The influence of DNA double-strand break repair on plant genome integrity

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von

Diplom-Biochemikerin **Nadiya Orel**

aus Ivano-Frankivsk, Ukraine

Dekan: Prof. Dr. M. Metzler
Referent: Prof. Dr. H. Puchta
Korreferent: Prof. Dr. A. Hartwig
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Table of Contents

Abbreviation

ATP	adenosine 5'-triphosphate
BAP	Benzylaminopurin
bp	base pair(s)
BSA	Bovine serum albumin
CaMV	Cauliflower mosaic virus
cDNA	complementary DNA
CIM	callus induction medium
Col-O	Columbia
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DSB	double-strand break
DMSO	dimethylsulfoxid
2,4-D	2,4-dichlorophenoxyacetic acid
dATP	deoxyadenosine 5'-triphosphate
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenedaminetetraacetic acid
EtOH	ethanol
Fe-EDTA	Fe- ethylenedaminetetraacetic acid
Fig.	Figure
GM	Germination medium
GT	Gene targeting
GUS	β-glucuronidase
HAc	the salt of acetic acid
HR	homologous recombination
IPTG	isopropyl- β-D-thiogalactopyranoside
IAA	indoleacetic acid
2iP	2-isopentenyl adenin
Kan	kanamycin
kb	kilo base pairs
LB	left border
M	Molarity (mol/l)
mRNA	messenger ribonucleic acid
MES	morpholinoethansulphonic acid
MS	Murashige and Skoog salt mixture
MW	Molecular weight
NAA	α-naphthaleacetic acid
NHEJ	non-homologous end joining
OD	optical density
ON	overnight
ORF	open reading frame
OSI	one-side invasion
P	promoter
PCR	polymerase chain reaction
pH	potentia Hydrogenii
RB	right border
Rf	rifampicin
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rotations per minute

RT	room temperature
SDS	sodium dodecyl sulphate
SM	Selection medium
T-DNA	transferred DNA
T	terminator
Tris	hydroxymethylaminomethan
U	Unit
UV	ultraviolet
vol.	volume
wt	wild type
X-Gluc	5-bromo-4-chloro-3-indolyl-ß-D-glucuronic acid

Units of Measure and Prefixes

Unit	Name
atm	atmosphere
Ci	Curie
°C	degree Celsius
D	Dalton
g	gram
h	hour(s)
L	Liter(s)
m	meter
min	minute(s)
psi	pounds per square inch
s	sec
V	volt

Symbol	Prefix (Factor)
k	kilo (10^{3})
c	centi (10^{-2})
m	milli (10^{-3})
µ	micro (10^{-6})
n	nano (10^{-9})
p	pico (10^{-12})

Abbreviation of species

A. tumefaciens	*Agrobacterium tumefaciens* (Smith and Townsend, 1907) Conn, 1942
A. thaliana	*Arabidopsis thaliana* (L.) Heynh
D. melanogaster	*Drosophila melanogaster* Meigen
E. coli	*Escherichia coli* (Migula,1895) Castellani and Chalmers, 1919
H. sapiens	*Homo sapiens* L.
N. plumbaginifolia	*Nicotiana plumbaginifolia* Viviani
N. tabacum	*Nicotina tabacum* L.
S. cerevisiae	*Saccharomyces cerevisiae* Meyen ex E. C. Hansen

1 Introduction

1.1 The evolution of plant genome size

125 Mbp to 110.000 Mbp represent the range of nuclear genome size, which has been observed among Angiosperms. Between these estimates for *Arabidopsis thaliana* and *Fritillaria assyriaca* (Lily family), a wide range of genome sizes and chromosome numbers are found among flowering plants (reviewed in Bennett et al., 2000). The variations are mainly due to different amounts of repetitive DNA sequences in these genomes. The abundance of repetitive sequences has been reported to be positively correlated with genome size (Bennetzen, 2000a; Bennetzen, 2000b). Polyploidy appears to be widespread in the plant kingdom, but it does not account for the large variations in genome sizes which are observed in higher plants.

The *Brassicaceae* family lends itself particularly well to comparative genome analysis studies. Small size of mature plants, a short generation time, prolific seed production from single plants and diploidy make *Arabidopsis thaliana* ideally suited for genetic and mutation analysis. For there reasons and due its small genome size, this crucifer has been chosen as model organism for molecular genetic studies in plants. The genome of *Arabidopsis thaliana* is the best-studied genome of higher plants and has been sequenced completely (The *Arabidopsis* Genome Initiative, 2000). The genomic sequence offers the raw material for comprehensive analysis of gene function in plants, and will provide further powerful opportunities for comparing genetic information with other plant species, prokaryotes, fungi and animals in future (Bennetzen, 2001). The *Arabidopsis* genome seems to have experienced many small genetic rearrangements far beyond that seen in any other sequenced genome. Small inversions, translocations, duplications or deletions of one or a few genes occurred with high frequency and mostly within the last millions years (O'Neill and Bancroft, 2000; Acarkan et. al., 2000). The six periods of large segmental duplications or the generation of polyploidy in the history of *Arabidopsis* lineage seem to

have occurred at discrete times between 200 and 50 millions years ago. (Vision et. al., 2000). Probably the numerous small rearrangements (particularly genic deletion) are tolerated because of the extensive genetic duplication present in the genome (Bennetzen, 2001). As the *Arabidopsis* genome is so small (125 Mbp) after the duplications, ample amount of DNA must have been lost during the recent evolution. Recently programmed sequence elimination in the first generations after allopolypolydiztion of wheat has been reported (Ozkan et. al., 2001; Shaked et. al., 2001), but further species-specific sequence loss over longer evolutionary time periods has to be postulated (Petrov, 2001). Indications for species-specific deletions were first found in insects. Studies of non coding retrotransposon-like sequences of insects indicated that DNA is lost at high rates from the *Drosophila* genome (Petrov, 1996). Indeed, the rate of deletions differed drastically between different insect genomes. The Hawaiian cricket *(Laupala)*, which has a 11 times larger genome than *Drosophila,* has a 40-fold lower rate of DNA loss than *Drosophila*. This applies for the number as well as the size of the deletions. These findings lead to the suggestion of a hypothesis that there is an inverse correlation between genome size and deletions size (Petrov et. al., 2000).

But what kind of process might be responsible for these deletions? Deletions may occur by different mechanisms: by replication slippage (as suggested by Capy, 2000), by unequal crossover (as suggested by Smith, 1976) or by double-strand break (DSB) repair.

Recently DSB-induced deletions, that result in a loss of function of a marker gene were compared between the two related dicotyledonous plant species *Arabidopsis* and tobacco. Whereas the mechanism of junction formation by illegitimate recombination itself was not different between the two species, the size classes of deletions in *Arabidopsis* and tobacco differed remarkably. In average the deletions were one third larger in *Arabidopsis* than in tobacco (Kirik et. al., 2000). In the same time it was not possible to find insertions

associated with deletions in *Arabidopsis* whereas this was the case in almost half of the repair events analysed in tobacco (Salomon and Puchta, 1998).

It was postulated that the loss of DNA by illegitimate recombination is the driving force for decreasing genome size of *Arabidopsis*. Devos et. al. (2002) in their study sustained this hypothesis by an "in silico" study which determined that at least fivefold more DNA is removed by illegitimate recombination then by unequal homologous recombination events during elimination of transposable elements (TEs). This study is based on the analysis of long terminal repeats (LTR)-retrotransposon families on the basis of homology of the LTRs rather than the open reading frames. Compared were not only complete elements but also solo LTRs and elements that underwent a variety of deletions. The presence of highly degraded retroelements also suggests that retrotransposon amplification has not been confined to the last 4 million years, as is indicated by dating of intact retroelements.

Arabidopsis thaliana and *Nicotiana tabaccum* are two related dicotyledonous plant species, which differ about 30 time in genome size. *Arabidopsis* with one of the smallest genomes among higher plants and the 2C DNA content has been determined at ≈0.30 pg (Arumuganathan and Earle, 1991). A much higher value of 8.7 pg has been determined for tobacco (Bennetzen et. al., 1997). The *Arabidopsis thaliana* genome contains a low amount of repetitive DNA (Meyerowitz and Somerville, 1994), in contrast, the tobacco genome contains about 55% repetitive sequences.

The possible reason for different deletion sizes during DSB repair could be due to dissimilar processing of DNA ends in both species. This question was addressed in the current study.

1.2 DNA Recombination

In generally, recombination involves the cutting and covalent joining of DNA sequences which results in the new combination of genetic information.

A number of models have been put forth over the years to explain how DNA recombination occurs. In principle, there are two different kinds of DNA-recombination (Leach, 1996; Hoffmann, 1994).

Homologous recombination (HR is the exchange of covalent linkage of DNA molecules that are identical or very similar in sequence) is classically thought to be a mechanism for promoting genetic diversity. For example, in diploid cell, meiotic recombination switches allele combinations along a linear chromosome, thereby producing a novel haploid for subsequent generation (Shinohara and Ogawa, 1995; Stahl, 1996).

It is commonly considered that homologous recombination is downregulated in somatic eukaryotic cells in preference to another recombination pathway, namely **illegitimate recombination or non-homologous end-joining** (NHEJ). NHEJ is the main mechanism in somatic cells for repairing of DNA double-strand breaks caused by surroundings physical and chemical reagents (Roth et. al., 1985; Lehman et. al., 1994; Mayerhofer et. al.,1991).

1.2.1 Homologous recombination

For all organisms, homologous recombination is important to ensure exchange of genetic material within a species, and is also a major pathway for the repair of DNA lesions, at least for lower eukaryotes and bacteria. Whereas many of the proteins and pathways involved in DNA repair and recombination are known in prokaryotes (bacteria), simple eukaryotes (yeast) and higher animal cells (West, 1997; Mosig, 1998; Haber, 2000; Wood et. al., 2001), comparably little is known about these components in plants. The ongoing sequence analysis of plant genomes revealed the presence of many genes similar to already

characterized recombination and repair genes from other organisms. This homology-based gene identification has several drawbacks: even if a plant gene is similar to a well-studied counterpart from a different organism, the exact biological function encoded protein might differ. In addition, plant-specific factors involved in DNA repair and recombination can not be identified by this approach.

Homologous recombination, in contrast to NHEJ, repairs damage precisely and thus the end products of this reaction are predictable. Because of this, one of the powerful uses of homologous recombination has been exploited for the modification of chromosomal genes - **"gene targeting"**.

Gene targeting signifies the integration of foreign (transfected) DNA molecules into homologous sequences of genomic DNA, thus allows the integration of DNA at predetermined positions. Therefore gene targeting is an important technology to study gene function. This technique has proved to be highly successful in yeast (Leung et. al., 1997) and mouse embryonic stem cells (Capecchi, 1989), but has been, so far, only of limited use in plants (Vergunst and Hooykaas, 1999; Mengiste and Paszkowski, 1999; Puchta, 2002; Hohn and Puchta, 2003; Britt and May, 2003), except in the moss, *Physcomitrella* (Schaefer, 2001). There are only several reports of successful targeting of endogenous natural genes in *Arabidopsis* (Kempin et. al., 1997; Hanin et. al., 2001) and rice (Terada et. al., 2002). The frequency of gene targeting in higher plants is too low (10^{-3}-10^{-5}) for practical application.

1.2.2 Illegitimate recombination or non-homologous end joining (NHEJ).

Illegitimate recombination or NHEJ is able to rejoin DNA ends in the absence of significant homology (Weaver, 1995; Lehman et. al., 1994). The pathway of NHEJ is conserved in eukaryotes as distantly related in *S. cerevisiae* and humans, and a similar

pathway is found in plants (Mayerhofer et. al., 1991; Gheysen et. al., 1991; Takano et.al , 1997; Salomon and Puchta, 1998).

First reports on NHEJ date back to the early eighties (Pellicer, 1980 ; Perucho et.al., 1980). In the following years, the mechanism of NHEJ was studied in detail in mammalian cells (Wilson et. al., 1982; Roth et. al., 1985), in extracts from *Xenopus* eggs (Pfeiffer, 1988; Thode et. al, 1990) and later in yeast (Goedecke et. al., 1994; Kramer et. al., 1994).

In yeast at least three different non-homologous end joining processes were characterised. One pathway is the precise joining of short overhanging, complementary ends, such as those produced by site-specific endonucleases. This is a highly efficient process, where most ends are successfully religated. However, when the ends are not complementary or when the presence of an endonuclease precludes precise re-ligation, there are two additional, but much less efficient, NHEJ processes. One, involving misalignment of overhanging ends, apparently occurs by pairing of one or a few base pairs, followed by filing-in by a DNA polymerase, and resulting in the ligation of ends with the insertion of a few base pairs. This process is strongly cell cycle regulated. Alternatively, annealing of microhomologies of two to a few base pair and removal of single-stranded tails, leads to the formation of deletions ranging from a few base pairs to several kb (Haber, 2000).

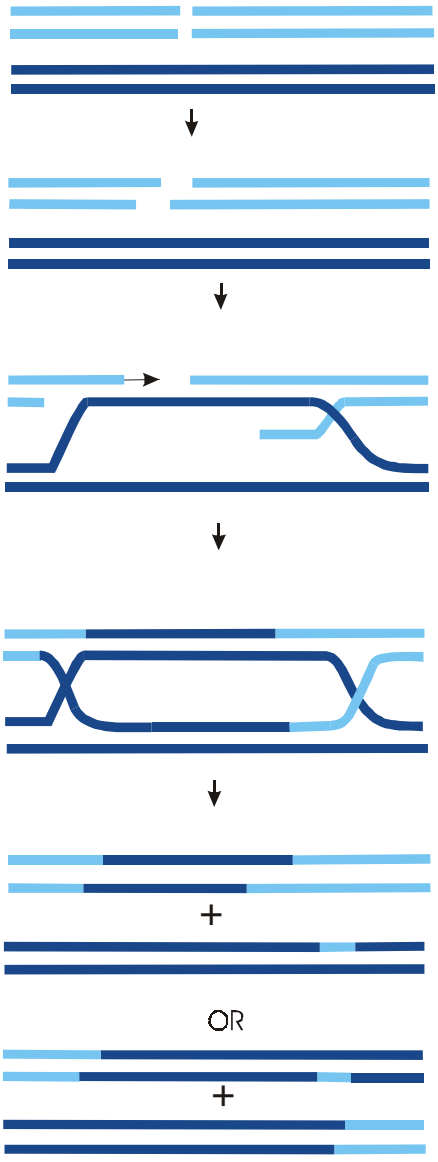
NHEJ, rather than homologous recombination, is the major pathway for DSB repair in plant somatic cells (Gorbunova and Levy, 1999). Characterisation of NHEJ in plants include the use of short homologies for rejoining, the occurrence of a short deletions at the broken site, and the presence of filler DNAs as well as direct or inverted repeats of nuclear sequences at the rejoined site. In plants NHEJ was characterized at the rejoined sites of restriction enzyme-digested plasmids in tobacco protoplasts (Gorbunova and Levy, 1997), at the rejoined sites of an integrated marker gene in tobacco cells that was cut before with I-*Sce*I (Salomon and Puchta, 1998), at the integration site or rearranged sites in transgenic rice and *Arabidopsis* after direct gene transfer (Takano et. al., 1997 ; Sawasaki et. al.,

1998; Kohli et. al., 1999), and at the border of T-DNA insertion sites (Gheysen et. al., 1991; Mayerhofer et. al., 1991; Laufs et. al., 1999).

1.3 The repair of DNA double-strand breaks – role in genome stability and genome evolution

The genomes of all living organisms are constantly subject to damage and decay. DNA double-strand breaks (DSB) may be induced by γ-irradiation, radio-mimetic chemicals or by breaks in single-stranded regions of DNA created during DNA replication (Kuzminov, 2001). DNA damage, in the form of DSBs, poses a considerable threat to genomic integrity and cell survival.

In early 80's classic model for the DNA double-strand break repair (DSBR) was postulated (Resnick, 1976; Szostak et. al.,1983), and since that time it has been applied as a general mechanisms of homologous recombination in all organisms examined (see Figure 1.1.). Nevertheless, beside DSBR model in last two decades alternative models were developed, recently namely single-strand annealing (SSA) (Lin et. al, 1984, 1990; Maryon and Carroll, 1991a, 1991b; Fishman-Lobell et. al., 1992), synthesis-dependent strand annealing (SDSA) (Richardson et.al.,1998) and one side invasion (OSI) (Belmaaza and Chartrand, 1994) model of recombination supplemented with new details and specified depending from object of study (Belmaaza and Chartrand, 1994; Nassif et. al., 1994; Puchta et. al., 1996; Holmes and Haber, 1999).



Double-strand breaks

Strand Resection.
One strand of the duplex is degraded by a nuclease starting from the DSB.

Strand Invasion, Annealing and Synthesis. The single stranded tail searches for a homologous sequence elsewhere in the genome. If it finds a homologous sequence it will invade the duplex and form a heteroduplex DNA. Strand synthesis initiates from the 3' ends. The other single-tranded tail can anneal to the displaced strand

Holliday Junction. DNA synthesis and strand displacement leads to formation of a second crossover structure called Holliday Junction.

Resolution of Holliday junctions and Crossing over. Cleavage of the Holliday junctions can occur via nucleases in two ways. Cleavage of both junctions along different axes will lead to a crossover of the distal arms. On the other hand, cleavage along the same axes will leave the distal arms unchanged.

Figure 1.1. The double-strand break repair (DSBR) model (Resnick, 1976; Szostak et. al.,1983).

The DSBR model was first proposed to explain plasmid gap repair and is the most accepted mechanism to explain the genetic relationship between gene conversion and crossover in meiotic recombination (Szostak et. al. 1983). Recombination is initiated by a DSB (Sun et. al., 1989; Cao et. al., 1990) and the 5’ ends at each side of the DSB are resected, exposing long 3’-ended single-stranded tails (Sun et. al., 1991), which invade the homologous double-strand DNA (dsDNA) and prime DNA synthesis. After pairing of the invading single-strand DNA

(ssDNA) and the invaded dsDNA, the reaction is followed by strand exchange. This generates a DNA heteroduplex (Goyon and Lichten, 1993; Nag and Petes, 1993), which is a key feature for gene conversion in all recombination reactions. The double-strand exchange leads to the formation of two four-stranded intermediates, termed Holliday junctions (Schwacha and Kleckner, 1994). The cleavage of these two Holliday junctions in either the same or the opposite direction results in non-crossover or crossover products, respectively.

It has been shown that, from yeast to animal cells, many mitotic gene conversion events are not always associated with crossovers (Gloor et. al., 1991; Nassif et. al., 1994; Puchta, 1999; Johnson and Jasin, 2000). Such findings led to an alternative model, termed **synthesis-dependent strand annealing** SDSA (Hastings, 1988; McGill et. al., 1989), in which, after 3' end invasion, the newly synthesized DNA strands are released from the invaded DNA template and returned to the acceptor molecule. Consequently, the only possible recombination product is a gene conversion not associated with crossover. SDSA is an important mechanism of mitotic recombination and it can also participate in the formation of meiotic gene conversions (Allers and Lichten, 2001). The SDSA model explains best the occurrence of gene conversion, a complex process ubiquitous in all organisms. This process maintains the integrity of eukaryotic genomes in somatic cells (Jasin, 2000) and reinitiates DNA replication after the replication fork passes through a single-strand nick in the DNA (Kuzminov, 1999). Furthermore, gene conversion is responsible for repairing the majority of DNA double-strand breaks that occur in the G2 phase of the cell cycle (e.g., in vertebrate cells, Takata et. al., 1998). Data suggest, that SDSA is the predominant mechanism in somatic cells (Nassif et. al., 1994; Chiurazzi et. al., 1996; Ray and Langer, 2002, Rubin and Levy, 1997; Puchta, 1998a). In the SDSA model (Figure 1.2), a broken DNA end invades a homologous template and primes DNA synthesis, producing long, 3' single-strand extensions. Since the template is homologous, the sequence of the 3' extension is complementary to the other broken end and therefore, after synthesis, strands from both ends

of the break may anneal together. Subsequent sequence trimming and/or gap filling, followed by ligation, complete the repair of the break. In somatic plant cells SDSA was also studied (Chiurazzi et al., 1996; Puchta, 1998a; Gorbunova and Levy, 1999). If the two 3' ends are not homologous the break will closed via illegitimate recombination, which lead to a change in genetic information (Figure1.2). SDSA model can be used for the description of repair processes that cannot easily be explained by the DSBR model (Puchta, 1999a).

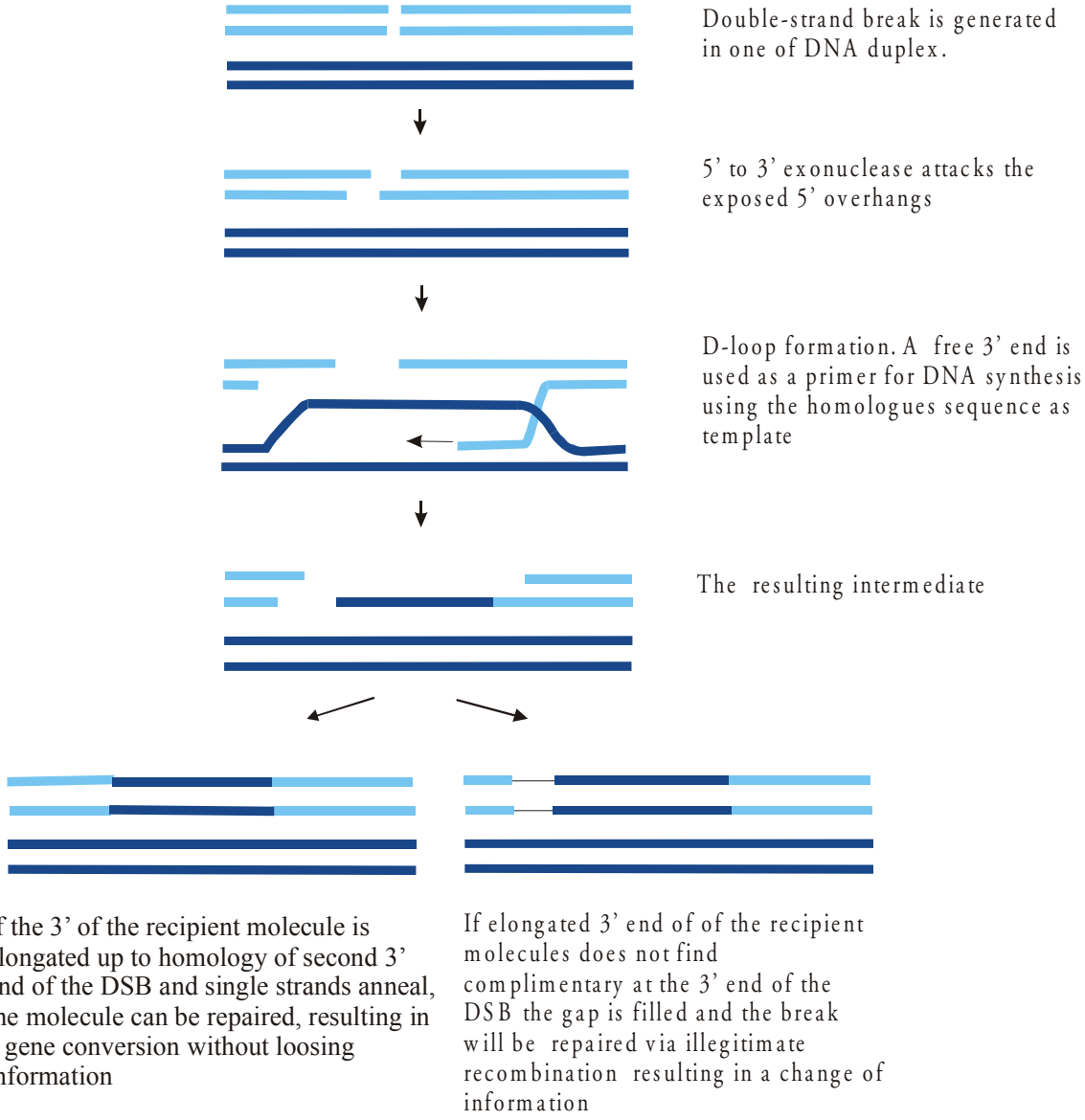


Figure 1.2. Model for the repair of genomic DSB based on the synthesis-dependent strand annealing (SDSA) model of recombination.

The single-strand annealing (SSA) model was first suggested for extrachromosomal recombination between plasmids in mammalian cells (Lin et. al., 1984; 1990). After induction of a DSB (Figure 1.3), the free double-stranded ends are resected by a single-strand specific exonuclease, leaving behind 3'-single-stranded overhangs. These single strands can anneal with each other at regions of complementarity, overhanging non-homologous sequences are digested or alternatively single stranded gaps could also be refilled by repair synthesis, and in a last step the double-strand is restored by religation of the remaining nicks. As a result a deletion occurs, the sequence information between the repeated sequences is lost. The model has been applied for describing extrachromosomal recombination in *Xenopus* oocytes (Maryon and Carroll, 1991a, 1991b) and yeast (Fishman-Lobell et. al., 1992). In plants it could be demonstrated that extrachromosomal recombination proceeds efficiently via single-strand annealing (Puchta and Hohn, 1991; Bilang et. al., 1992; de Groot et. al., 1992; Puchta and Meier, 1994).

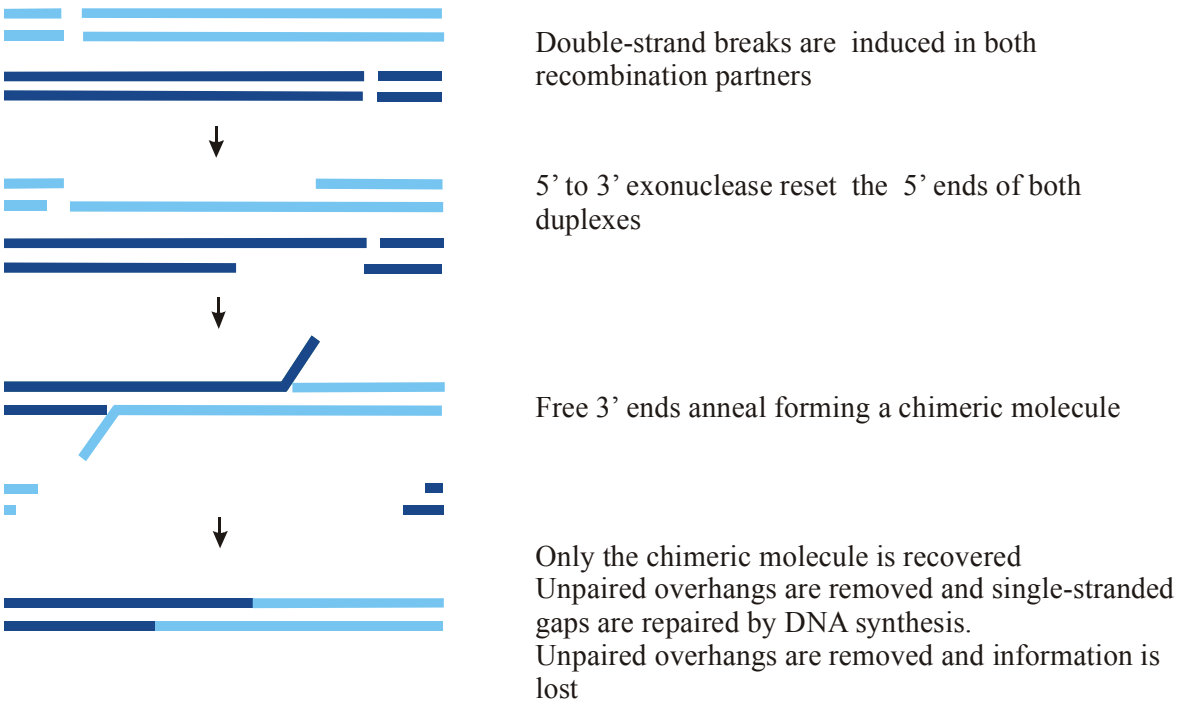


Figure 1.3. Single Strand Annealing (SSA) mechanism as models for the repair of DSBs

The repair of DNA double-strand breaks by **homologous recombination** requires that the two interacting DNA molecules have very similar sequences. The amount of homology required for repair has been studied in two main contexts. One focuses on the fraction of sequence identity shared between the recipient and donor DNA sequences. The frequency of gene conversion is greatest when the two sequences are identical and declines as the percentage identity between the two sequences decreases. In *E. coli* and *S. cerevisiae*, and presumably also in other organisms, two different systems evaluate the sequence identity between the interacting molecules (Kuzminov, 1999; Paques and Haber, 1999). During the search for homology the initial match made by the recA/RAD51 protein is promoted (at least in vitro) between sequences with about 10% sequence difference (Bazemore et. al., 1997). The mismatch repair system then tests for sequence matching and rejects those that contain mismatched base pairs (Modrich and Lahue, 1996; Evans and Alani, 2000). Thus, the mismatch repair system is responsible for most of the sequence specificity during double-strand-break repair by gene conversion.

The homology requirements for DSB repair have also been examined in the context of the extent of donor sequence homology. This is often expressed in terms of the minimal efficient processing segment (MEPS). This is the smallest amount of contiguous identical sequence between the donor and recipient sites that are required to initiate efficient double-strand-break repair by gene conversion. The MEPS varies from 25–50 bp in *E. coli* and bacteriophage T4 to 200 bp in eukaryotic systems (Singer et. al. 1982; Rubnitz and Subramani, 1984; Shen and Huang, 1986; Liskay et. al. 1987; Thaler and Noordewier, 1992; Jinks-Robertson et. al., 1993). In these analyses various heterologous sequences were used as markers for the occurrence of gene conversion, but the size and sequence of the heterologous sequences were not systematically varied.

Using transposable elements or highly specific restriction endonucleases for induction of breaks at specific loci within eukaryotic genomes (for general reviews on endonucleases

induced repair see Paques and Haber, 1999; Jasin, 2000) it has been possible to characterize DSB-induced homologous recombination in somatic plant cells (for review see Gorbunova and Levy, 1999). In previous studies besides enhancing integration of T-DNAs at homologous loci (Puchta et. al., 1996; Puchta, 1998b; Reiss et. al., 2000), repair of a DSB with homologous sequences close to the break (Chiurazzi et al., 1996; Xiao and Peterson, 2000; Siebert and Puchta, 2002) or at an ectopic position in the genome (Shalev and Levi, 1997; Puchta, 1999a) were analyzed. Recently it was shown that a DSBs can also be repaired by homologous recombination using transgenic allelic sequences on the homologous chromosome with a frequency about 10^{-4} (Gisler et. al., 2002).

In general, NHEJ seems to be the main mode of DNA repair also in somatic plant cells (Salomon and Puchta, 1998; Kirik et. al., 2000).

1.4 Proteins involved in the processing of chromosomal DSBs

Non-homologous end joining and homologous recombination pathways appear to compete with one another. Because of the fundamental differences in their enzymology, it is likely that at some step each DSB repair pathway becomes irreversible committed after early steps, that are common to both pathways.

In yeast DSB can provide a substrate for at least two multiple-enzyme complexes in the pathways to HR: the Rad50-Mre11-Xrs2 and the Rad51-Rad54-Rad55-Rad57 complex (Paques and Haber, 1999). The Rad50-Mre11-Xrs2 (RMX) complex has an end-tethering function and clear, although variable, defects in both HR and NHEJ pathways (Petrini, 1999; De Jager et al., 2001). The RMX complex first assembles on the DNA ends and resects the ends by exonuclease activity to expose single strands. Concomitant with resection, the complex is thought to interact with Rad52, the defining member of the required epistatis group of genes, encodes a multimeric protein that possesses DNA-end-binding function (Van Dyck et. a1., 1999; Stasiak et. al., 2000). Rad52 assembles the

Rad51-Rad54-Rad55-Rad57 complex, which leads to the DNA-sequence-homology-dependent pathways. SDSA requires continued participation of Mre11 and Xrs2 (Moore and Haber, 1996) but DSBR is more dependent on Rad51 (Rattray and Symington, 1994). Rad52 and Rad59 can also act to catalyze the direct annealing of the two 3' nucleoprotein filaments independently of the other epistasis group members, to ultimately create a deletion in a pathway known as single-strand annealing (Ivanov et. al., 1996; Shinohara et. al., 1998; Sugawara et. al., 2000).

In yeast, Rad52 is essential for any repair by HR and presumably has functions in addition to recruiting the Rad51 complex. (Van Dyck et. al., 1999). If Rad52 is unavailable, then a Ku70-Ku80 complex binds the ends and helps to recruit the DNA-depended protein kinase. Rad52 is thought to compete with Ku70-Ku80 for DNA binding. There seems to be no strong Rad52 homologous in plants, although there are recognizable homologs of most other members of the Rad52-epistasis group of proteins. The function of Rad52, thought to be involved in the regulation between HR and NHEJ, is probably accomplished by an unknown component in plants. It was postulated that the absence of the Rad52 homolog is related to low levels of HR in mitotic plant cells (Ray and Langer; 2002). Ku70-Ku80 association of DNA ends triggers mitotic check-point arrest by signalling though DNA-dependent protein kinase (Lee et. al., 1998) and allows the recruitment of at least two other proteins that are essential for NHEJ: DNA ligase IV (DnL4) and its accessory cofactor LIF1(XRCC4) (Chen et. al., 2001). NHEJ in mammalian cells has an analogous requirement: Ku70-Ku80, DNA ligase IV and XRCC4 (Van Dyck et a1., 1999). In meiotic cells of mice, the levels of Ku70-Ku80 are much lower then those in somatic cells are (Goedecke et. al., 1999). This implies that HR, which repairs the chromosomal breaks during early meiosis require for efficient processing a downregulation of the NHEJ activity. In meiosis-competent yeast cells, LIF1 expression is reduced, again suggesting that the yeast NHEJ is low when HR is high (Valencia et. al., 2001). Certain alleles of rad50 in yeast

display mitotic hyper-recombination and reduced NHEJ, suggesting that the yeast NHEJ pathway is more sensitive to detect in the initial Rad50-Mre11-Xrs2 complex than the HR pathway (Moore and Haber, 1996; Malone et. al., 1990). Analogously, an *Arabidopsis rad50* mutant display somatic hyper-recombination (Gherbi et. al., 2001), but it is not known whether this has reduced levels of NHEJ. Plant DnL4 and XRCC4 homologs are functionally similar to the corresponding sequence homologous yeast proteins (West et. al., 2000), confirming the existence of a conserved NHEJ mechanisms in plant, yeast and mammals.

1.5 Aims of the current work

In the current study two different, but related topics about DSBR processes in plants were investigated.

1. Species-specific differences in the processing of double-strand breaks by NHEJ in *Arabidopsis* and tobacco.

Recently species-specific differences in non-homologous end-joining (NHEJ) of genomic double-strand breaks (DSBs) were reported for the two dicotyledonous plants *Arabidopsis thaliana* and *Nicotiana tabacum* (Kirik et. al., 2000). In *Arabidopsis* deletions were on average larger than in tobacco and not associated with insertions. This was postulated to be due to differences in the processing of DNA ends in both species.

The specific aim of the study was to analyse and compare the processing of free DNA ends in both plant species by biolistic transformation of leaf tissue with linearized plasmid molecules.

2. Different pathways of homologous recombination are used for the repair of double-strand breaks within tandemly arranged sequences in the plant genome.

Beside the NHEJ (Salomon and Puchta, 1998; Gorbunova and Levy, 1999; Kirik et. al., 2000; Ray and Langer, 2002) homologous recombination can be used for repair DSBs in somatic plant cells (Shalev and Levy, 1998; Puchta, 1999a; Gisler et. al., 2002). The fraction of homologous repair events in relation to NHEJ rises if homology close to the break is available (intrachromosomal recombination) (Siebert and Puchta, 2002; Xiao and Peterson, 2000). Depending on the structure of the respective chromosomal locus at least two different kinds of pathways could be used to repair the break. One pathway results in

the formation of a deletion between the repeats (as described by SSA model) and the other is a gene conversion event (as described by the SDSA model) (Fischman-Lobell et. al., 1992). Recent results indicated that deletion formation is highly efficient pathway in plants that can be used in up to a third of the cases for the repair of a DSB in proximity to homologies (Siebert and Puchta, 2002; Kirik, doctoral thesis, 2001). It was important to find out under comparable conditions to which extent the gene conversion pathway could be used for the repair for DSBs in somatic plant cells.

2. Materials

2.1. Chemicals and reagents

Most of the chemicals and reagents were purchased from Roth (Karlsruhe), Serva (Heidelberg), Fluka (Deisenhofen), Merk (Darmstadt), Sigma (München), Duchefa (Haarlem, NL). Chemicals or reagents obtained from other companies are listed below:

Bacto-Agar, Beef extract	Difco, Detroit MI, USA
Yeast extract, Pepton , EDTA	Gibco-BRL, Eggenstein
Sephadex^{TM} G-25	Amersham Pharmacia Biotech UK
Fe-sulphate	Riedel-de-Haen, Seelze
Natrium-EDTA	UD, Cleveland, USA
Silwet L-77	Lehle Seeds, Round Rock, USA
Tris-(hydroxymethyl)-aminometan (Tris), Tris-(hydroxymethyl)-aminometan - HCl (Tris-HCl), SDS	USB, USA

Solution were prepared with deionised, destilated and sterile water. Medias, buffers and solutions were sterilized per autoclaving at 120˚C/ 20 min .

2.2 Enzymes used in molecular biology experiments

Exonuclease-free Klenow	USB, USA
Restriction endonucleases	BioLabs, Amersham
Ribonuclease A (RNase A)	Sigma, München
DNA-Polymerase TaKaRa ExTaq	TaKaRa, Japan
T4 DNA-ligase	Gibco-BRL
Shrimp Alkaline Phosphatase	USB, USA

2.3 Kits

High Pure PCR Purification Kit	Roche, Mannheim
Quiagen Midiprep	Qiagen
QIAEX Gel Extraction kit	Qiagen
Qiagen Plasmid Isolation kit	Qiagen
Plant DNA Mini kit	Peqlab, Erlangen
Salmon Sperm DNA kit	Stratagene
Megaprime DNA Labelling system	Amersham-Pharmacia Biotech UK

2.4 Disposables

GB58 Blotting paper	Heinemann Labortechnik
Nylon membrane *Hybond-N+*	Amersham-Pharmacia Biotech UK
Rapture Disks, 1,100 psi	BioRad, USA
Macrocarriers	BioRad, USA
Stopping Screens	BioRad, USA
1 μm gold Microcarriers	BioRad, USA

2.5 Equipment

Biolistic PDS-1000/Helium Particle Delivery System	BioRad, USA
Centrifuge 5415C, 5415, 5417	Eppendorf, Hamburg
GeneAmp PCR System 2400	Perkin Elmer, Langen
Electroporator	EasyjecT Prima, EquiBio
Electrophoresis power supply Power Pac 3000	BioRad, München
Electrophoresis horizontal system EASYCAST	Owl Scientific, Inc. USA
Freezer (-80˚C)	Froilabo, France

Hybridization ovens	Biometra, Götingen
Incubators	Heraeus, Hanau
Laminar	Kendro
Microscopes	Zeiss, Germany
Megafuge 1.OR	Heraeus, Hanau
pH-meter, HI 9321	Schütt, Goettingen
Phosphor screens	Fujifilm
Shaker	GFL, Germany
Spectrophotometer Spectronic 1201	Milton Roy, Rochester, USA
Sterilisator Varioclav 400	H+P Labortechnik, Oberschisheim
Thermomixers 5436, 5437	Eppendorf, Hamburg
Waterbath-thermostat D1	Haake, Karlsruhe

2.6 Electronic data management

Software

Adobe Photoshop 6.0	Adobe System
DNASTAR	Lasergene
Tina 2.09g	
Chromas 1.45	
Microsoft Excel 2000	Microsoft Corporation
Microsoft Word 2000	Microsoft Corporation
Microsoft PowerPoint	Microsoft Corporation
Netscape Communicator	Netscape
CorelDRAW 9	

2.7 WWW-sites

National Center for Biotechnology Information

http://www.ncbi.nlm.nih.gov:80/

Biochemistry and Molecular Biology Journals

http://www.geocities.com/~jrbeasley/biochem/journals.html#P

The *Arabidopsis* Information Resource (TAIR)

http://www.arabidopsis.org

2.8 Biological material

2.8.1 Bacteria strains

Escherichia coli strain:

DH5α	Gibco BRL, Eggenstein (Sambrook et. al., 1989)

Agrobacterium tumefaciens strain:

C58 Cl Rf	pGV 2260 in C58C1 (Deblaere et. al., 1985)

2.8.2 Plants

The *Arabidopsis thaliana* ecotype Colambia and *Nicotiana tabacum* SR1 were used for plant transformation and bombardment.

2.9 Plasmids

pGUS23	Puchta and Hohn, 1991
DGU.US	Orel et.al., 2003b
pZpp-ISceI	Kirik A., Ph.D thesis, 2001
pPM6000k	Rossi et al., 1993
pCH23	Puchta, unpublished
pGEM-T Easy Vector system	Promega

2.10 Oligonucleotide primers

The oligonucleotide primers were designed on the basis of sequence information and synthesis from Metabion company.

231S	5'-gtacagcatgcgggcccttaattaaccgcggaggcctgttaacatccataccggttctaga-3'
231AS	5'-gtacttctagaaccggtatcgatgttaacaggcctccgcggttaattaaggggcccgcatgc-3'
Chrisoben	5’-ctcgagattaccctgttatccctagtcgac-3’
Chrisunten	5’-gtcgactagggataacagggtaatctcgag-3’
p35SX	5’-gccgtctagaggagtcaaagattcaaatagaggacc-3’
pTH	5’-gccgaagcttattccgatctagtaacatagatagcagg-3’
pHZD	5’-gccgggtaccgcggccgccgcgaaaactgtggaattgatcagcg-3’
pRZD	5’-gccgggtaccgctctttaatcgcctgtaagtgcg-3’
pGUSR	5’-gcgttaattaattcgagctcggtagcaatttcgaggc-3’
GUSHIN2	5’cggaagcttctcagactaagcaggtgacgaacg-3’

2.11 Media, buffers and solutions

2.11.1 Bacteria media

Luria-Bertani (LB) medium

(Sambrook et. al., 1989)

Bactotrypton - 10g/l

Yeast extract - 5g/l

NaCl - 10 g/l

pH 7,0 with NaOH

for plates, medium was supplemented with 15 g/l agar and appropriate antibiotics

Yeast-broth (YEB) medium

Beef extract - 5 g/l

Peptone - 5 g/l

Sucrose - 5 g/l

Yeast extract - 1 g/l

$MgSO_4 \cdot 7H_2O$ – 0.5 g/l

pH 7,2 with NaOH

for plates, medium was supplemented with 15 g/l agar and antibiotics

SOC

Bactotrypton - 20 g/l

Yeast extract - 5 g/l

NaCl - 0,58 g/l

KCl - 0,186 g/l

Glucose -20mM

pH 7,4 with NaOH

2.11.2 Plant media

Germination medium (GM)

B5 Basal salt mix

B5-vitamins

Fe-EDTA – 74,6 mg/l

MES - 1g/l

Sucrose - 10 g/l

pH 5,7 with KOH

for plates, medium was supplemented with 8 g/l agar

Callus induction medium (CIM)

B5 Basal salt mix

B5-vitamins

Glucose - 20 g/l

MES - 1g/l

pH 5,74 with KOH

after autoclaving

2,4-D - 1 mg/l, kinetin - 0,2 mg/l, NAA - 1 mg/l,

biotin -1 mg/l,

BAP - 1mg/l

Selection medium (SM)

Murashide and Skoog (MS) inorganic salts

B5-vitanims

pH 5,7 with KOH

for plates, medium was supplemented with 8 g/l agar and antibiotics

Murashige and Skoog (MS) medium

MS salts

B5-vitamins

Fe-EDTA - 74,6 mg/l

MES - 1g/l

Sucrose - 30 g/l

pH 5,7 with KOH

for plates, medium was supplemented with 8 g/l agar

Infiltration medium (IM)

1/2xMS salts

B5-vitamins

Sucrose - 50 g/l

BAP - 0,44 µM

3,5-Dimethoxy-4-hydroxyacetophenon -100 mg/l

adjust to pH 5,7 with KOH and add

Silwet L-77 - 400 µl/l

2.11.3 Buffers

1M Na-phosphate buffer pH7,0

1M $NaH_2PO_4 \cdot H_2O$ buffer pH 9,1 was mixed with 1M $Na_2HPO_4 \cdot 7H_2O$ pH 4,0 in the ratio 2:1

20xSSC

0,3M Na citrate

3 M NaCl

pH 7 with NaOH

10xTAE buffer pH 8	Tris - 48,4 g/l
	acetic acid - 11,4 ml/l
	EDTA – 0.3 g/l
DNA loading buffer (10x)	30% glycerol, 1xTAE
	0,025% bromophenol blue
	0,025% xylene-glycol
20xSSPE buffer	3M NaCl
	0.2M $NaH_2PO_4 \cdot H_2O$
	0.02M EDTA pH 7.4
	pH 7.4 with 10N NaOH
Church Hybridisation buffer	$NaH_2PO_4 \cdot H_2O$ – 1.76 g/l
	$Na_2HPO_4 \cdot 2H_2O$ – 66.2 g/l
	SDS – 70 g/l
	BSA – 10 g/l

2.12 Stock solutions for antibiotics, hormones and vitamins

	Concentration stock	Solvent	Final con-centration	Application
Ampicillin*	50 mg/ml	H_2O	50 mg/l	LB for *E. coli*
BAP**	1 mg/ml	NaOH/H_20		Infiltration medium
Biotin**	1 mg/ml	NaOH/H_20	1 mg/l	CIM
B5-vitamins(100x)** (Gamborg et.al., 1968)	0.1% Nicotinic asid 0.1% Pyridoxine-HCl 1% Tiamine-HCl 10% Myoinositol	H_2O	1x	Plant medium
Carbenecillin*	50 mg/ml	H_2O	200 mg/l	LB for *E. coli*
2,4-D **	1 mg/ml	NaOH/H_20	1 mg/l	CIM
Gentamycin*	100 mg/ml	H_2O	60 mg/l	Medium for *Arabidopsis*
Hydroxyacetophenon*	100 mg/ml	DMSO	100 mg/l	Infiltration medium
Hygromycin*	50 mg/ml	H_2O	30 mg/l	Medium for *Arabidopsis*
Kanamycin*	50 mg/ml	H_2O	50 mg/l	Medium for *E.coli* and *Arabidopsis*
Kinetin*	0.2 mg/ml	NaOH/H_20	0,2 mg/l	CIM
NAA***	1 mg/ml	NaOH/H_20	0,1 mg/l	CIM
Rifampicin*	50 mg/ml	DMSO	50 mg/l	Medium for *Agrobacterium tumefaciens*

All solution were filter sterilised (0.2 µm).

*- Storage at –20°C. **- Storage at 4°C. ***- Storage at RT.

3 Methods

3.1 Plant culture

3.1.1 Sterilization of *Arabidopsis* seeds

Seeds were put in Eppendorf reaction tube and 1 ml of the following reagents were added: 70% EtOH - 1 min, 4% NaOCl - 15 min, sterile water - 4 times (1 min, 2min, 5 min, 5 min).

Removing of solution was done after brief centrifugation by 6000 rpm. For the last step seeds were resuspended in 0,5 ml sterile 0.1% agarose and poured onto plates. Seeds were spread evenly over the entire area and after drying for 10 min. the plates were sealed with surgical tape and were placed overnight for +4°C and then in a growth chamber (Schmidt-Puchta, et. al., in press).

3.1.2 Plant growth

Seeds were sowed in lightweight plastic pots prepared with soil and plants were grown under conditions of 16 hours light/ 8 hours dark at 20 °C to 22 °C, fertilizing from below with *Arabidopsis* fertilizer once a week.

3.1.3 Transformation of *Arabidopsis* by vacuum infiltration

400 ml *A. tumefaciens* cultures grew at 28°C at 200 rpm till OD_{600}>2. The cells were centrifuged (6000 rpm, 10 min, RT) and after harvesting, were resuspended in 800 ml infiltration medium (OD_{600} approx. 0,8).

Then above-ground part plants (5-7 weeks old) were dipped in infiltration medium for 5-8 s, with gentle agitation. Dipped plants were placed under a cover for 16 to 24 hours to maintain high humidity. Then plants were set upright, keeping watering and grown

normally till seeds become mature (Clough S.J. and Bent A.F.,1998). Dry seed were harvested.

3.1.4 Genetic analysis of transgenic plants

Sterilized seeds (3.1.1) were resuspend in 0,5 ml sterile 0.1% agarose and poured onto SM plates containing the appropriate selection. After one week in the growth chamber, when the first true leaves started developing, it was possible to select putative transgenic plants (green) from untransformed plants (bleached). Putative transgenic plants were transferred onto a second selection plate to reduce the possibility of selecting false positives. 2-3 week old seedlings were planted to soil.

3.1.5 Crossing *Arabidopsis* plants

Arabidopsis plants were grown until the flowers were at a stage right before the white petals become visible. All flowers were cut out, that were too old or too young from the inflorescence and 2-6 flowers were left per pollination. All flower parts except the ovary were removed. From a donor plant pollen were obtained and were placed on the tip of the exposed ovary. This was repeated at least twice to ensure proper pollination. Each pollinated ovary was labelled and let for developing to maturity. When the siliques were dry the seeds were harvested.

3.1.6 Inoculation of *Arabidopsis* seedlings with *A. tumefaciens*

After centrifugation of 50 ml fresh overnight culture of *A. tumefaciens*, grown in YEB medium containing corresponding antibiotics, the pellet was washed with 20 ml Basic medium and resuspended in Basic medium to a final OD_{600}=1.0.

2 weeks old *Arabidopsis* seedlings were added to bacterial suspension supplied with

300 µg/ml acetosyringon. The mixture was exposed to reduced pressure (0,15 atm) in sterile vacuum chamber for 5 min.

The seedlings were placed on CIM plates and further cocultivated for three days in a growth chamber. After cocultivation they were analysed with the histochemical GUS assay.

3.1.7 Histological staining for GUS expression

Arabidopsis and tobacco leaves or *Arabidopsis* seedlings were put into ß-glucuronidase staining solution (0,1M sodium phosphate buffer pH 7.0 with 0,05% X-Gluc dissolved in dimethyl formamide, in presence of 0,1% sodium azide) and after application of vacuum (0,15 atm., 5 min.) the incubation was continued at 37˚C for two days. The plantlets were bleached with ethanol at 65˚C for 1h. Sectors on the leaves were counted under binocular.

3.1.8 Non-destructive GUS-assay

To detect recombination events in plants and to propagate them further, ß-glucuronidase staining was done under non-destructive conditions. After 10 days growing on germination medium under sterile conditions, seedling were transferred to 0,1M possatium phosphate buffer (pH=7.0) with 0.05% X-Gluc dissolved in dimethyl formamide. After 1-5 hours, plants showed blue staining were identified. Part of them, with completely blue roots, were put to the planes with GM and one week later to the greenhouses. From others - tissue, that was identified by blue staining as recombinant, was cut out of seedlings and transferred to plates with GM harbouring plant hormones. This recombined material was propagated for future analysis via callus culture.

3.2 Molecular biology

All standard methods of molecular biology were performed according to Sambrook et. al. (1989) if not otherwise indicated.

Electrophoresis of DNA was carried in 1% agarose gels with ethidium bromide (final concentration 2,5 μg/ml) in 1xTAE buffer in horizontal apparatus. DNA samples were resuspended in loading buffer.

Restriction endonuclease digestion of DNA was carried out under the salt and buffer requirements as recommended by suppliers.

DNA molecular weight determination was performed using SmartLadder (Eurogentech).

3.2.1 Transformation of *E.coli*

The transformation of *E.coli* was performed using heat shock method. For transformation an aliquot of competent cells (150 μl in a tube) was thawed on ice and added to 2-10 μl of ligation reaction, directly to the cold tube. All reactions were mixed by tapping the tubes, and incubated on ice for 30 min. After this cells were transferred for 120 s in a 42^{o}C water bath and cooled on ice (2 min). 700 μl of SOC medium were added to each reaction and mixed. Tubes were incubated on shaker (200 rpm) at 37^{o}C for 1 h. 30-200 μl of each transformation were pipetted on LB+ antibiotic plates and cells were spread with bent glass rod. Plates were placed inverted and incubated at 37^{o}C overnight and then at 4^{o}C until picking colonies.

3.2.2 Plasmid DNA extraction

For plasmid isolation bacteria grew in overnight culture (3 ml for miniprep extraction up to 10 μg and 50 ml for midiprep up to 500 μg plasmid DNA) at 37°C by shaking (200 rpm). The cells were harvested by centrifugation and plasmid DNA was extracted using Qiagen Plasmid Isolation kit or Quiagen Midiprep kit following the supplier's protocol.

3.2.3 Transformation of *A. tumefaciens* by electroporation

For each transformation 50 µl of competent cells of *A. tumefacies* strain C58 Cl Rf, thawed on ice, were mixed with 3 µl of plasmid DNA (about 10 ng) and transferred to the electroporation cuvette. Electroporation was done at 2,5 kV on electroporator EasyjecT Prima (EquiBio). The cells were mixed in 1 ml SOC medium and placed on YEB media containing selective agent and incubated at 28°C for 24 h.

3.2.4 Plant DNA extraction

Total plant DNA was isolated from *Arabidopsis* and tobacco leaves and calli according to Fulton et. al. (1995).

3.2.5 Southern analysis

For Southern analysis, 5 µg of purified genomic DNA was digested at 37 °C with corresponding enzymes ON and fragments were fractionated on agarose gel. Agarose gel electrophoresis was performed with 1% agarose gels in 1xTAE buffer submerged in a horizontal electrophoresis tank containing 1xHAC buffer according to the methods described by Sambrook et. al. (1989). After electrophoresis the DNA fragments were nicked by soaking agarose gel for 15 min. in 0,25 M HCl. The DNA fragments were bound by capillary transfer to a nylon hybridisation membrane "Hybond N+" with 0,4 M NaOH overnight. The membrane was washed twice 10 min with 2xSSPE and prehybrized at hybridization temperature for 1 to 2 hours in Church buffer.

Plasmid DNA or DNA fragments were labelled using a random priming labelling kit (Amersham, UK) by [α-^{32}]dCTP (Amersham, UK). Hybridization was performed according to manual instructions at 65ºC.

3.2.6 PCR reaction

As substrate for PCR reaction plasmide templates, amplified DNA and plant genomic DNA were used. General reaction mixture for PCR reaction (total 50 µl): TaKaRa Ex Taq (5 units/µl)-0,5 µl; 10x ExTaq buffer – 5 µl; dNTP Mixture (2,5 mM) – 4 µl; template - < 1 µg; both primers –1 µM (final concentration); sterilized distilled water up to 50µl. The standard PCR condition was as follows: denaturing at 94°C for 5 min for the first cycle and 30 s for the subsequent cycles; primer annealing at 55-65°C for 30 s; elongation at 74°C for 1-2 min; and final elongation step at 74°C for 7 min. The sequences of oligonucleotides used in this study are listed in 2.10.

3.2.7 Sequencing.

DNA sequencing was done with use of automated A.L.F.-sequencing equipment (Pharmacia) on sequencing laboratory (S. König, B. Bruckner, IPK, Gatersleben).

3.3 Transformation by particular bombardment

3.3.1 Preparation of DNA

From the resulting clone pGUS 231 plasmid DNA was prepared using the restriction enzymes *Xba*I, *Stu*I, *Sma*I, *Aat*I, *Sca*I and *Bsa*I. All restriction enzymes were obtained from BioLab or Amersham and the digestion was performed in the buffer systems and temperature conditions as suggested by the manufacturers. Plasmid DNA was digested for 12-16 h.

Completion of digestion was tested by retransformation in to *E.coli*. For experiments plasmid was used with >99% efficiency of digestion.

3.3.2 Plant material and preparation of leaves

Wild type *Arabidopsis thaliana* (ecotype Colambia-O) and *Nicociana tabacum* (SR1) plants were grown under standard conditions on GM in Sigma boxes.

Plant leaves (tobacco - 1, *Arabidopsis* - 8-9) were arranged to form 3cm-diameter circle in the centre of a petri dish containing callus induction medium and incubated 24 h before bombardment in growth chamber.

3.3.3 Covering gold particles with plasmid DNA and bombardment

Gold particles (5 mg - sufficient for 16 bombardment; 1 μm diameter) were kept overnight in 1 ml absolute ethanol. After washing two times with sterile water, the particles were resuspended in 100 μl of 50% glycerol.

For each shooting about 200 ng plasmid DNA was used. The mixture used for 4 bombardments was prepared as follows: 25 μl (X) of gold particle suspension was mixed with 10 μl of plasmid DNA (Y) and 35 μl (X+Y) of 1M Ca $(NO_2)_3$. After 10 min incubation at room temperature and brief centrifugation supernatant was discarded and coated gold was resuspended in 100 μl of absolute ethanol. 25 μl of plasmid/gold suspension were pipetted onto each macrocarrier disk. Leaves were bombarded after complete evaporation of ethanol using the Biolistic PDS1000-He Particle Delivery System from BioRad - 1100 psi, 1/4" gap.

4 RESULTS

4.1 Species-specific differences in the repair of double-strand breaks by NHEJ in *Arabidopsis* and tobacco

4.1.1 Cloning procedure of pGUS231

Plasmid pGUS23 is a pUC7 derivative that carries the β-glucuronidase ORF under the control of the 35S promoter (Puchta and Hohn, 1991). Two oligonucleotides 231S and 231AS were designed (Figure 4.1.). After annealing specific polylinker was inserted into *Acc65*I site of pGUS23 between 35S promoter and ORF of ß-glucuronidase gene.

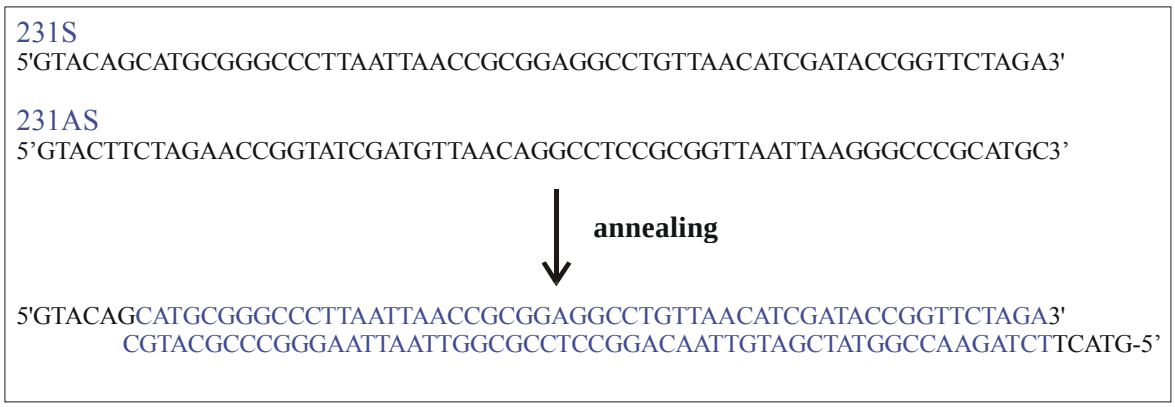


Figure 4.1. Cloning polylinker for insertion in pGUS23.

The polylinker contains recognition sites for 9 different restriction enzymes (Table 4.1.). Insert of the polylinker into the pGUS231 in 3 plasmid was proved by restriction with *Acc*65I, *Sph*I and *Xba*I (Figure 4.2.).

Restriction enzyme	Sequence for cleavage site	Kind of restricted ends
*Sph*I	GCATGC CGTACG	5′- overhangs
*Apa*I	GGGCCC CCCGGG	5′- overhangs
*Pac*I	TTAATTAA AATTAATT	5′- overhangs
*Sac*II	CCGCGG GGCGCC	5′- overhangs
*Stu*I	AGGCCT TCCGGA	blunt end
*Hpa*I	GTTAAC CAATTG	blunt end
*Bsp*DI	ATCGAT TAGCTA	3′- overhangs
*Age*I	ACCGGT TGGCCA	3′- overhangs
*Xba*I	TCTAGA AGATCT	3′- overhangs

Table 4.1. Types of restriction sites in polylinker, inserted in pGUS23.

From the resulting clone pGUS231 (Fig. 4.3.) plasmid DNA was prepared by Qiagen plasmid Maxi Kit and then linearized using one of the restriction enzymes. Completion of digestion was tested by retransformation of the linearized plasmid DNA into *E. coli*. For plant bombardment experiments only plasmid preparations were used in which the number of colonies was decreased by more than 99% compared with untreated plasmid preparation.

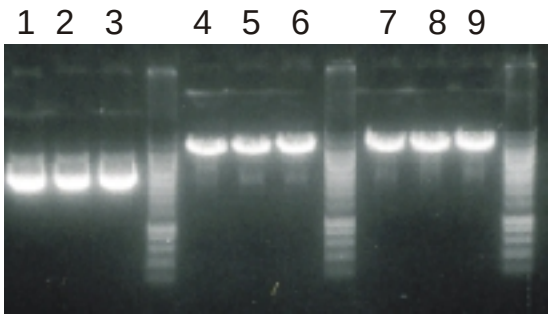


Figure 4.2. Identification of 3 clones of pGUS231 plasmid (clone 1 - lines 1,4,7; clone 2 - 2,5,8 and clone 3 - 3,6,9) by restriction with *Acc*65I (1-3), *Sph*I (4-6) and *Xba*I (7-9).

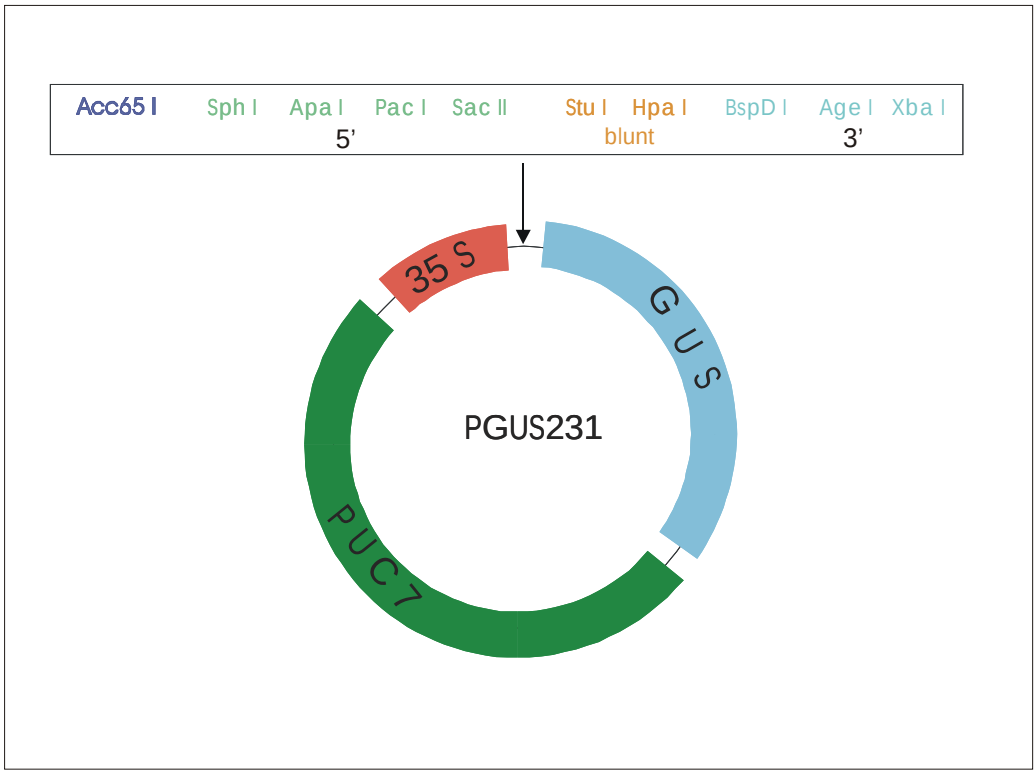


Figure 4.3. The plasmid pGUS231. The 35S promoter and open reading frame (GUS) of ß-glucuronidase cassette are indicated as well as the cloned polylinker used for this study.

4.1.2 Stability of linearized plasmid DNA in *Arabidopsis* and tobacco

To test the hypothesis that the differences found in DSB repair between *Arabidopsis* and tobacco are due to differences in the processing of DNA ends it was decided to test the stability of plasmid DNA transformed into leaves cells of both organisms. For this purpose four different restriction sites within the plasmid pGUS231 were chosen - *Xba*I and *Stu*I in the polylinker of plasmid pGUS231 between 35S promoter and ß-glucuronidase ORF and *Sma*I and *Aat*II which cut in the pUC backbone of pGUS231 (Figure 4.4). Thus all different kinds of DNA restricted ends could be tested: *Stu*I and *Sma*I, result in linearized plasmid DNA with blunt ends, *Aat*II results in linearized DNA with 5'-overhangs and *Xba*I results in linearized DNA with 3'-overhangs.

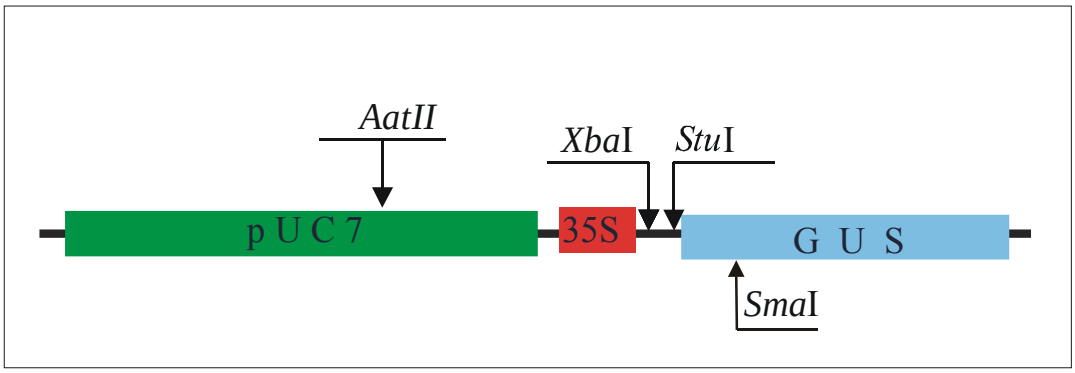


Figure 4.4. The plasmid pGUS231. The promoter and open reading frame of the ß-glucuronidase expression cassette are indicated as well as the recognition sites of restriction enzymes applied for study stability of linearized plasmid DNA in *Arabidopsis* and tobacco.

4.1.2.1 Particle bombardment of linearized pGUS231 into *Arabidopsis* and tobacco leaves

To transfer the DNA into tobacco and *Arabidopsis* leaves particle bombardment was used (Seki et al., 1999). Gold particles (1 µm diameter) were covered with linearized pGUS231 DNA. For each shooting ~200 ng plasmid DNA was used. Leaves were bombarded using the Biolistic PDS1000-He Particle Delivery System from BioRad - 1100 psi, 1/4" gap. To avoid variation due to differences in the DNA preparations in all cases the same batch of the DNA-gold preparation was used simultaneously for bombardment of *Arabidopsis* and tobacco cells. For this purpose leaves of 8 week old tobacco seedlings and leaves of 6 week old *Arabidopsis* seedlings, both in a similar phase of their development, were cultured for one day on CIM medium and then bombarded with gold particles coated with DNA in parallel. Then, the leaves were cultivated for two days on CIM medium before further analysis on the stability of the transferred DNA either by Southern blotting or by transient expression studies was performed.

4.1.2.2 Southern blot analysis of DNA extracted from leaves after biolistic transformation

In a first set of experiments plasmid stability was analysed by Southern blotting. To elucidate how different types of breaks influence the stability of a DNA molecule in plant cells, pGUS231 with 3'-(*Xba*I), 5'-(*Ata*II) overhangs or blunt ends (*Sma*I, *Stu*I) was prepared. After incubation for two days the leaves were harvested and total DNA was extracted. This DNA was digested overnight with the restriction enzyme *Hind*III that has no recognition sequence within pGUS231. After gel electrophoresis and Southern blotting respective membrane was hybridised with a plasmid-specific probe under high stringent conditions. The obtained radioactive signals on the blot were quantified by the use of a phosphoimager.

4.1.2.3 Indications for differences in DNA end processing in *Arabidopsis* and tobacco

The intensity of two kinds of signals were determined – the signal of the linearized plasmid (see bracket a in Figure 4.5.) and signal of degradated plasmid (see bracket b in Figure 4.5).

The signal of the linearized plasmid DNA was set into relation to the smear produced by digested DNA, in the same lane (relation shown in Figure 4.6). The data obtained from tobacco and *Arabidopsis* were then set into relation. In pilot experiments different incubation times of the leaves after bombardment were tested. After 8, 24 and 48 hours besides degraded DNA the linearized plasmid band was clearly visible for both plant species, whereas after 72 hours the resulting band became too weak for a reliable quantification. For further analysis we therefore chose the longest possible incubation period for which a reliable quantification could be performed (two days). In general the intensity of the linear band was between 1 and 5 % of the total intensity of the signal in the respective lane. These percent values obtained for tobacco and *Arabidopsis* were then set

into relation. E.g. if in case of a particular experiment the linearized band represented 3 % of the total signal intensity in tobacco and 2 % of the total signal intensity in *Arabidopsis*, a ratio of 1.5 was determined. Thus any value over 1 indicates that the full-length linear DNA was more stable in tobacco than in *Arabidopsis*. The higher the values the more linear plasmid molecules remained undegraded in tobacco in comparison to *Arabidopsis*. A value of 2 would indicate that twice as much linear plasmid molecules remained undegraded in tobacco than in *Arabidopsis*. Series of experiments were performed using differently restricted plasmids. For each restriction enzyme nine independent experiments are depicted in Table 4.2. In spite of quite large variation between single experiments, which are typical for transient transformation assays (e.g. Puchta and Hohn, 1991), our results clearly demonstrate that, independent of the respective DNA end, in most cases (29 out of 36) the linearized DNA was less degraded in tobacco than in *Arabidopsis* (Table 4.2). Linearized plasmid DNA was on average 1.4 to 1.9 times more stable in tobacco than *Arabidopsis*. No major differences between blunt and overhanging DNA ends could be detected (Table 4.2). However, due to the fact that the subtraction of the standard deviations from the means, given in Table 4.2, result in only two of four cases in a value above 1, these results can only be taken as a first indication of differences in processing of DNA molecules in the two plant species and needs to be further confirmed by transient expression analysis.

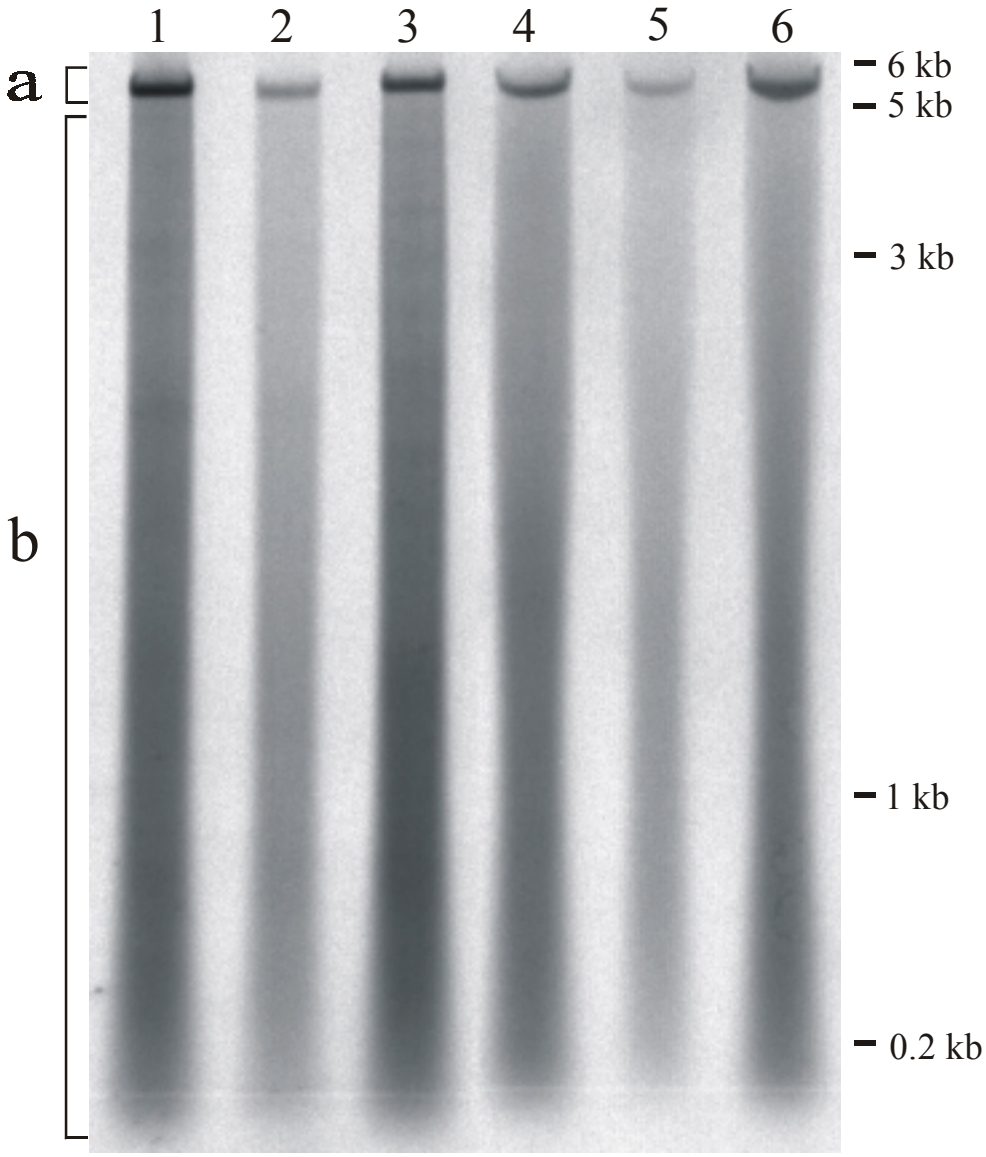
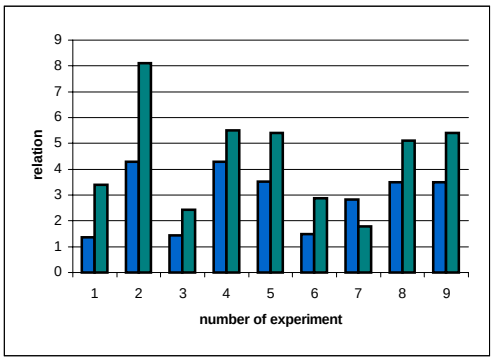
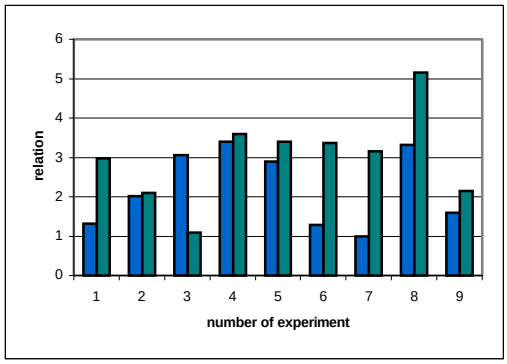


Figure 4.5. Representative Southern blot of DNA extracted from leaves two days after biolistic transformation with linearized DNA of the plasmid pGUS231. After isolation the DNA was restricted with *Hind*III, blotted and hybridised with ^{32}P labelled pGUS231. The intensity of the signals was determined by the use of a phosophoimager. The linear band (a) was put into relation with the smear resulting from degraded DNA (b). In lanes 1-3 *Arabidopsis* DNA and in lanes 4-6 tobacco DNA was loaded. Lanes 1, 4 : DNA of plant leaves bombarded with pGUS231 restricted with *Xba*I; Lanes 2, 5: DNA of plant leaves bombarded with pGUS231 restricted with *Sma*I; Lanes 3, 6: DNA of plant leaves bombarded with pGUS231 restricted with *Ata*II.



A.



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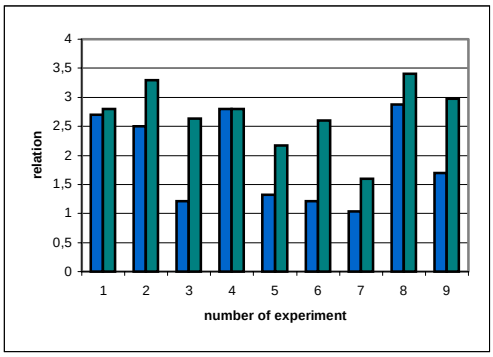
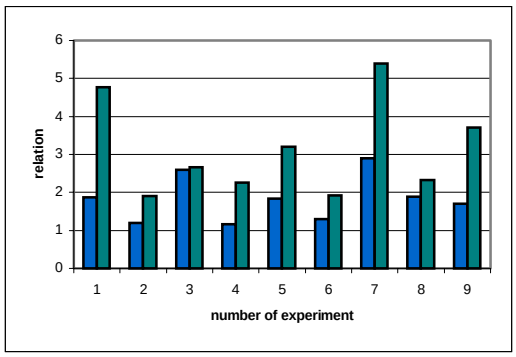


C.



D.

Figure 4.6. Relation between signal of the linearized plasmid band (see bracket a in Figure 4.5.) to the signal of the degradated plasmid smear (see bracket b in Figure 4.5.) in *Arabidopsis* and tobacco A. *Xba*I; B. *Stu*I; C. *Sma*I; D. *Aat*II

*Xba*I 3'-overhang	*Stu*I blunt	*Sma*I blunt	*Ata*II 5'-overhang
2.48	2.25	0.96	2.64
1.88	0.97	1.32	0.63
1.70	2.80	2.17	0.97
1.28	0.94	1.00	1.95
1.52	1.17	1.64	1.74
1.92	2.63	2.15	1.48
0.63	3.15	1.54	1.86
1.46	1.55	1.18	1.23
1.54	1.34	1.75	0.46
1.60 $\pm$ 0.50 (9)	1.91 $\pm$ 0.92 (9)	1.52 $\pm$ 0.45 (9)	1.42 $\pm$ 0.66 (9)

Tabl. 4.2. The relative stability of linearized DNA molecules of the plasmid pGUS231 in leaves cells of tobacco in relation to *Arabidopsis.*

4.1.3 Study of transient expression in *Arabidosis* and tobacco

The previous analysis demonstrated that DNA molecules are degraded to different extents in tobacco and *Arabidopsis*. However, the results obtained above could be due to differences in the activity of DNA exo- or endonucleases. To further clarify the nature of the nuclease-specific differences a transient expression assay after biolistic bombardment of *Arabidopsis* and tobacco with linearized pGUS231 was performed. The idea was that in case of endonucleolitic degradation, there should be no difference in the relative rate of transient expression of marker gene between *Arabidopsis* and tobacco irrespective where the break was induced in the plasmid molecule. However, in case of exonucleolitic degradation different positions of the break were expected to reveal differences between the two species. The closer the break site is to a DNA sequence required for expression the

more the activity should be reduced. Therefore on one side the *Xba*I and *Stu*I restriction sites that were lying between promoter and ORF of the ß-glucuronidase gene and on the other the *Sca*I site, that is located within the pUC backbone (Figure 4.7) were used.

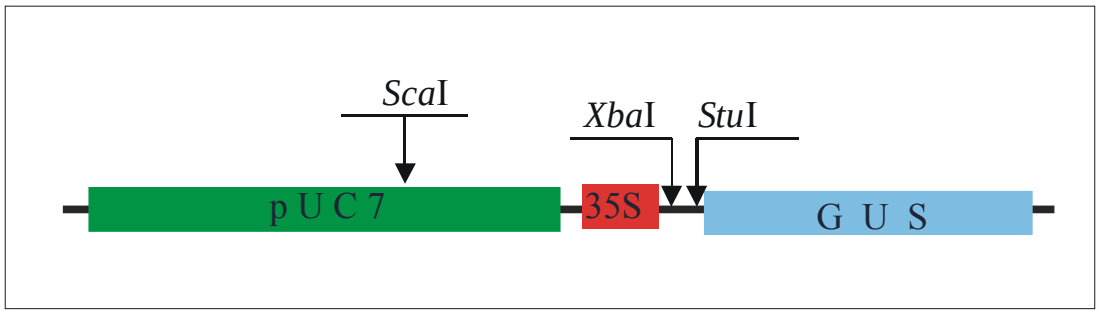


Figure 4.7. The plasmid pGUS231. The promoter and open reading frame of the ß-glucuronidase expression cassette are indicated as well as the recognition sites of restriction enzymes applied for study transient expression in *Arabidopsis* and tobacco.

Whereas in case of *Xba*I and *Stu*I exonucleolitic degradation of less than 100 bp at each end would most probably result in a loss of function of the ß-glucuronidase gene, in case of *Sca*I only degradation of more than 1 kb on one or more than 2 kb on the other would result in a loss of function of the enzyme. As both *Stu*I and *Sca*I digestion result in blunt ends, not the nature of the break itself but only the position of the break differs between the respective DNA molecules. In contrast, *Xba*I-digested (3' ends) and *Stu*I-digested (blunt ends) DNAs differ strongly in the nature of the DNA ends but hardly in the position of the break site. Thus we were able to test whether the respective position or/and the nature of the break site would influence the outcome of the reaction.

After plasmid linearization with respective enzymes the one was transformed into *Arabidopsis* and tobacco leaves using the Biolistic PDS1000-He Particle Delivery System (4.1.2.1.). Bombardment with circular plasmid DNA was included as transcription control. Histochemical staining of leaves material of both species was done (Figure 4.8). The number of blue sports per cm^2 were calculated for leaves transformed with circular, non digested pGUS231 and for leaves transformed with pGUS231 digested with one of enzymes - *Xba*I, *Stu*I or *Sca*I. Data are shown in Table 4.3.

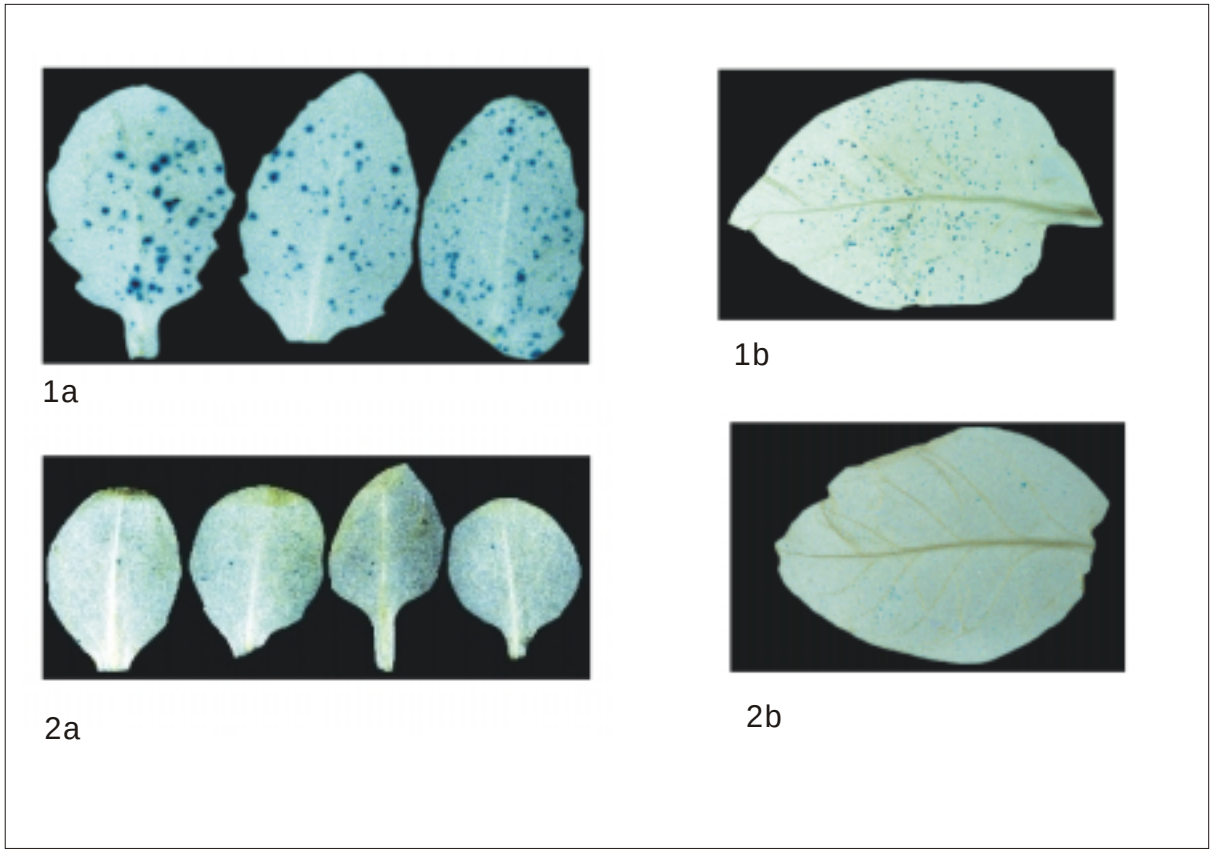


Figure 4.8. Transient GUS expression in *Arabidopsis* and tobacco leaves. 1a and 1b: *Arabidopsis* and tobacco leaves transformed with circle pGUS231 plasmid; 2a and 2b: *Arabidopsis* and tobacco leaves transformed with pGUS231 digested with *Xba*I.

Experi-ments	Plasmid	Species	Spots per cm^2	Relation to circular pGUS231	Relation tobacco/*Arabidopsis*
1.	circular	tobacco	260		
		Arabidopsis	44,2		
	*Xba*I	tobacco	70	0.27	**1.40**
		Arabidopsis	8.4	0.19	
	*Sca*I	tobacco	240	0.92	**1.03**
		Arabidopsis	39.4	0.89	
2.	circular	tobacco	500		
		Arabidopsis	131		
	*Xba*I	tobacco	122.2	0.24	**2.00**
		Arabidopsis	15.2	0.12	
	*Sca*I	tobacco	290	0.58	**1.14**
		Arabidopsis	67.4	0.51	
3.	circular	tobacco	157.5		
		Arabidopsis	52.7		
	*Xba*I	tobacco	24.6	0.16	**1.60**
		Arabidopsis	5.4	0.10	
	*Sca*I	tobacco	73	0.46	**0.50**
		Arabidopsis	48.5	0.92	
4.	circular	tobacco	103		
		Arabidopsis	40		
	*Xba*I	tobacco	30.3	0.29	**1.38**
		Arabidopsis	8.5	0.21	
	*Sca*I	tobacco	86.4	0.83	**0.71**
		Arabidopsis	47	1.17	
5.	circular	tobacco	185.7		
		Arabidopsis	34.7		
	*Xba*I	tobacco	28.1	0.15	**2.10**
		Arabidopsis	2.29	0.07	
6.	circular	tobacco	260		
		Arabidopsis	44.2		
	*Xba*I	tobacco	73	0.28	**1.50**
		Arabidopsis	8.4	0.19	
	*Sca*I	tobacco	120	0.46	**0.50**
		Arabidopsis	39.4	0.89	
7.	circular	tobacco	292.8		
		Arabidopsis	132.7		
	*Sca*I	tobacco	158	0.54	**1.23**
		Arabidopsis	58.6	0.44	
	*Stu*I	tobacco	25	0.085	**2.43**
		Arabidopsis	4.6	0.035	
8.	circular	tobacco	67.7		
		Arabidopsis	9.3		
	*Stu*I	tobacco	19.3	0.29	**2.4**
		Arabidopsis	1.17	0.12	
9.	circular	tobacco	368.5		
		Arabidopsis	72		
	*Stu*I	tobacco	15.5	0.040	**2.35**
		Arabidopsis	1.2	0.017	
10.	circular	tobacco	185.7		
		Arabidopsis	34.7		
	*Stu*I	tobacco	28.1	0.152	**2.15**

		Arabidopsis	2.29	0.0706	
11.	circular	tobacco	258		
		Arabidopsis	55		
	*Stu*I	tobacco	168.5	0.654	**2.18**
		Arabidopsis	17	0.300	
12.	circular	tobacco	280		
		Arabidopsis	120		
	*Stu*I	tobacco	11.5	0.0410	**1.97**
		Arabidopsis	2.5	0.0208	

Table 4.3. Number of blue spots per cm^2 and frequency of ß-glucuronidase restoration after bombardment of linearized DNA molecules of the plasmid pGUS231 in leave cells of tobacco and *Arabidopsis*.

The average number of spots per cm^2 of both plant species was set in relation to each other. The value obtained representing the efficiency of expression of the respective construct in the two plant species. In general independent of plant species the highest numbers of spots were detected after shooting with circular pGUS231, followed by the plasmid cut outside of the marker gene. Religation of pGUS231 harbouring sticky ends occurred quite efficiently, depending on experiment in the range of 10 to 30% of the molecules the ORF was restored, whereas religation of the blunt-ended plasmid gave in most experiments lower rates. All values were above 1%, the putative maximal "background" value, due to traces of undigested plasmid DNA in the preparation, as determined by retransformation of the respective linearized plasmid preparations into *E. coli*. To directly compare the results obtained from tobacco and *Arabidopsis* the values obtained with the linearized plasmid molecules were set into relation to the circular ones (Table 4.3, second last row). By this procedure values were obtained that were independent of transcription-based differences between tobacco and *Arabidopsis*. These values were then set into relation to one another (Table 4.3, last row). E.g., a value of 2 indicates the presence of twice as many functional ß-glucuronidase genes in tobacco than in *Arabidopsis* after bombardment with the respective DNA. In repeated experiments (for the statistical evaluation see Table 4.4) this

relation was found to be around 1 in case of the *Sca*I digested pGUS231 and around 2 for both *Xba*I and *Stu*I digested pGUS231. This indicates that in case of a break between promoter and ORF on average two times more functional ß-glucuronidase expression cassettes were restored in tobacco than in *Arabidopsis* irrespective of the nature of the DNA ends. The calculated standard deviations indicate that the detected differences are highly significant.

Kinds of plasmid linearization		
***Stu*I**	***Sca*I**	***Xba*I**
2.43	1.03	1.40
2.40	1.14	2.00
2.35	0.50	1.60
2.15	0.71	1.38
2.18	1.23	2.10
1.97	0.50	1.50
2.25±0.18 (6)	0.85±0.32 (6)	1.66±0.31 (6)

Table 4.4. The relative expression of ß-glucuronidase gene after bombardment of linearized DNA molecules of the plasmid pGUS231 in leave cells of tobacco in relation to *Arabidopsis.* In the bottom line the means and standard deviations are given.

These results demonstrate that if the break is near to the marker gene (*Stu*I/*Xba*I) ß-glucuronidase expression is lower in *Arabidopsis* in comparison to tobacco, in contrast to the situation, when break is far from ORF for marker gene (*Sca*I).

Thus, DNA ends are less stable in *Arabidopsis* then in tobacco, at least under our experimental condition.

4.2 Double-strand break repair by homologous recombination in *Arabidopsis thaliana*

4.2.1 Cloning procedure

For the analysis of the efficiency of homologous DSB repair in the presence of nearby homology, the binary vectors pDU.GUS and pIU.GUS were constructed. Standard cloning procedures were used (Sambrook et. al., 1989). In pDU.GUS and pIU.GUS, a non-functional internal fragment of the GUS gene of 1087 nucleotides was cloned in direct or inverted orientation to a ß-glucuronidase gene into which in about the middle of the gene a linker with I-*Sce*I and *Xho*I sites was incorporated rendering it non-functional. For creation of the I-*Sce*I restriction site, a specific polylinker with the sequence:

5'-CTCGAGATTACCCTGTTATCCCTAGTCGAC-3' was cloned into the *Msc*I–digested pGUS23 vector (Puchta and Hohn, 1991) (Fig.4.9), resulting in the plasmid pGUS23I, carrying I-*Sce*I restriction site inside of the non-functional GUS gene. Homologous overlaps of 537 and 550 bp, interrupted by the linker, are shared by both GUS sequences. The *Sal*I site within the linker was removed by cutting the plasmid and consecutive blunting of the obtained ends using the Klenow enzyme followed by religation. The obtained plasmid was cut by *Acc65*I, treated with Klenow enzyme for filling in and religated. Via PCR, using p35SX and pTH primers from the resulting plasmid pGUS23I+, a fragment containing GUS gene interrupted by I-*Sce*I was isolated and cloned into *XbaI* and *Hind*III sites of plasmid pCH (Tinland et al., 1994) – resulting in the plasmid pCH23 (Fig.4.10). Subsequently, the non-functional internal fragment of the GUS gene (1087 nucleotides) was amplified from pGUS23 with pHZD and pRZD primers and inserted in the *Acc*65I site of pCH23, resulting pCH23I. The orientation of the fragment was determined by *Not*I digestion. Two plasmids were identified containing a recombination substrate with part of the GUS gene in direct (pDU.GUS) and in inverted (pIU.GUS) orientation (Fig.4.11).

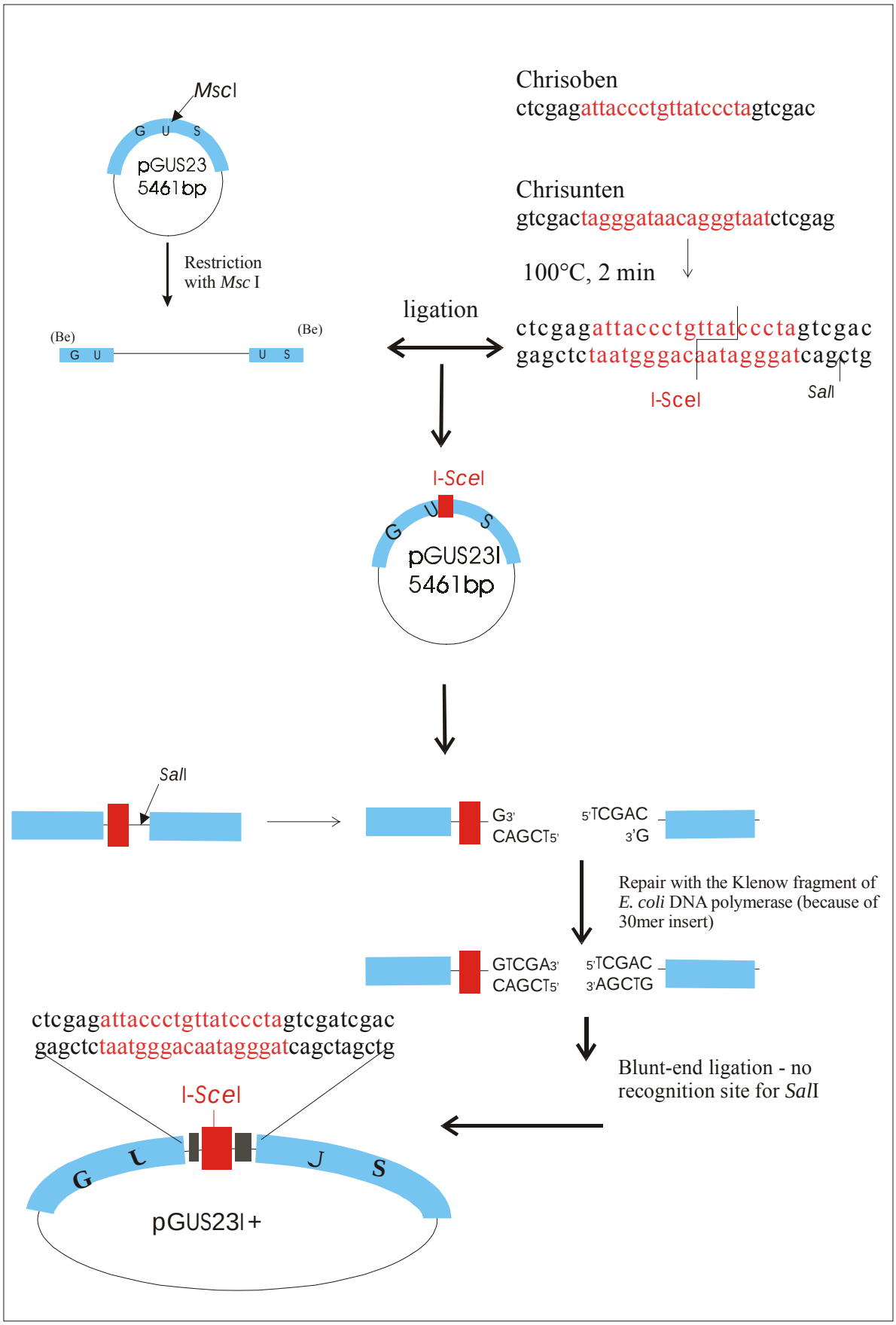


Figure 4.9. Scheme for construction pGUS23I+ carrying a I-*Sce*I restriction site inside of the non-functional GUS gene.

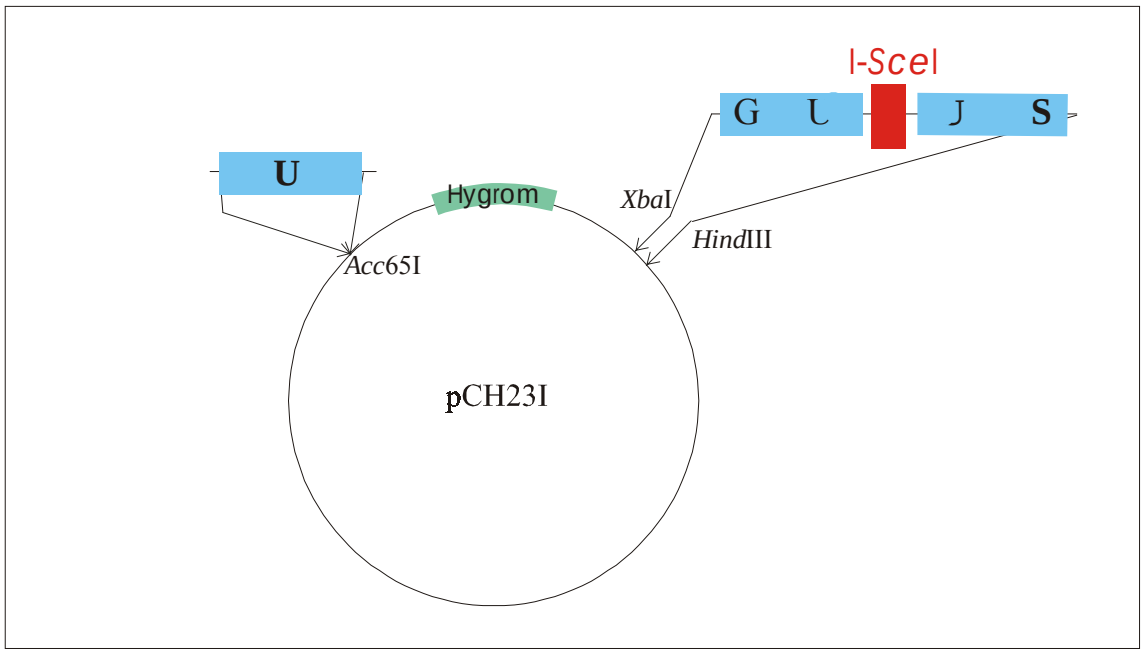


Figure 4.10. Construction of pCH23I vector. (For details see the text).

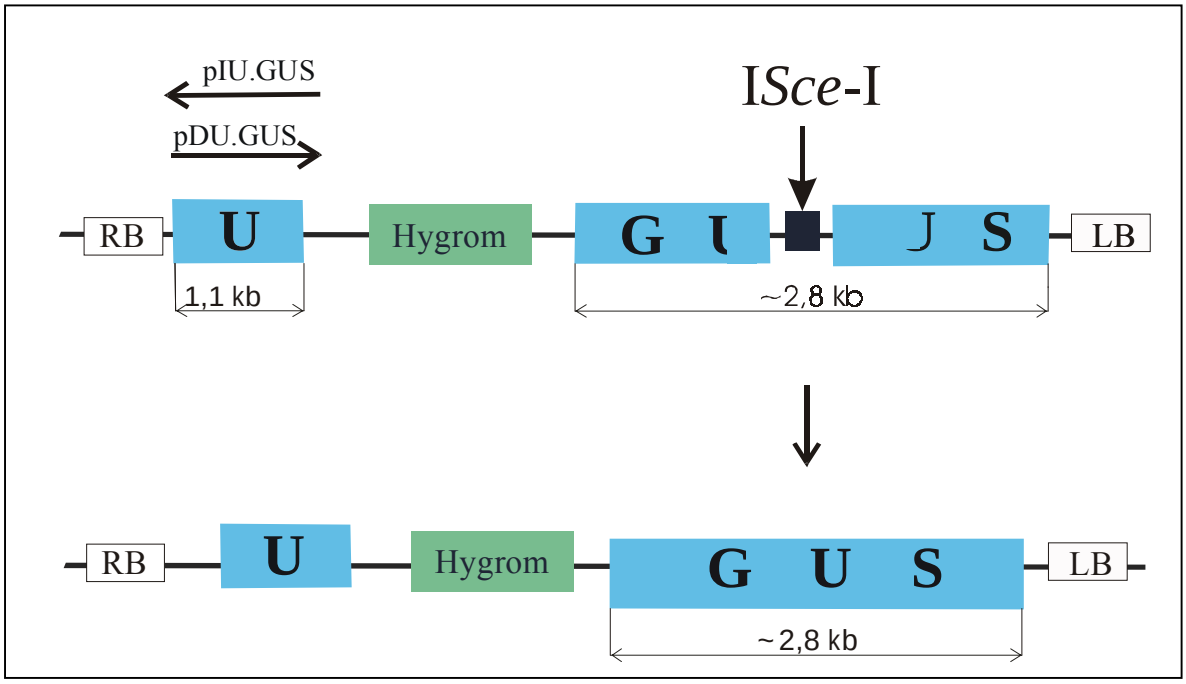


Figure 4.11. Schematic representation of the recombination substrate and structure of the expected product of the homologous recombination.

4.2.2 *In planta* transformation of *Arabidopsis thaliana* by vacuum infiltration

The plasmids carrying recombination substrate were transferred into *Agrobacterium tumefaciens* via electroporation. The resulting strains containing the pDU.GUS and pIU.GUS constructs, were used to transform five-week-old *Arabidopsis thaliana* plants ecotype Colombia-O by vacuum infiltration (see 3.1.3).

4.2.3 Segregation analysis and characterisation of T-DNA insertions in transgenic lines.

24 and 21 individual hygromycin resistant plants were obtained from transformations with pIU.GUS and pDU.GUS, respectively. The initial hygromycin resistant plants are referred to hereafter as F1. The progeny obtained from a F1 transgenic plant (by self-cross) will be referred to as F2. F2 plants were analysed for the integration of the foreign gene by calculating the ratio of the number of hygromycin tolerant plants to the number of non-tolerant plants. Under selective conditions, progenies of nine and nine lines of F2 plants developed in the proportion of three resistant to one sensitive, that is characteristic for the segregation of a single locus (3:1). From each of the selected lines, at least 10 F2 resistant plants were grown in soil.

To confirm the presence and number of T-DNA insertions, genomic DNA from nontransformed (wild type Col-O) and transgenic plants was extracted. 10 µg of DNA was digested with *Hind*III, fractionated in an agarose gel, blotted and the resulting Southern Blot was hybridised with the [α-^{32}P]-dCTP-labeled 2kb *Kpn*I/*Sac*I fragment of the pGUS23 plasmid, harbouring the GUS gene. The results, shown in Figure 4.12 indicated, that two of the nine pDU.GUS lines contained one copy of insert (lines 4 and 6 in Figure 4.12) and three from nine pIU.GUS lines contained one copy of insert (data not shown).

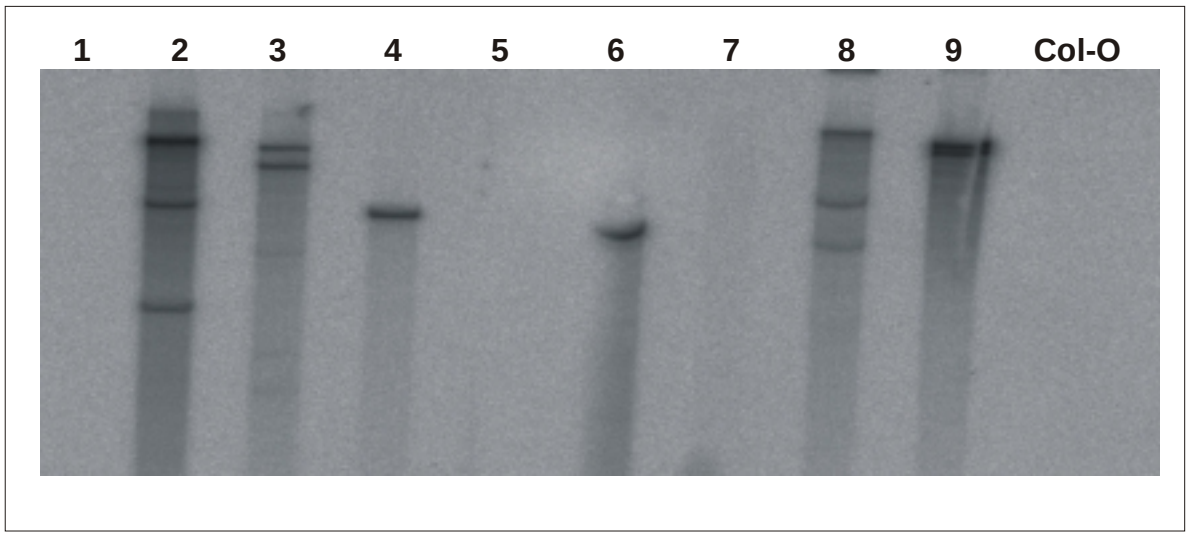


Figure 4.12. Southern blot analysis for the presence and number of T-DNA insertions.

10 µg of genomic DNA from the wild type and transgenic lines were digested over-night with *Hind*III and subsequently fractionated in an agarose gel (0.8%). The DNA was blotted onto a nylon filter and hybridised with radioactive labelled GUS probe. Lines 1-9 – plant DNA from transgenic lines pDU.GUS; line Col-O – DNA from wild-type Colombia-O *Arabidopsis thaliana.*.

4.2.4 Induction of homologous recombination via transformation of seedlings

DSBs by I-*Sce*I expression in plants can be induced in two ways: either by transient expression via transformation of seedlings or by stable transformation of the I-*Sce*I expression cassette in plants.

To induce recombination at the cotyledon stage of the transgenic lines with the recombinant substrate, homozygous for the transgene seedlings were inoculated with an *Agrobacterium* strain pZpp-I*Sce*I, carrying the I-*Sce*I open reading frame under the control of 35S promoter on its T-DNA to achieve transient expression of the enzyme (Puchta et. al., 1996). In parallel experiments seedlings were inoculated with the same *Agrobacterium* strain devoid of binary vector (pPM6000k). To identify putative homologous recombination events, seedlings were histochemically stained for GUS expression after two days of incubation. Numbers of blue

sectors (recombination events) in transformed and nontransformed plants were calculated. Table 4.5. shows the number of sectors per seedling and enhancement of recombination events.

Transgenic line	Sectors per seedling			Enhancement of intrachromosomal homologous recombination
	without inoculation	after inoculation with pPM6000k	after inoculation with pZpp-I*Sce*I	
pDU.GUS 6	0.53 0.87 1.1	0.1 2.5 4.6	0.18 2.1 3.7	0.3 2.4 3.4
pDU.GUS 8	0.95 2.0 1.4	0.47 2.1 1.44	0.94 3.14 1.6	0.99 1.27 1.1
pIU.GUS 1	1.29	5.68	3.14	2.4
pIU.GUS 7	0.73 4.2	0.54 6.6	0.7 5.6	0.9 1.3
pIU.GUS 8	0.54 2.4 2.05	0.98 1.4 4.1	1.41 1.4 4.1	2.6 0.58 2

Table 4.5. Recombination frequencies in different transgenic lines with and without induction of DSBs by seedling inoculation.

Our experiments do not indicate a strong recombination-enhancing effect by the transient expression of I-*Sce*I.

This can be due to the fact that owing to the our transformation procedure only in few cells of the seedlings I-*Sce*I was expressed. To achieve expression of the enzyme as much as possible an approach with the stable transformation of expression cassette was done.

4.2.5 The use of different kind of recombination substrate for the analysis of homologous DSB repair.

For analysing efficiency of different kinds of homologous DSB repair in the presence of nearby homologous sequences two addition transgenic *Arabidopsis* lines were used. Line pDGU.US1 and pDGU.US2 (A. Kirik, PhD thesis) contain a construct, in which I-*Sce*I site

is flanked by two halves of ß-glucuronidase gene harbouring an overlap of 557 bp (Tinland et. al, 1994). See Figure 4.13.

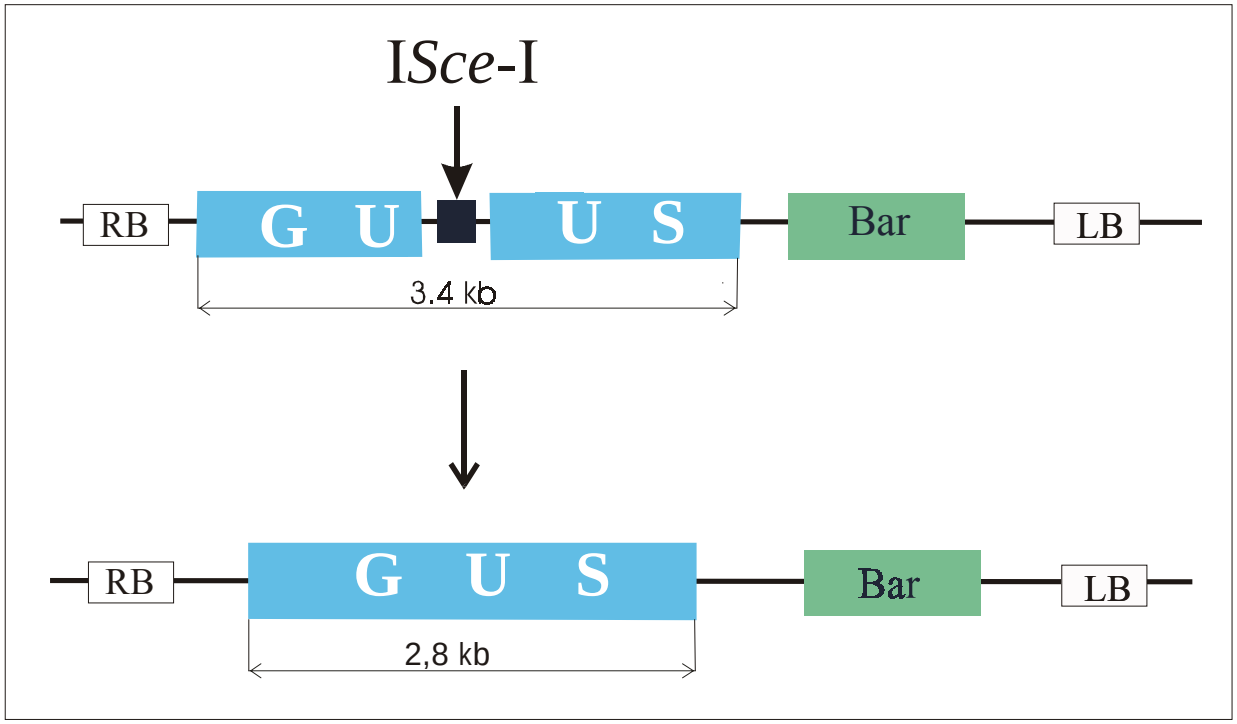


Figure 4.13. Schematic representation of the recombination substrate line pDGU.US and structure of the possible recombination product.

4.2.6 Determination of recombination frequencies with and without DSB induction *in vivo*.

To study homologous recombination after induction of DSB *in vivo,* lines of transgenic plants that contain the three different recombination substrates, were crossed with homozygous plants from line pDISceI1. This line was produced by *Agrobacterium*-mediated transformation with the binary plasmid pDISceI for plant expression of an artificial I-*Sce*I ORF (Puchta et. al., 1993) under the control of the DMC1 promoter and gentamycin as a selective marker (Klimyuk and Jones, 1997).

The obtained progeny was grown for two weeks on germination medium with antibiotics and then subjected to a histochemical staining using X-Gluc to determine the number of

recombination events. At the same time, seedlings of transgenic plants which are homozygous for the recombination substrates, were stained to determine the level of DNA recombination without DSB induction. Results obtained for F1 generation after crossing are depicted in Table 4.6. and presented in Figure 4.14.

Line	Number of seedlings	Number of blue sectors	Sectors per seedling	Enhancement
IU.GUS 7	39	40	1.03	
IU.GUS 7 x DISceI	29	373	12.86	12.49
IU.GUS 8	32	60	1.88	
IU.GUS 8 x DISceI	21	711	33.86	18.01
DU.GUS 6	37	21	0.57	
DU.GUS 6 x DISceI	25	381	15.24	26.7
DU.GUS 8	32	39	1.22	
DU.GUS 8 x DISceI	28	400	14.28	11.7
DGU.US1	31	47	1.5	
DGU.US1 x DISceI	22	2380	108.2	72.13
DGU.US2	17	12	0.7	
DGU.US2 x DISceI	14	894	63.86	91.2

Table 4.6. Determination of recombination frequencies in different transgenic lines with and without induction of DSB.

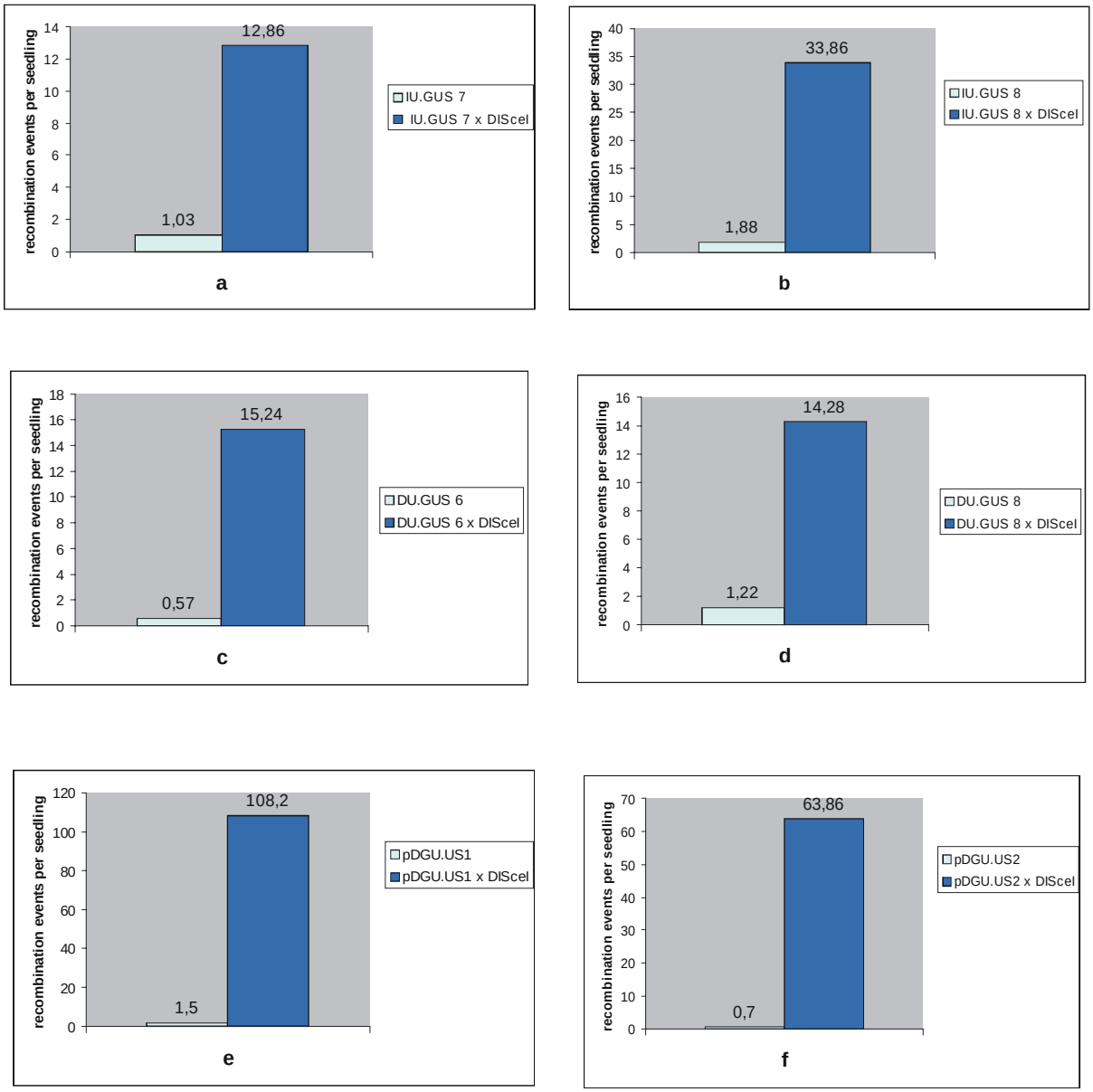


Figure 4.14. Enhancement of somatic recombination frequency by *in vivo* DSB induction in different F1 transgenic lines containing a recombination substrate.

Without DBS-induction all lines with different recombination substrates revealed a similar number of sectors in a range from 0.57 to 1.88 per seedling. Whereas in about half of the seedlings no recombination event could be detected, in others from 1 to 5 blue sectors could be identified (Table 4.7.). No major differences were found for the three particular

substrates. Different lines harbouring the same construct did not differ more than by a factor of two. The means of the three lines harbouring the same kind of transgene conformation differed by less than a factor 2 for the three constructs. Thus, without DSB induction no significant differences between the three recombination substrates could be detected.

After crossing with the line pDISceI-1 the number of blue sectors increased drastically (Figure 4.15). The presence of the I-*Sce*I expression cassette resulted in about ten to thirty times more sectors for DU.GUS and IU.GUS and up to almost one hundred times more for DGU.US. As in the seedlings from the crossings the recombination substrates are present in a hemizygous state the rate of induction might be underestimated by a factor of two. It has been shown before (Puchta et. al., 1995) that the frequency spontaneous intrachromosomal recombination is reduced to about half in hemizygous in comparison to homozygous plants, which have been used as a control in the current experiment. For line DGU.US 1 between 55 and 210 and for line DGU.US 2 between 34 and 84 blue sectors were detected per individual seedling. Similarly to the experiment without DSB induction, no major difference could be found between the lines harboring the repeat in direct or inverted orientation. Also the distribution of numbers of recombination events between the individuals was similar for these lines. Between 6 and 22 blue sectors were detected per plant for lines DU.GUS 6, DU.GUS 8 and IU.GUS 7. Only in the case of line IU.GUS 8 the values were somewhat higher (9 to 59 sectors) (Table 4.7). If we compare the distribution of non-induced to induced recombination events of line IU.GUS 8 with DU.GUS 6, DU.GUS 8 and IU.GUS 7 the χ^2 values (1.607, 3.297 and 1.911) indicate that the differences are statistically not significant (rejection $p<0.05$). This clearly demonstrates, that the efficiency of gene conversion is not influenced by the orientation of the donor sequence in respect to the acceptor. In contrast, a comparison of IU.GUS 8 with lines DGU.US 1 and DGU.US 2 reveals statistical significant differences ($p<0.0005$; χ^2 values 37.777 and 23.897), whereas

the two DGU.US lines do not differ significantly between themselves ($p>0.6$, χ^2 value 0.245).

Line	Number of blue sectors	Number of seedlings	Sectors per seedling	Enhancement
DGU.US1	0	14		
	1	6		
	2	3		
	3	2		
	4	1		
	5	5		
			47/31=1.5	
DGU.US1 x DISceI	55	1		
	62	1		
	63	1		
	65	3		
	75	1		
	85	2		
	87	1		
	90	1		
	95	1		
	105	2		
	108	1		
	110	2		
	150	1		
	180	1		
	200	1		
	210	2		
			2380/22=108.2	72.13
DGU.US2	0	8		
	1	7		
	2	1		
	3	1		
			12/17=0.7	
DGU.US2 x DISceI	34	1		
	45	1		
	57	1		
	58	1		
	59	1		
	62	1		
	65	3		
	70	1		
	74	2		
	82	1		
	84	1		
			894/14=63.86	63.86

IU.GUS 7	0 1 2 3 4	19 10 3 4 3		
			40/39=1.03	
IU.GUS 7 x DISceI	5 6 7 9 10 11 12 13 14 15 17 18 19 20 22	2 1 1 1 6 2 2 3 2 2 1 1 1 2 2		
			373/29=12.86	12.49
IU.GUS 8	0 1 2 3 4 5	12 5 3 3 5 4		
			60/32=1.88	
IU.GUS 8 x DISceI	9 18 28 29 32 35 37 39 44 48 59	1 1 2 3 3 3 3 2 1 1 1		
			711/21=33.86	18.01
DU.GUS 6	0 1 2 3 4	26 5 3 2 1		
			21/37=0.57	

DU.GUS 6 x DISceI	6 9 12 13 14 15 16 18 19 20	1 1 2 2 3 3 5 4 3 1		
			381/25=15.24	26.7
DU.GUS 8	0 1 2 3 4 5	16 6 3 3 2 2		
			39/32=1.22	
DU.GUS 8 x DISceI	6 7 10 11 12 13 14 15 16 18 19	1 1 1 1 2 2 6 5 4 2 3		
			400/28=14.28	11.7

Table 4.7. Distribution of recombination events in *Arabidopsis* seedlings carrying a recombination substrate without and with induction of DSBs.

4.2.7 Molecular analysis of recombinants

To demonstrate that the restoration of the ß-glucuronidase activity is indeed due to homologous recombination, events were analyzed on a molecular level. A ß-glucuronidase assay under nondestructive conditions was performed on seedlings from the third generation after crossing. Tissue that due to light blue staining was putatively identified as recombinant, was cut out of the respective seedlings and transferred to GM harboring plant hormones so that the recombinant material could be propagated *via* callus culture for further analysis.

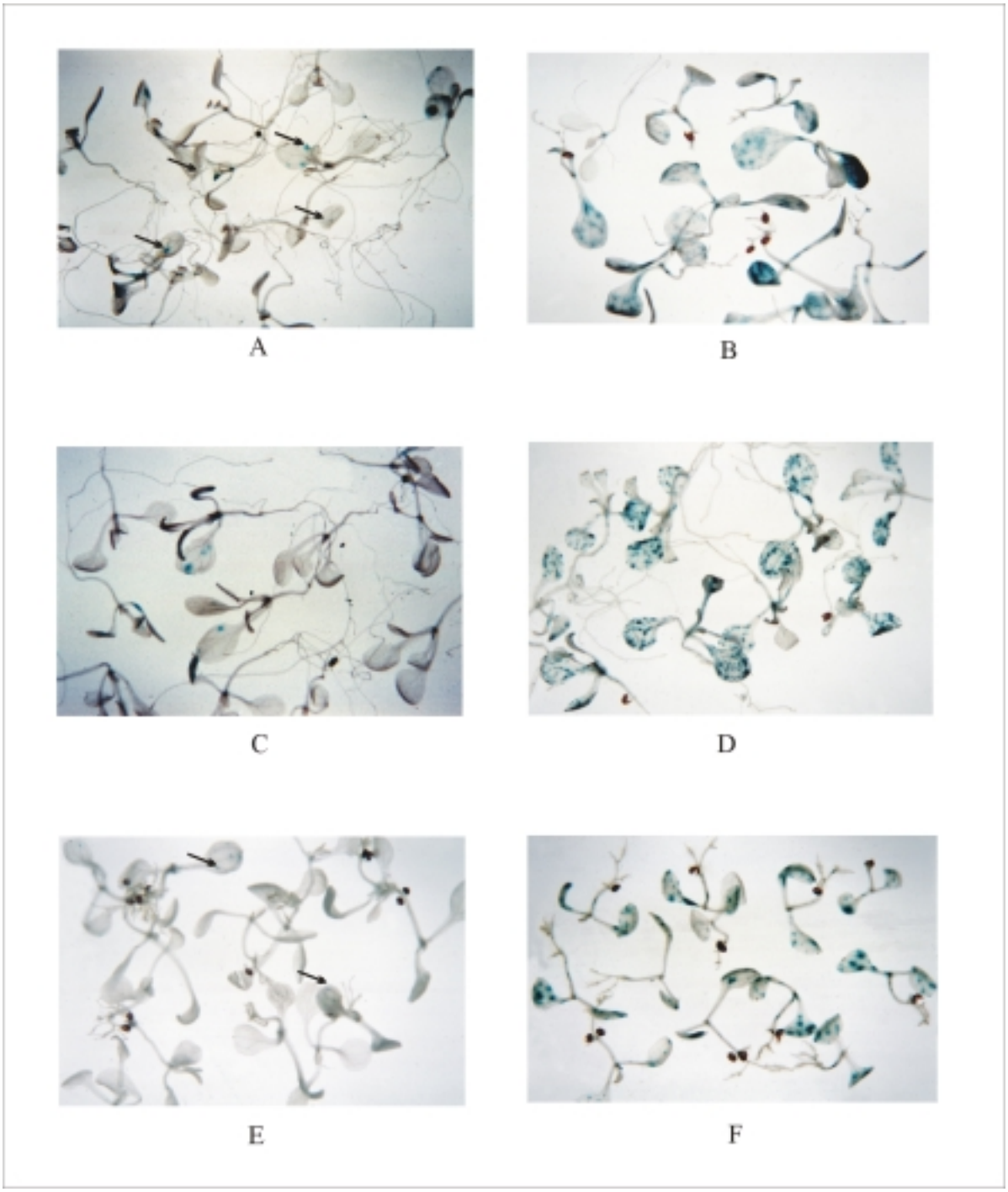


Figure 4.15. ß-Glucuronidase assay of transgenic seedlings harbouring a recombination substrate before and after crossing with the line expressing DI-SceI1. Recombination events, that, due to HR, restore the marker gene, result in blue staining of the corresponding cell and its progeny. On the left - seedlings of the lines IU.GUS 7 (A), DGU.US 1 (C) and DU.GUS 8 (E), and on the right - seedlings of the same lines crossed with line D-I*Sce*I1, IU.GUS 7 X DI*Sce*I (B); DGU.US 1 X DI*Sce*I (D); DU.GUS 8 X I*SceI* (F).

As the efficiency of regeneration with this procedure is quite low (less than 1% of the calli, Swoboda et. al., 1994) we used for this analysis also progeny from several crossed lines that harbored both the recombination substrate and the I-*Sce*I expressing cassette. These plants showed a similar enhancement of recombination as the parental lines. DNA was extracted from callus material. When staining indicated a recombination event early in development (bigger parts of the plants stained blue) in some cases such seedling were transferred directly to soil for getting offspring. After propagation part of the obtained plant material was stained with X-Gluc to confirm expression of the recombined GUS gene and lines which had completely blue plants were selected. From another part DNA was extracted for Southern blot and PCR analysis. The analysis sustained in all cases that the restoration of the marker was due to HR. Results of the molecular analysis for all three constructs are given below.

4.2.7.1 PCR analysis of recombinants

Via PCR the restoration of the ß-glucuronidase gene could be demonstrated.

In case of line and IU.GUS 7 R1, calli were completely blue after several hours staining, indeed that the material was propagated from cell with single recombination event. The remaining part of the calli was used for DNA extraction and the DNA was digested with I-*Sce*I. Using this material, a fragment of the GUS gene (1,9 kb) was amplified by PCR with primers binding outside of the *Acc*65I site (pGUSR+GUSHIN2) (Figure 4.16.). The product of the reaction was extracted from gel and shortly reamplified (20 cycles). Restriction of this PCR product with I-*Sce*I (*Xho*I) indicated that the I-*Sce*I restriction site was absent in the part of PCR templates. For further characterization, the amplified band was cloned into the *Sma*I site (blunt ends) of the pUC19 vector. Restriction analysis indicated that a similar number of plasmid clones contained inserts with and without I-*Sce*I site. Sequence analysis (with primers pRZD and pHZD) demonstrated that the removal of the I-*Sce*I was indeed

correlated with the restoration of the functional GUS gene, whereas the original transgene sequence, including the I-*Sce*I, site was conserved in the other plasmids (Figure 4.16.).

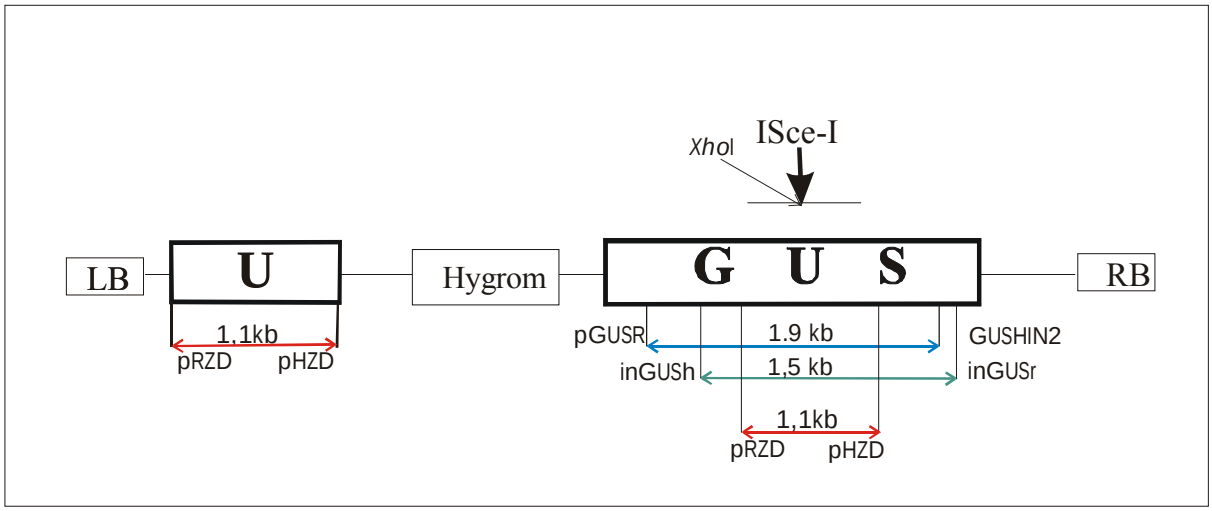


Figure 4.16. Schematic drawing of a line with the recombined substrate with pairs of primers and sizes of possible PCR products.

DNA from the original line (IU.GUS 7) was also amplified and cloned in the same way into pUC19 vectors. For sequence analysis the PCR products from amplification with pRZD and pHZD primers was used.

The analysis detected that in case of the original line (line IU.GUS 7) the I-*Sce*I restriction site was still present with its original boundaries, whereas for the recombined line (line IU.GUS 7 R1) – the I-*Sce*I restriction site had been removed and the sequence of GUS gene was restored.

A similar analysis was performed for recombination events of line IU.GUS 8 with an identical outcome. We were able to regenerate plant material from four recombination events of line IU.GUS 8. In all four cases, PCR followed by sequence analysis revealed that the marker gene was restored by a gene conversion event that removed the linker sequence within the GUS gene.

As the outcome of the recombination reaction with the substrate DU.GUS was expected to be identical to the one of IU.GUS, a molecular analysis similar to the one described above was performed for the line DU.GUS 8R1. We were able to regenerate two more recombination events from line DU.GUS 8R (DU.GUS 8R2 and 3) after DSB induction and in both cases restriction of the amplified PCR fragment with *Xho*I sustained the loss of the linker sequence within the GUS gene (Figure 4.17. A and B lines 2, 3, 5). This observation could be further confirmed by sequence analysis.

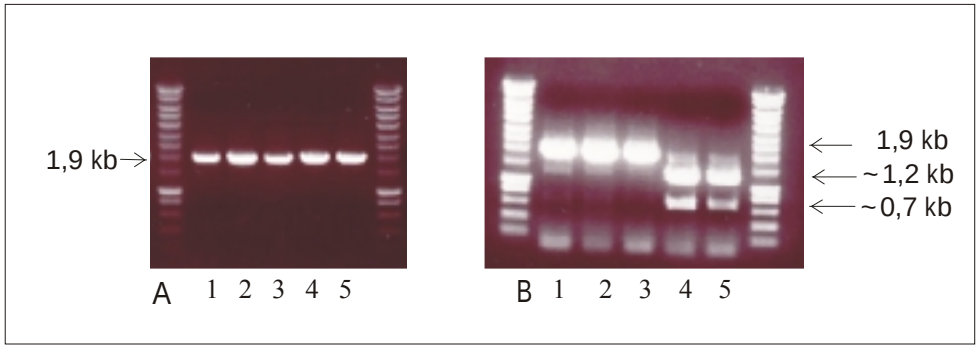


Figure 4.17. A – fragment obtained after a PCR reaction with pGUSR and GUSHIN2 primers; B – digestion of this PCR products with *Xho*I. Lines: 1 – IU.GUS 8 R1; 2 - DU.GUS 8 R2; 3 - DU.GUS 8 R3; 4 - IU.GUS 8; 5 - DU.GUS 8.

4.2.7.2 Southern blotting analysis of recombinants

To full characterization the recombined lines in detail Southern blot analysis was performed. Plant DNA from the original lines and the recombined lines was digested with different enzymes and hybridized with a GUS-specific radioactive labeled probe. A schematic representation of the expected fragments in Southern blotting analysis is depicted in Figures 4.18 whereas Figures 4.19, 4.20, 4.21. and 4.22 shows the result from the blotting experiments.

The analysis confirmed that the restoration of the marker was due to HR in all three recombination substrates.

Southern blotting of DNA from the original line IU.GUS 8 and from the recombined line IU.GUS 8 R1 (Figure 4.20) indicated that - as expected for a gene conversion event - no detectable changes in size of the transgene occurred (lane 1 and 2, *Hind*III digested DNA). We also found no indication for any change within the donor sequences (lane 5 and 6, the size of the small *Acc65*I fragment did not change between parental and recombined line). However the *Xho*I/*Hind*III digest indicated that the linker sequence was removed due to gene conversion (lane 4), resulting in the restoration of the functional GUS gene as detected by histochemical staining. IGU.US 8R1 is homozygous for the recombination substrate and therefore beside the upper band in lane 4 that could not be restricted by *Xho*I, two smaller bands corresponding to the bands of the same size in IGU.US 8 were also present. Thus, the second copy of the transgene did not undergo gene conversion.

A similar analysis was performed for a DSB-induced recombination event of line IU.GUS 7 (IU.GUS 7 R1) with an identical outcome (Figure 4.19).

We were able to regenerate plant material from three more recombination events of line IU.GUS 8 X DI-*Sce*I1 (IU.GUS 8 R2, 3 and 4) and one more for line IU.GUS 7 X DI-*Sce*I1 (IU.GUS 7R2). In all cases, PCR followed by sequence analysis revealed that the marker gene was restored by a gene conversion event that removed the linker sequence within the GUS gene.

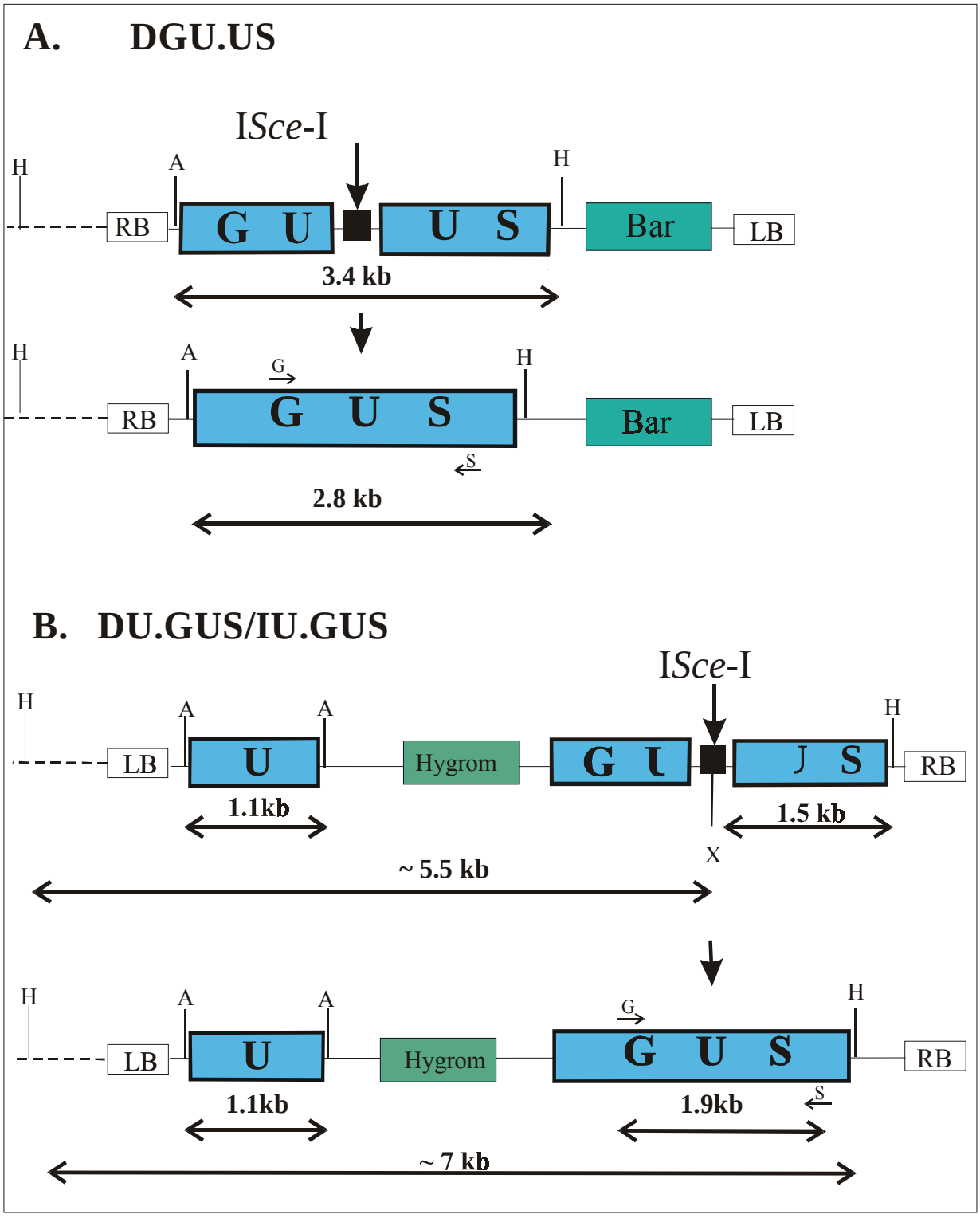


Figure 4.18. Schematic map of the recombination substrates used in this study and predicted outcome of the HR reaction. (Details see in text).

As the outcome of the recombination reaction with the substrate DU.GUS was expected to be identical to that of IU.GUS, corresponding analyses were performed for the line DU.GUS 8. As shown in Figure 4.21, Southern blotting revealed no major changes in the recombinant DU.GUS 8R1 in comparison to the parental line DU.GUS 8 as to the transgene construct beside the elimination of the inserted linker with the *Xho*I and I-*Sce*I sites. As in case of the recombinant IU.GUS 8 XR1 the analyzed line DU.GUS 8R1 was homozygous for the marker construct but only one copy underwent gene conversion. We were able to regenerate two more recombination events (DU.GUS 8R2 and 3) and in all cases restriction of the amplified PCR fragment with *Xho*I sustained the loss of the linker sequence within the GUS gene. The loss of the complete linker sequence could be confirmed by sequence analysis.

In case of the lines DGU.US1 and DGU.US2, restoration of the GUS gene was expected to result from a deletion of the interrupting sequence including the I-*Sce*I site. Southern blotting of the recombinant line DGU.US1 R1 revealed that this was indeed the case. After the recombination event the GUS-specific fragment (*Hind*III-*Acc*65I) was reduced in size from 3.4 to 2.8 kb (Figure 4.22, lanes 5 and 6; 11 and 12). A similar size reduction is indicated by *Hind*III digest (Figure 4.22, lanes 1 and 2; 7 and 8). The removal of the linker is demonstrated by the fact that only the *Hind*III fragment of the original line, but not of the recombination can be restricted by I-*Sce*I (compare Figure 4.22 lane 3 with lane 4 and line 9 with line 10).

In addition to the described case six more recombination events (three from line DGU.US 1, DGU.US1 1R 2 to 4 and three from line DGU.US2, DGU.US2 1R 1 to 3) could be isolated. PCR analysis of the respective lines revealed in all cases that the restoration of the marker gene was coupled with the elimination of I-*Sce*I restriction site.

Thus, the molecular characterisation confirm that the events that lead to the restoration of the ß- glucuronidase gene are due to the homologous recombination and this applies for all constructs used in this study.

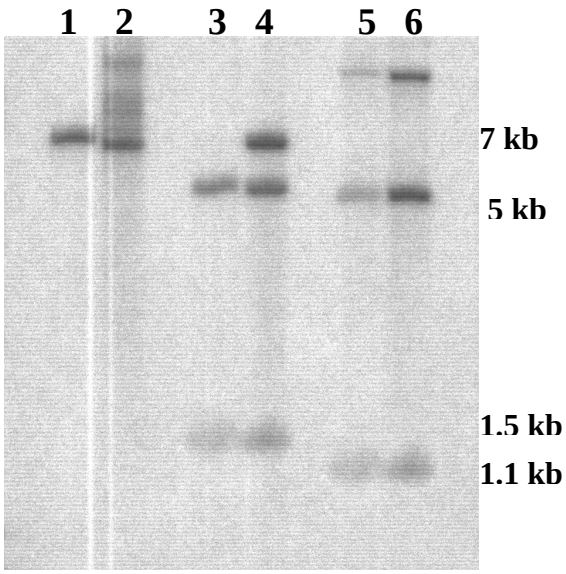


Figure 4.19. Southern blot with restriction digested DNA of the plant IU.GUS 7 and a recombinant IU.GUS 7R1. Lanes 1 and 2: *Hind*III restricted DNA; lines 3 and 4: *Hind*III and *Xho*I restricted DNA; lanes 5 and 6 : *Acc65I* restricted DNA. The DNA was hybridized with a ß-glucuronidase specific probe.

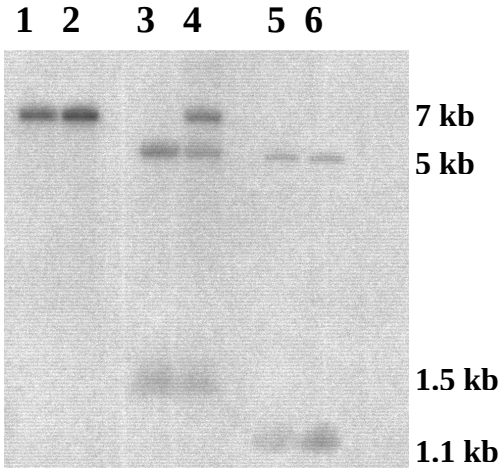


Figure 4.20. Southern blot with restriction digested DNA of the plant line IU.GUS 8 and a recombinant IU.GUS 8R1. Lanes 1 and 2: *Hind*III restricted DNA, 3 and 4: *Hind*III and *Xho*I restricted DNA and lanes 5 and 6 *Acc65I* restricted DNA. The blot was hybridized with a ß-glucuronidase specific probe.

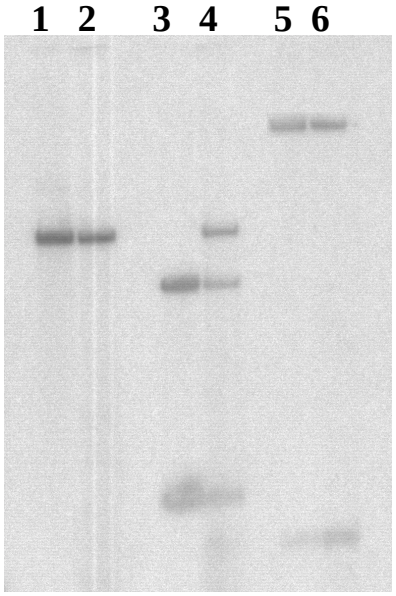


Figure 4.21. Southern blot with restriction digested DNA of the plant line DU.GUS 8 and a recombinant DU.GUS 8R1. Lanes 1 and 2: *Hind*III- restricted DNA, 3 and 4: *Hind*III and *Xho*I restricted DNA and lanes 5 and 6 *Acc65I* restricted DNA. The blot was hybridized with a ß-glucuronidase specific probe.

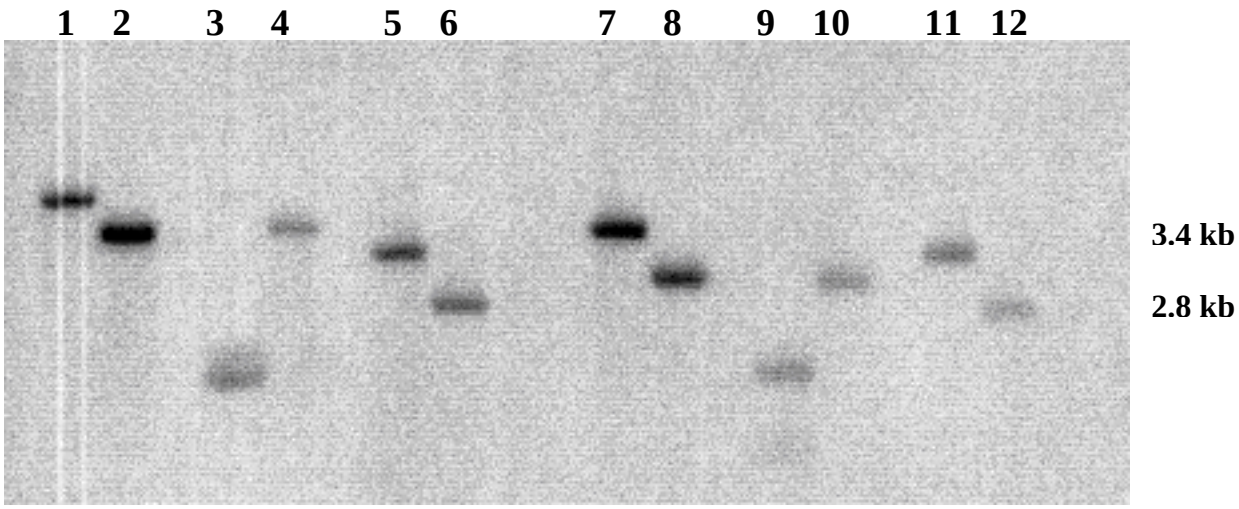


Figure 4.22. Southern blot with restriction digested DNA of the plant line DGU.US1 and a recombinant DGU.US 1R1 (lines1-6), DGU.US 2 and a recombinant DGU.US 2R2 (lines 7-12). Lanes 1 and 2 (7 and 8) *Hind*III-restricted DNA, 3 and 4 (9 and 10) *Hind*III and I-*Sce*I restricted DNA and lanes 5 and 6 (11 and 12) *Hind*III and *Acc65I* restricted DNA. The blot was hybridized with a ß-glucuronidase specific probe. The 2.8 kb band in lines 6 and 12 indicates that the I-*Sce*I site was removed by deletion that resulted in a restoration of the ß-glucuronidase gene.

5 Discussion

5.1 The role of NHEJ in genomic DSB repair in different plant species and genome evolution in plants

Double-strand breaks (DSBs) are critical lesions in genomes. Efficient repair of DSBs is necessary for the survival of all organisms. In principle, DSBs can be repaired *via* non-homologous end joining (NHEJ) or *via* homologous recombination. Although homologous recombination is used for DSB repair in plants (Chiurazzi et. al., 1996; Shalev and Levy, 1997; Puchta, 1999a; Xiao and Peterson, 2000; Siebert and Puchta, 2002) NHEJ seems to be the main mode of DSB repair (for reviews see Puchta and Hohn, 1996, Vergunst and Hooykaas, 1999; Gorbunova and Levy, 1999; Mengiste and Paszkowski, 1999). Error prone DSB repair may result either in deletions, insertions or various other kinds of genomic rearrangements (Pipiras et. al., 1998; Salomon and Puchta, 1998; Jasin, 2000). In plants genomic alterations in somatic cells of meristems can be transferred to the offspring (Walbot, 1996) and are therefore relevant for evolution. Thus, somatic DSB repair might in evolutionary terms have an impact on genome size and genome organization.

The reason for the large differences in the nuclear DNA content of eukaryotes, particularly plants, known as “C value paradox”, has been a matter of debate since long (Cavalier-Smith, 1985; Dove and Flavell, 1988). Even closely related species with similar phenotype may significantly differ as to their diploid genome size. One mechanism responsible for these differences could be due to a species-specific increase/reduction of repetitive sequences. In principle, genomes may become larger *via* duplications and insertions or smaller *via* deletions. Species-specific spread of retrotransposons was postulated as a main route enlarging plant genomes (SanMiguel et. al., 1996; SanMiguel et. al., 1998; Bennetzen and Kellog, 1997). Alternatively, deletions might reduce genome size and counterbalance enlargements (Petrov, 2001). Recently, an elegant theoretical study (Petrov et al., 2000) has demonstrated that deletions of significantly different extension within retroelements yielded

species-specific genome size alterations in related insect species over evolutionary time periods. It was demonstrated experimentally that the mean size of deletions obtained from DSB repair is on average a third larger in *Arabidopsis* than in tobacco (Kirik et. al., 2000). Thus, for plants and insects an inverse correlation between genome size and deletion size was found. However, one has to be careful to draw final conclusion from the comparison of only two species each, but it is tempting to speculate that the phenomenon might be due to a general principle of genome evolution.

During DSB repair the size of a deletion depends on the processing of DNA ends. If broken ends are not religated directly the processing of such ends might result in the loss of DNA at the break site (Salomon et. al., 1998; Kirik et. al., 2000). Depending on the efficiency of DNA degradation more or less information will be lost. In the current work differences as to the stability of linearized plasmid DNA in tobacco and *Arabidopsis* cells was detected. Although the results obtained by Southern blotting indicate that on average plasmid molecules transformed into *Arabidopsis* cells are less stable than in tobacco cells, it of course cannot be excluded that this difference might not be caused by degradation in the nucleus, as plasmid molecules are also transferred into other compartments of the cell. However, as two other assays, that are clearly nuclear-based (chromosomal DSB repair [Kirik et al., 2000] and the transient expression assay described in this report) demonstrate a similar difference using unrelated methodologies, it is possible to speculate that this is indeed caused by the same kind of phenomenon.

The results presented in the current report indicate that the difference in the processing of DNA ends between the two plant species is at least mainly due to an enhanced exonucleolytic degradation of DNA in *Arabidopsis*. If a break was induced between promoter and open reading frame of a marker gene the expression level was reduced by a factor of two in *Arabidopsis* in relation to tobacco. This is in contrast to the similar expression levels obtained for both species when the break was introduced distantly from the marker gene. Whereas in

the first case exonucleolytic digest of few nucleotides would result in destruction of the expression cassette, in the latter case the removal of more than thousand base pairs would be a prerequisite for the destruction of the ORF. Because endonucleolitic degradation would destroy all information on a plasmid at the same rate independent of a preexisting break site, no differences should be found in presence of such a nuclease activity. However, since the restoration of the ß-glucuronidase ORF in case of the *Stu*I and *Xba*I restricted plasmid requires a ligation step, the data of the expression assay alone do not suffice to discriminate between a more efficient exonucleolytic destruction of the marker gene or a less efficient ligation reaction in *Arabidopsis* cells. As Southern blotting revealed differences in the degradation of plasmid DNA linearized with the exactly the same enzymes between *Arabidopsis* and tobacco, one can assume that at least most of the effect is due a more efficient exonucleolytic degradation of DNA in *Arabidopsis*. However one question remains: What is the reason for the enhanced DNA degradation in *Arabidopsis*? This could be either due to less active DNA exonucleases or due to the better protection of DNA ends from degradation e.g. by DNA end binding factors (Liang et. al., 1996) in tobacco.

Purposely, the stability of circular plasmid DNA in both plant species was not tested, as this approach would not have allowed us to discriminate between exo- and endonucleolytic degradation. Linearization of the plasmid DNA by an endogenous endonuclease would be a prerequisite of exonucleolitic degradation, however if the plasmid is linearized, exonuclease could immediately work on it. Thus, it would be hard to discriminate to which extent what kind of enzyme activity would contribute to DNA degradation in *Arabidopsis* and tobacco. Using linearized molecules the work was done with a more defined system as endonucleases could work on all DNA molecules immediately after transformation and independent of a second reaction.

Theoretical calculations on the evolution of genome sizes have to take into account the rate of duplication and the rate of loss of genomic sequences (Petrov, 2001). DSB repair is one

mechanism that may result in sequence deletions. To evaluate the possible role of DSB repair in this process one has to take into account two factors: the number of breaks induced and the loss of sequence information per repair event. The loss of sequence information is strongly influenced by the stability of the broken DNA ends. The more efficient the ends are recessed by exonucleolitic degradation the more the genome size is supposed to shrink over an evolutionary time period. In line with this argumentation we found indications that in a plant with a small genome free DNA ends are less stable than in a plant with a more than twenty times larger genome.

In a recent investigation of retroelements in *Arabidopsis thaliana* the hypothesis about reduction genome size through illegitimate recombination was confirmed (Devos et. al., 2002). The data indicate the absence of LTRs older then a few millions years, which allows to conclude that they are gradual degradated over time. Illegitimate recombination associated with deletion seems to play a more prominent role than homologous recombination in the elimination of DNA for the *Arabidopsis thaliana*: at least fivefold more DNA has been removed by NHEJ than unequal homologous processes.

5.2 The role of homologous recombination in genomic DSB repair in plants

The main mode of repair of double-strand breaks (DSBs) in plants, like in other higher eukaryotes, proceeds via non-homologous end-joining (Gorbunova and Levy, 1999; Salomon and Puchta, 1998; Kirik et. al., 2000). However, homologous sequences can potentially be used for repair. In several studies by the use of the rare cutting restriction endonuclease I-*Sce*I it was analyzed to what extend the position of the homologous sequence in relation to the DSB determines the proportion of breaks repaired by HR. Only one out of about 10.000 breaks undergoes HR using homologous sequences in ectopic or allelic positions (Puchta 1999a; Gisler et. al., 2002; for results obtained by a transposon-based approach see Shalev and Levy, 1997). In contrast, breaks within a duplicated region can be repaired in up to one

third of the cases by formation of a deletion involving homologous regions (Siebert and Puchta, 2002; for a transposon based approach see also Xiao and Peterson, 2000; Xiao et. al., 2000). These differences might be due to different mechanisms of DSB repair acting under these experimental conditions. Whereas the synthesis-dependent strand-annealing (SDSA) model seems to be the most appropriate to describe gene conversions between allelic or ectopic homologous sequences, the formation of a deletion due to HR between homologous sequences in close proximity is best described by the single-strand annealing (SSA) model. Both models are depicted in Figure 1.2 and Figure 1.3.

The SDSA model describes transfer of information from a homologous donor sequence to the break site (Gloor et. al., 1994) without a loss of sequences at the donor locus. This process does not result in the loss of genomic sequences as the donor locus is conserved. The SSA model (Lin et. al., 1984; 1990) describes a non-conservative reaction resulting in a loss of information that is positioned between the annealing repeats. SSA-like models have also been postulated for explaining the loss of information during NHEJ (e.g. Nicolas et. al., 1995). It is tempting to speculate that the enzyme machinery involved in both mechanisms differs in at least some of the factors. For the formation of a D-loop structure and the switch of the template during DNA synthesis probably more factors are required than for a simple annealing reaction. According to the SDSA model the orientation of the donor sequence to the break has no influence on gene conversion.

Transgenic *Arabidopsis thaliana* plants contains interrupted sequences of ß-glucuronidase gene as recombination substrate were used to assay homologous recombination events. As expected, both with and without DSB induction, no difference was found as to the frequency of ß-glucuronidase gene restoration for both orientations of the constructs detecting gene conversion events (DU.GUS; IU.GUS). Without DSB induction, the frequency of HR found with the DGU.US lines was similar to that observed for DU.GUS and IU.GUS. The picture changes when we look at the frequencies of DSB-induced marker gene restoration. Here, the

SSA pathway seems to be more efficient (~5 fold) than the gene conversion pathway. A possible explanation for this phenomenon would be that the kind of lesion leading to the spontaneous HR events might differ from the DSB induced by I-*Sce*I. One might speculate that spontaneous events are linked to replication. A prominent recombination reaction linked to replication is sister chromatid exchange (SCE). Indeed, besides the recombination mechanisms discussed above (SSA and SDSA) the ß-glucuronidase gene could be restored by crossing over using information from the sister chromatid. This applies for all three different recombination substrates used in this study. By crossing the transgenic lines produced in this study with insertion mutants of *Arabidopsis* deficient in the expression individual members of the repair and replication machinery (for a recent review on mutants see Hays, 2002) it might be possible to differentiate between these reactions by identifying the factors involved.

Although our results clearly show that classical gene conversion events are less efficient than SSA events under similar conditions, one has to keep in mind that according to the SDSA pathway besides classical conversion events other products might arise, too. Earlier work demonstrated that DSBs can also be repaired by a combination of HR and NHEJ (Puchta et. al., 1996; Puchta 1998a; Puchta, 1999a). However, with the assay system applied in this study such events cannot be detected, as only repair of both ends of the break *via* HR result in a functional marker. The previous results indicated that repair of one and of both ends of a break by HR occur at about the same frequency (Puchta, 1998a) so that the SDSA-like pathway of DSB repair seems to be in any case less efficient than the SSA-like pathway in plants.

If one take all previous results into account and compare them with the results obtained in this study one is able to obtain a detailed picture on how DSBs are repaired in somatic plant cells. The most efficient way to repair a break is by non-homologous end-joining. If homologous sequences are available close to the break, the repair can take place in up to a third of the cases by a single-strand annealing pathway (Siebert and Puchta, 2002). If gene

conversion is five times less efficient than deletion formation, as it was demonstrated in this study, then one can expect that in about one out of fifteen breaks, repair could also proceed *via* the SDSA pathway. This is two to three orders of magnitude more efficient than a gene conversion event that uses homology from an allelic (Gisler et. al., 2002) or ectopic site (Puchta, 1999a). Thus DSB-induced gene conversion between members of tandemly arranged gene families might well play an important role in plant genome evolution. Various resistance genes are organized in tandem arrays in plant genomes and gene conversion events between the various members have been demonstrated by sequence analysis (e.g. Parniske et. al., 1997, Parniske and Jones, 1999). Our results indicate that such events might not necessarily be due to meiotic recombination but may - at least partially - also occur during vegetative growth. It has been shown before that somatic changes in meristems can be transferred to the “germ line” in plants (for discussion see Puchta and Hohn, 1996). Indeed, pathogen attack is inducing intrachromosomal recombination between tandemly repeated sequences (Lucht et. al., 2002). Moreover, certain sites in plant genomes might be less stable for repeated sequences than others. This is documented by finding that certain transgene sequences are deleted from the genome at high rates (Zubko et. al., 2000), a phenomenon which has been postulated to be due to transient DSB-induction (Puchta, 2000). It is tempting to speculate that the various gene conversion events, detected in tandemly arranged resistance gene clusters, might be correlated with higher rates of transient breaks in these regions of the plant genome (Ramakrishna et. al., 2002).

Summary

To establish the molecular basis of differences in the repair genomic double-strand breaks (DSBs) by non-homologous end-joining (NHEJ) in *Arabidopsis thaliana* and *Nicotiana tabacum* the fate of free DNA ends in both plant species was analysed by biolistic transformation of leave tissue with linearized plasmid molecules. Total DNA was isolated and Southern blotting was performed. Hybridisation against labelled plasmid DNA indicated, that irrespective of nature of the ends (blunt; 5' or 3' overhangs) linearized full-length DNA molecules were less stable in *Arabidopsis* than in tobacco. To clarify nature of degradation (exo- or endonucleotic) measument of activity of a ß-glucuronidase gene of the restricted plasmid DNA was performed. When the break was at distance to the marker gene the relative expression of the ß-glucuronidase was similar in both plant species. If the DSB was introduced between promoter and open reading frame, transient expression was reduced by half in *Arabidopsis* in comparison to tobacco. These results indicate that DNA ends are more stable in tobacco than in *Arabidopsis*, either due to less DNA exonuclease activity or due to a better protection of the DNA ends or both. Exonucleotic degradation of DNA ends might therefore be a driving force in the evolution of genome size as the *Arabidopsis* genome is over twenty times smaller than the tobacco genome.

To study the efficiency of the different pathways of homologous recombination in somatic cells six transgenic lines carrying three different recombination substrates were produced. The transgenes contain a recognition site for the restriction endonuclease I-*Sce*I either between direct GUS repeats to detect deletion formation (DGU.US), or within the GUS gene to detect gene conversion using a nearby donor sequence in direct or inverted orientation (DU.GUS and IU.GUS). Without expression of I-*Sce*I, the frequency of homologous recombination (HR) was low and similar for all three constructs. By crossing transgenic lines, carrying different recombination substrate with an I-*Sce*I expressing line, recombination was induced by one to two orders of magnitude. The frequencies obtained with the DGU.US construct

were about five times higher than with DU.GUS and IU.GUS, irrespective of the orientation the of donor sequence. Molecular characterization of recombinants was shown by restoration of the marker due to HR. These results indicate that recombination associated with deletions is the most efficient pathway of homologous DSB repair in plants. However, DSB-induced gene conversion seem to be frequent enough to play a significant role in evolution of tandemly arranged gene families like genes which are responsible for resistance against pathogens.

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Publications

1. Orel N., Puchta H. (2003) Differences in the processing of DNA ends in *Arabidopsis thaliana* and tobacco: possible implications for genome evolution. *Plant Mol Biol.* 51(4):523-31.
2. Orel N., Kyryk A. and Puchta H.(2003) Different pathways of homologous recombination are used for the repair of double-strand breaks within tandemly arranged sequences in the plant genome. *Plant Journal*. 35:604-612.
3. Schmidt-Puchta W., Orel N., Kyryk A. and Puchta H. (2003) Intrachromosomal homologous recombination in *Arabidopsis thaliana. Methods Mol Biol*. Humana Press. (in press).

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Lebenslauf

Name	Orel
Vorname	Nadiya
Geburtsdatum	30.03.1976
Geburtsort	Ivano-Frankivsk, Ukraine

Die Ausbildung

1983-1993	Grundschule und Gymnasium N18 in Ivano-Frankivsk (Ukraine). Abgeschlossen mit Auszeichnung.
1993-1998	Studium der Biochemie an der Lwiwer Staatsuniversität von I. Franko (Ukraine). Diplomprüfungen in der Fächern: Biochemie, Philosophie. Diplomarbeit "Influence of point substitute non-polar amino acid on the forming and function of the protein molecules". Diplom mit Auszeichnung als Biochemikerin sowie als Lehrerin
1999-2003	Doktorarbeit in der Gruppe DNA-Rekombination von Herrn Prof. Dr. H. Puchta am IPK, Gatersleben (Sachsen-Anhalt)