Similarly, single-stranded binding protein RecO (functional counterpart of Rad52) requires a minimum of 13 nt of ssDNA for efficient annealing (Kantake et al., 2002). It is possible that the invading DNA is 13–26 nt long. We previously showed that the point mutations as the

result of DSB processing are most frequent at a distance no larger than 25 nt from the break (Kovalchuk et al., 2004).

The main reason for a point mutation increase could be polymerase mistakes accumulated during the re-synthesis step of SDSA (Pages and Fuchs, 2002). We previously ruled out the contribution of nucleotide excision repair to the occurrence of reversions (Kovalchuk et al., 2004). The fact that we observed no differences in the reversion rates between the circular vectors and vectors cut at a distance from the stop codon, suggested that the NER machinery is not involved in the strand processing (Kovalchuk et al., 2004). We cannot deny that the radical-induced damage placed at the vicinity of the DSB is processed with less efficiency. A “neglected” BER repair would increase the chance of the stop codon reversion in our assay. It is difficult to imagine how both (BER and NHEJ) repair machineries will fit their protein complexes into less than 25 nt (the distance from the break) stretch of DNA.

The pretreatment of the tissue (protoplasts and detached leaves) with the UV-C increased the activity of the *uidA* gene both in the case of transfected circular and linear vectors (Figure 6(A) and (B)). UV-C could increase the level of dimers targeting the mutated nucleotide (Britt, 1999). This would happen either on circular or linear DNA with the same frequency. Since we irradiated the plant tissue prior to the delivery of DNA, the difference in the mutation frequency cannot be attributed to the direct modification of DNA by UV-C. On the other hand, one of the effects of the ultraviolet light is the production of free radicals (Hollosoy, 2002). Free radicals have a short life and most of them apparently cannot survive for periods longer than 15 minutes. This time however would be sufficient to oxidize organic molecules and trigger multiple cellular

responses (Hollosoy, 2002), and scavenging radical products would efficiently block the effect of UV-C. When we pre-treated the protoplasts and tobacco leaves with the potent radical scavenging agent - N-acetyl-L-cysteine (NAC) we did not observe the UV-C-induced increase of reversions (He and Hader, 2002). We previously observed a similar effect when plants pretreated with NAC did not react with an increase of double-strand breaks upon the incubation with the radical-inducing compound Rose Bengal (Kovalchuk et al., 2003). NAC has been shown to be an important peroxide scavenger (Junn et al., 2000; Ding et al., 2002). In our experiments we found that NAC pretreatment resulted in lower levels of oxidized lipids ("organic" peroxide) after subsequent UV-C exposure of protoplasts.

Ultraviolet light is known to create cross-linking and oxidation of organic molecules preventing their normal function (Kunkel et al., 2003). It is difficult to explain why there was a significant difference in the level of point mutations stemming from the plant tissue transformed with plasmids disrupted at close proximity to the stop codon *versus* plasmids disrupted at a distance (Figure 4.3). One could suggest that the difference was due to the involvement of different types of proteins in the DNA repair processes. The reversions distant from the break were primarily due to the mistakes of NER and BER, whereas those that were close to the generated break were due to the NHEJ repair mistakes.

A great number of mistakes created during repair are due to the malfunction of polymerase proofreading activity (Pages and Fuchs, 2002; Kunkel et al., 2003). Polymerases are employed in most repair mechanisms (Lindahl and Wood, 1999). Would the recruitment of different polymerases to the NER and NHEJ explain the different UV-

C sensitivity of these two types of repair? There is little information available from plant research but detailed information in mammalian models suggesting the involvement of similar polymerases δ and ϵ in filling the gap of long patch BER replicative DNA synthesis in NER as well as in inaccurate type repair in NHEJ (Wood et al., 1996). By contrast, short patch of BER involves DNA pol β (Hoeijmakers, 2001). It is possible that polymerase β is less sensitive to UV-C and thus functions more efficiently generating fewer mistakes upon the repair of damaged bases at a distance from the break.

An attractive model is one that proposes extensive degradation of the extrachromosomal DNA ends. In this case the primary involvement of either 5' or 3' endonuclease would result in stronger degradation activity on one of the strands. This would create a long stretch of ssDNA. When the error-prone polymerase fills the gap it would incorporate additional mutations. This model is still based on the involvement of an error-prone polymerase.

There could be other explanations for the phenomenon of mutational increase in the DNA delivered to the irradiated tissue. The introduction of the linear plasmids into irradiated cells initiated a massive strand break repair process. The number of the NHEJ proteins involved in the sealing of all breaks was high and significantly exceeded the number of BER or NER proteins involved in the repair of spontaneously arising nucleotide damages. The probability of making a mistake in the repair of a significantly larger number of NHEJ substrates is of course much higher. It is also possible that the irradiation of the tissue created a long-lasting free radical burst that damaged the transfected DNA in parallel with the damage of genomic DNA.

Although our experiments shed light on the mechanism of NHEJ in plants more intensive studies of DNA repair in plants are necessary. It would be important to identify the largest possible distance from the stop codon able to create DSB allowing the increase of reversions. This would likely reflect the size of the ssDNA that the repair proteins have to bind in order to promote efficient repair. It would also be interesting to detect UV-sensitivity of various proteins and protein complexes in *in vitro* repair assays.

5.3. Generation of DNA repair deficient *Arabidopsis* lines carrying point mutation substrate

5.3.1. AtAtm

AtATM deficiency in *Arabidopsis* suppresses the DNA damage induced expression of several DNA repair genes (AtRAD51, AtPARP1, ATGR1 and AtLIG4). Also mutant plants have been shown to be partially sterile, which points to the fact that ATM in *Arabidopsis* doesn't lead to complete meiotic arrest in contrast to mammalian systems. Also Arabidopsis appears to lack a p53 homologue that is the ATM target in mammals (see literature review).

Based on the aforementioned information one could suggest that the lack of ATM expression can cause severe disruption of DSB repair in *Arabidopsis* via both the HR and NHEJ pathways. Lack of a strong cell cycle checkpoints together with a possible involvement of the ATM protein in triggering DSB-induced apoptosis, allows plant cells to bypass DNA damage and resume cell divisions. This is supported by the fact that ATM mutants grow much faster early in developmental as compared to wild type plants

exposed to mutagens. It is possible that wild type plants have a slower growth rate due to the necessity to stop the division and repair accumulated DNA damage.

Analysis of the HR frequency in ATM should this be capital letters mutant could provide additional evidence to explain the lower frequency of point mutations in AtATM deficient plants.

5.3.2. AtXpb

According to the model proposed by Jenkins et al. (1997), AtXPD protein works as the negative regulator preventing the PCD from triggering until a certain damaging signal threshold is reached. In AtXPD(-/-) plants this threshold is much lower therefore they respond to weaker signal that should lead first to increased stress response which might include DNA repair and then to PCD that result in accelerated cell death and morphological changes.

Apparently mutant plants mobilize their defensive mechanisms faster than the wild type when exposed to UV light. This could explain the lower mutation frequency in mutated plants exposed to UV.

There is no evidence that could elucidate the role of AtXPD in DSB repair *Arabidopsis*. In our experiment we obtained results that points at significant influence of AtXPD on DSB repair as well.

5.3.3. AtKu80

The Ku80 protein is part of the KU heterodimer that binds with high affinity to the exposed DNA ends of broken chromosomes, protects them from exonuclease activity,

juxtaposes the ends to facilitate subsequent ligation and recruits additional DNA repair proteins to the site of the lesion. KU is one of the major players in NHEJ – the main DNA repair pathway in plants.

The *atku80* mutant shows hypersensitivity to bleomycin and menadione. Bleomycin is a radiomimetic agent that causes single- and double-strand breaks in DNA. Hypersensitivity of *atku80* mutant to bleomycin is consistent with its role in NHEJ pathway. Menadione causes oxidative damage by generation of peroxide and superoxide, agent that cause both single- and double-strand breaks (Reicheld et al., 1999; Shi et al., 1994). In our experiment *atku80* mutant displayed two times higher frequency of point mutation events upon the UVC treatment. UV light is known to induce reactive oxygen species that might cause DSBs (de Gruijl et al., 2001; Ravanat et al, 2001). UVC light is apparently less potent source of reactive oxygen species than menadione as *atku80* plants display no hypersensitivity to UVC in comparison to wild-type (West, 2003). However it still could lead to a slight increase of DSB frequency that doesn't affect viability of the cells. The higher point mutations frequency (Table 4.6) in this case might be explained as the consequence of an increased degradation of broken DNA ends by exonucleases in *Ku80* deficient plants. The other possibility is that in *atku80* mutant DSB repair large part of DSBs are processed via HR pathway instead of NHEJ. Proteins involved in HR could be affected by UVC, which lowers the fidelity of the repair process and leads to frequent nucleotide misincorporations as we have shown. It is also possible that both are involved as *atku80* mutant still display slight increases of point mutations frequency in the case of the MMS treatment without any influence of UV that can affect DNA repair machinery.

Additional experiments are required to prove whether the shift between NHEJ and HR indeed occurs.

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