DSB repair by illegitimate and homologous DNA recombination in *Arabidopsis thaliana*.

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List of abbreviations

A.thumefaciens	*Agrobacterium thumefaciens*	LB	Left border
ABA	abscisic acid	M	molarity
Amp^r	ampicillin resistance	m	mili
bp	base pair	min	minutes
BAP	6-Benzylaminopurin	μ	micro
°C	grad Celsius	n	nano
DNA	deoxyribonucleic acid	NAA	α-naphthaleneacetic acid
DAP	days after polination	OD	optical density
DSB	double-strand break	ORF	open reading frame
DMF	formamid	P	Promoter
DMSO	dimethylsulfoxid	PCR	Polymerase chain reaction
2,4D	2,4-dichlorophenoxyacetic acid	pH	potentia Hydrogenii
E.coli	*Escherichia coli*	r.p.m.	revolutions per minute
EDTA	ethylenediamine tetraacetic acid	RB	right border
Fig.	Figure	RT	room temperature
5-FC	5-fluorocytosin	sec.	seconds
g	gram	SDS	sodium dodecyl sulphate
GUS	β-glucuronidase	T-DNA	transferred DNA
h	hour	T	terminator
IPTG	Isopropyl-β-D-thiogalactopyranoside	Tris	hydroxymethyl-aminomethan
IAA	indoleacetic acid	wt	wild type
2iP	2-isopentenyl adenine	Km	kanamycin

1. INTRODUCTION

Recombination is a process of DNA strand exchange that leads to new combinations of alleles on chromosomes. Recombination is especially noticeable during meiosis, where homologous chromosomes undergo crossover (Heyting, 1996; Roeder, 1997; Bascom-Slack *et al*., 1997). In sexually reproducing organisms, homologous recombination increases genetic diversity in gametes and ensures proper chromosome segregation (Klecker, 1996; Smith and Nicolas, 1998).

In somatic cells recombination is induced by DNA damage and is used for repair. In addition somatic recombination can be responsible for creation of genetic diversity in plants. Mitotic or somatic recombination events are shown to be transmitted to the offspring (Das *et al*., 1990). In *Arabidopsis thaliana* mature seeds a small number of cells from 1 to 4 are shown to contribute to the next generation (Li and Redei, 1969). Lineage analyses in several plant species demonstrated that meristematic cells proliferate in a predictable manner to form the differentiated tissues of the mature shoot system (Irish and Sussex, 1990; Irish, 1991; Jenik and Irish, 2000).

Recombination serves different roles in different stages of the life cycle. It stabilises genome via repairing of damaged chromosomes and is a factor that promotes evolution, through the creation of new genes and linkages.

Different types of recombination can be distinguished (Low and Porter, 1978):

i) homologous recombination – if the two parental molecules are extremely similar, have identical sequence of bases for at least 200 bases pair (bp);

ii) site–specific recombination that occurs between two very limited sites with defined sequences;

iii) transposition – involves the interaction of the two ends of a specific DNA sequence, called transposable elements, with a third, more or less random site on the same or another chromosome;

iiii) illegitimate recombination – when rearrangements of DNA molecules result from an apparent end-to-end fusion process or else a chromosomal crossover, which does not appear to involve extensive sequence homology. Sometimes, the term "illegitimate" recombination is used to describe site-specific and transposition recombination events (Anderson, 1987).

1.1. Homologous recombination and gene targeting

Homologous recombination is distinguished from other types of recombination by its ability to produce genetic exchange at any point along homologous DNA sequences. Homologous recombination is one of the basic mechanisms for the rearrangement of genetic material. It is well studied in prokaryotes (Smith, 1989) and simple eukaryotes (Petes and Hill, 1988). In higher organisms three different approaches have generally been used to study homologous recombination: extra-chromosomal recombination between transferred DNA molecules, chromosomal recombination between repeated genes stably integrated in genome and recombination between introduced DNA molecules and homologous sequences in the chromosome ("gene targeting") (Bollag *et al*., 1989).

Gene targeting, which is the directed integration of introduced DNA into the genome via homologous recombination, can be a valuable tool for many molecular genetic studies. In prokaryotes and some lower eukaryotes, where introduced DNA is predominantly integrated via homologous recombination, gene targeting has become a

standard technique. Initially observed in *Saccharomyces cerevisiae* (Hinnen *et al*., 1978), targeting event is a prerequisite in reverse genetic studies, gene disruption and allele replacement (Berg, 1991). With such approaches, virtually any cloned gene, even of unknown function, can be specifically mutagenised *in vitro* and reintroduced to its own chromosomal location by exchange of the endogenous counterpart (Struhl, 1983).

In higher eukaryotes however the introduced DNA is predominantly integrated at random position. The efficiency of homologous recombination is in the range of 10^{-3} to 10^{-5} in comparison with illegitimate recombination. Thus, the efficiency of gene targeting is very low. However, in some mammalian systems gene targeting frequencies of more than 1% of transformation events were obtained (Capecchi, 1989). The procedure entails the introduction of the mutated gene in embryonic stem (ES) cells, which after fusion to recipient blastocysts give rise to chimerical animals that may transmit the mutated allele to their offspring.

Targeting events have also been reported to occur in plant cells, however at low frequency of about $5 \cdot 10^{-4}$ (Offringa, 1992; Ohl *et al*., 1994). These results have been obtained both after direct DNA-transfer (Halfter *et al.*, 1992; Paszkowski *et al.*, 1988) and after *Agrobacterium* mediated T – DNA transfer (Lee *et al*., 1990; Offringa *et al*., 1993) to plant cells. Different model systems were used in these experiments. Restoration of a defective genomic copy of the gene via homologous recombination with a complementing part of the gene on the targeting vector was detected by restored antibiotic-resistance. Target genes, neomycin phosphotransferase (Paszkowski *et al.,* 1988; Offringa *et al.,* 1990; Risseeuw *et al.,* 1995) and hygromycin phosphotransferase (*htp*) (Halfter *et al.,* 1992) were used to allow selection of rare homologous recombination events.

In another study the acetolactate synthase gene (*ALS*) (Lee *et al.,* 1990) was used as an endogenous selectable target gene. Positive selection for recombination event was possible by introducing a specific point mutation in the targeting DNA, conferring resistance to the herbicide chlorsulfuron.

Thykjaer *et al.,* (1997) used the cytosine deaminase (*codA*) gene as a negative marker to enrich gene-targeting events of two endogenous genes in combination with positive selection in *Lotus japonicus.* The negative selection was carried out by applying 5-fluorocytosine (FC), which is converted into toxic 5-fluorouracil (FU) by the codA gene product. Gene targeting experiments were performed with the *LjGln1* (gene coding for a cytosolic glutamine synthetase) and with the *LjPzf* (gene corresponding to the cDNA for the plant transcription factor with a zincfinger motive) loci, using *Agrobacterium tumefaciens* T-DNA replacement vectors. Large flanking regions up to 22.9 kb surrounding a positive selection marker (geneticin) were presented as substrates for homologous recombination. For easier detection of putative recombinants the negative selectable marker was inserted outside the homologous region. Only in case of homologous recombination in the target loci the negative selection marker could be lost. A combination of positive and negative selection allowing double-recombinants to grow while counter-selecting random insertion was used to select putative targeting events. However, no recombinants were identified among over 18.000 transformed plants.

Besides codA, others negative selectable markers have been used for application in plants (Czako′ and Marton, 1994). In gene targeting experiments the *iaaH* gene from the T-DNA of the *Agrobacterium* Ti-Plasmid was used (Offringa, 1992). The iaaH gene product converts α-naphthalene acetamide (NAM) into the auxin naphthalene acetic acid (NAA), which is toxic at high concentration. The cells in which gene

targeting had occurred could be enriched several fold under the proper culture conditions.

The moss *Physcomitrella patens* has much higher gene targeting efficiency in contrast to higher plants (Schaefer and Zryd, 1997). The efficiency of gene targeting determined in these experiments was above 90%. DNA molecules integrated 10 times more efficiently by homologous recombination than by illegitimate recombination. This is the only example where a plant has frequency of the homologous recombination similar to yeast. The authors suggested a possible correlation between the efficiency of gene targeting and the length of a haplophase in eukaryotes. However, experiments with haploid tissue of higher plants did not corroborate this hypothesis (Schmidt-Puchta and Puchta, unpublished).

1.2. Double-strand break (DSB) repair

DNA Double-strand breaks (DSBs) are a form of DNA damage that can arise spontaneously in genome or may be induced by ionizing radiation, chemicals, oxidative stress, errors in DNA synthesis or metabolic processes (Camerini-Otero and Hsieh, 1995; Hoffman, 1994; Taghian and Nickoloff, 1997; Shinohara and Ogawa, 1995; Wood, 1996). If misrepaired or unrepaired, DSBs may be mutagenic or lethal for the cells.

DSBs can be repaired by either homologous or non-homologous (illegitimate) recombination. In yeast and bacteria DSBs repair occurs preferentially by homologous recombination, but in higher plants (Puchta and Hohn, 1996; Puchta *et al.*, 1996) and animals (Roth and Wilson, 1988) DSBs are repaired mainly via the illegitimate pathway.

1.2.1. DSB repair by homologous recombination

Genomic DSBs are key intermediates in the process of homologous recombination (Szostak *et al*., 1983; Lin *et al*., 1984; Belmaaza and Chartrand, 1994). Alternatively to the effect of harmful exogenous agents DSBs may be introduced in genomic DNA by endonucleases during normal cellular processes such as meiosis or gene conversion.

In yeast the meiotic recombination in most cases is initiated by DSBs (Wu and Lichten, 1994). Mitotic gene conversion in yeast was enhanced by DSBs at the recognition site of HO endonuclease (Haber, 1992). The induction of the HO nuclease expression and cleavage of the HO recognition site resulted in enhanced of intra- and intermolecular recombination (Haber, 1995).

The yeast mitochondrial I-SceI endonuclease (Perrin *et al*., 1993) with an 18-bp recognition site was used to induce recombination reactions in various organisms (Colleaux, *et al.,* 1988). In previous studies performed with I-SceI, specific DSBs were induced *in vivo* in plasmid DNA fragment transfected or injected into eukaryotic cells (Plessis *et al*., 1993; Segal and Carrol, 1995). I-SceI–mediated induction of genomic DSBs and their repair by homologous recombination were described for mouse cells (Rouet *et al*., 1994; Choulika *et al*., 1995). Induction of site-specific genomic DSBs was also performed in plants (Puchta *et al*., 1993; Puchta *et al*., 1996; Puchta, 1999b).

The basic features of recombination are conserved from bacteria through yeast to humans. The machinery for mitotic DSB repair is formed on the basis of meiosis-specific recombination complex. By chromosomal analysis of cells with a repressible

version of the *Rad51* gene, Sonoda *et al*. (1998) suggested that DSBs, which occur during normal DNA replication, are repaired by a recombination pathway involving the Rad51 gene product. Rad51 known as yeast analog of bacterial RecA is an essential component of homologous recombination (Nickoloff *et al*., 1998). Several genes, which implicated in DSB repair by homologous recombination on the basis of their sequence homology to yeast *Rad51,* were isolated: the *AtRad51* gene from *Arabidopsis* (Doutriaux *et al*., 1998) and the $XRCC_2$ gene from hamster (Johnson *et al*., 1999).

Johnson *et al*., (1999) showed the essential role of $XRCC_2$ gene for efficient repair of DNA DSBs by homologous recombination between sister chromatids. The authors found that hamster cells deficient in $XRCC_2$ show more that 100-fold decrease in the frequency of homologous recombination induced by double-strand break compared with the parental cell line.

The Rad51 protein in yeast forms a complex with Rad54, Rad55, Rad57 that is implicated in homology-dependent DSB repair, (Hays *et al*., 1995; Golub *et al*., 1997). The activity of Rad51 can be modulated by the RPA (replication single-stranded DNA binding protein) (Sugiyama *et al*., 1997). Recently, a novel protein Pir51 that strongly interacts with Rad51 was isolated (Kovalenko *et al*., 1997).

The DSBs are produced in genome of yeast during meiosis in a normal natural recombination process by the Spo11 protein (Keeney *et al*., 1997). Homologous of yeast *Spo11* gene were also isolated from others eukaryotes: *Drosophila melanogaster, Mus musculus, Arabidopsis thaliana* (McKim and Hauashi-Hagihara, 1998; Keeney *et al*., 1999; Hartung and Puchta, 2000).

The Rad52 DNA binding protein has an essential role in homologous recombination and stimulates DNA strand annealing in yeast. Mutations in Rad52 lead to defects in both mitotic and meiotic recombination (Mortensen *et al.*, 1996).

The Rad59 protein, which has homology with Rad52, appears to act in a salvage mechanism that can promote recombination when the Rad51 filament is not functional (Jablonovich *et al*., 1999).

1.2.2. DSB repair by illegitimate recombination

Whereas homologous recombination requires extensive tracts of sequence homology, illegitimate recombination is distinguished by its ability to join sequences with little or no homology. As a general rule, organisms with relatively compact genomes (bacteria and yeast) are mostly repairing DNA breaks via homologous recombination. In yeast for instance the illegitimate recombination is observed only in cells defective in the Rad52 pathway or in cells where no homology is available for repair by homologous recombination (Kramer *et al*., 1994; Moore and Haber, 1996a; Sugawara and Haber, 1992).

Organisms with larger genomes tend to repair breaks via illegitimate recombination regardless of how much homology is provided. In mammalian cells, for example, the repair of double-strand breaks predominantly occurs by illegitimate recombination (Roth and Wilson, 1988; Sargent *et al*., 1997).

Illegitimate recombination in plants has been mainly investigated by transgene integration especially via T-DNA (Hiei *et al*., 1994; Ohba *et al*., 1995; Papp *et al*., 1996; Iglesias *et al*., 1997; Takano *et al*., 1997). In many cases T-DNA integration

was shown to be associated with complex rearrangements including deletion and filler DNA insertions (Takano *et al.*, 1997; Salomon and Puchta, 1998).

Non-homologous end joining in plants was also analyzed in a more direct way. Linearized plasmid DNA was transformed in tobacco cells and novel joints that formed between the plasmid ends were sequenced (Gorbunova and Levy, 1997). In another study DSBs were induced at the I-SceI recognition site within a negative selectable marker gene on a chromosome and newly recombined junctions were sequenced (Salomon and Puchta, 1998). These investigations allow following conclusions:

1. Some degradation of the broken ends (deletions) usually occurs before ligation.

2. Microhomologies are often utilized during illegitimate recombination.

3. Filler DNA can be copied into the break. These may be recognizable sequences from elsewhere in the genome, duplications or insertions of local sequences or simple sequences that seem to be generated at random.

Seven proteins are known to be required for the DSB repair by non-homologous recombination: Ku70, Ku80, DNA ligase IV and the associated $XRCC_4$ protein, Mre11, Rad50 and Xrs2/p95 (Ivanov *et al.*, 1994; Jonzuka and Ogawa, 1995; Moore and Haber, 1996a; Critchlow and Jackson, 1998, Haber, 1999).

Ku proteins partially protect DNA ends from digestion by exonucleases (Holmes and Haber, 1999). Thus, if Rad52 protein may play a role of “gatekeeper” in homologous recombination, the Ku proteins seem to favor non-homologous end joining (Van Dyck *et al.*, 1999).

Tsukamoto *et al.*, (1997) has shown that the Ku70 protein interacts with Sir4 (silencing information regulator) protein, which binds to histones. Sir2, Sir3 and Sir4 are involved in regulation of transcriptional silencing at telomeres. The authors

proposed that the Ku70 protein binds to DSBs and then attracts Sir4 to form a heterochromatin-like structure that is necessary for the functioning of Rad50, Mre11 and Xrs2 in end joining.

Mre11, which interacts with Rad50, (Jonzuka and Ogawa, 1995) is involved in exonucleolytic processing of the DSBs ends.

Other enzymes, such as Rad1, Rad10 are also active in DSB repair by removing non-homologous ends from DNA (Ivanov and Haber, 1995; Sugawara *et al.*, 1997), while DNA ligase mediates both precise and non-precise illegitimate DNA end-joining (Schär *et al.*, 1997; Wilson *et al.*, 1997).

1.3. Recombination mechanisms. Models of recombination

The models of recombination are based on a occurrence of DSBs in DNA followed by repair of the break by homologous or non-homologous recombination.

The four mostly referred models are: the double-strand break repair (DSBR) model (Szostak *et al.*, 1983), the single-strand annealing (SSA) model (Lin *et al.*, 1984), the synthesis-dependent strand annealing (SDSA) model (Nassif *et al.*, 1994) and the one-sided invasion (OSI) model (Belmaaza and Chartrand, 1994).

The double-strand break repair (DSBR) model. The recombination reaction is initiated between two homologous sequences by a double-strand break in the molecule that is the acceptor of genetic information (Fig. 1, Ia). Further action of nucleases leads to increase of the break and to free 3´-ends formation (Fig. 1, Ib). Free 3´-ends at each side of the gap initiate recombination by invasion of the partner double helix (Fig.1, Ic). Repair synthesis heals the gap and creates a pair of cross-bridges – thus a double Holliday junction is formed (Fig.1, Id). The first cross-bridge

is resolved by a cut in the other strand. The remaining Holliday structure may be resolved by cleavage of the cross-bridge giving recombination of flanking markers (crossover) or retention of the parental linkage (gene conversion) (Fig.1, Ie). This mechanism is conservative, because no genetic information is lost during the recombination process. The DSBR model is used to describe the meiotic recombination, somatic intrachromosomal recombination events such as gene conversion, crossover between inverted repeats, crossover between direct repeats and the recombination events occurring between an induced genomic double-strand break and an extrachromosomal piece of DNA.

The single-strand annealing (SSA) model. The SSA model describes non-conservative events in which genetic information from one of the participating DNA molecules is lost (Fig.1, II). The double-strand breaks are induced in both recombination partners (Fig.1, IIa). After exonuclease–catalyzed digestion free single-stranded 3´-overhangs are formed (Fig.1, IIb). These overhangs anneal at a site of micro- or longer homology. If the resulting DNA molecule (Fig.1, IIc) contains non-complementary overhanging ends they are removed. Then DNA polymerase fills remaining single-stranded regions and the repair process is completed by ligation. As a result, one repaired molecule is restored out of two DNA molecules (Fig.1, IId).

The SSA model explains the extrachromosomal recombination in somatic plant cells and some intrachromosomal recombination events (recombination of closely linked chromosomal direct repeats).

The synthesis–dependent strand annealing (SDSA) model. According to this model, no stable heteroduplex DNA and Holliday junctions are formed (Fig.1, III). After induction of a DSB in the acceptor molecule (Fig.1, IIIa) 3´ single-strand overhangs

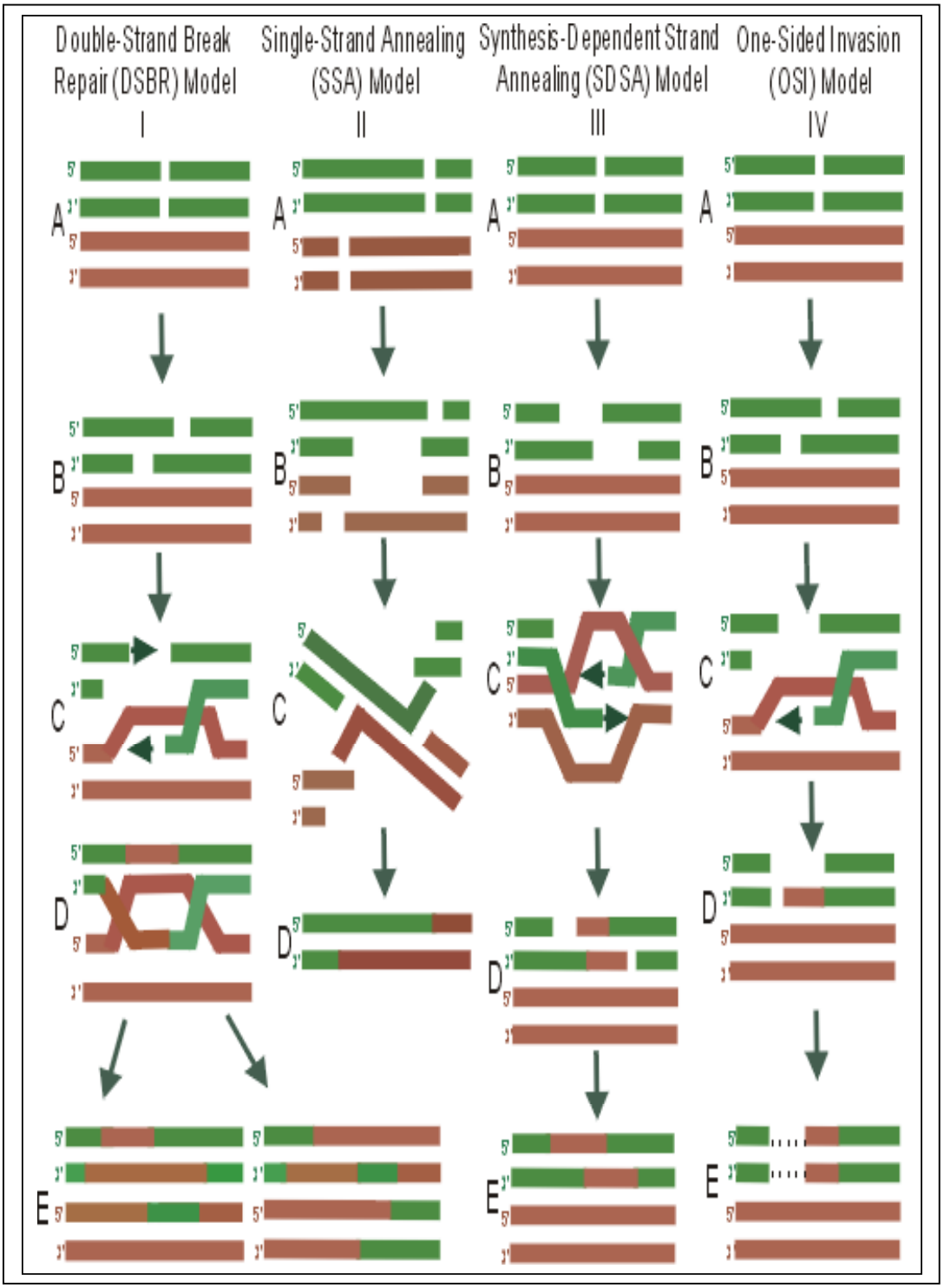


Fig. 1. Schematic representation of recombination models (Puchta and Hohn, 1996; Vergunst and Hooykaas, 1999).

are produced (Fig.1, IIIb). The ends invade independently and displace only a local loop or bubble of DNA (Fig.1, IIIc). In this bubble the association between the template and the newly synthesized strand is weak. This facilitates abortion of synthesis and template switch (Fig.1, IIId, IIIe). If a homologous template is invaded and gap repair occurs without template switch this will result in gene conversion. Such mechanism provides a good explanation for DSB repair via homologous recombination in somatic tissues.

One-sided invasion (OSI) model is used for recombination events, which are a combination of homologous and illegitimate recombination reactions (Fig.1, IV). After induction of double-strand breaks in the acceptor molecule (Fig.1, IVa) and formation of 3´single-stranded DNA ends (Fig.1, IVb), one end invades the donor sequence, a stable heteroduplex is built and repair synthesis occurs (Fig.1, IVc). Resolution can be carried out by unwinding of the newly synthesized strand or its displacement due to migration followed by ligation to the non-invading end of the recipient molecule (Fig.1, IVd). This leads to a change in genetic information – the mechanism is non-conservative. Alternatively, resolution can occur as homologous repair of the second end (Fig.1, IVe), the arising recombination product would be identical to that produced in DSBR model. However, no double Holliday junction is formed and only gene conversion products arise. DSB-induced recombination and gene targeting in somatic plant cells can be described by OSI model.

Because the SDSA model can explain recombination events resulting in homologous sequence on both sides of DNA break by gene conversion and DSBR model cannot explain events where the homology to only one side of the DSB is sufficient to initiate recombination (the DSBR model explains two-sided homology), Puchta (1998) proposes an integrative model based on the SDSA and OSI models for DSB repair in

somatic cells. After induction of a DSB in the recipient molecule, 3′-single-strand overhangs are produced via exonuclease catalyzed digestion. By forming a D-loop, a free 3′ end invades into the homologous double-stranded donor, a non-stable heteroduplex is formed and repair synthesis occurs. For the future processing of the intermediate two possible outcomes can be envisaged: if the 3′ of the recipient molecule is elongated up to the homology of the second 3′ end of the DSB and the single-strand anneals the molecule can be repaired resulting in a gene conversion without loss of information. If the elongated 3′-end of the recipient molecule does not bind complementarily at the 3′-end of the DSB, the break is closed via illegitimate recombination. This process frequently is accompanied by insertions or/and deletions (Puchta, 1998; Puchta, 1999a).

1.4. Research objectives

Double-strand breaks (DSBs) are naturally occurred lesions in genome. Their efficient repair is critical for the survival of a living organism. DSBs can be repaired via illegitimate or homologous recombination. In higher eukaryotes including plants, illegitimate recombination seems to be the main mode of the DSB repair (Puchta and Hohn, 1996; Gorbunova and Levy, 1999; Mengiste and Paczkowski, 1999; Vergunst and Hooykaas, 1999). One the other hand, the plant genome contains large amounts of repeated DNA sequences, as satellites, ribosomal RNA genes and multigene families. These sequences provide targets for homologous recombination, which could result in continuous alteration of the genome.

Alterations of the plant genome due to illegitimate and homologous recombination occur with specific frequencies for the given loci (Assaad and Signer, 1992; Swoboda

et al., 1993). These frequencies can be elevated via DSB induction in plants by external factors (e.g. UV-radiation, chemical mutagens) and by DSB induction in target loci of T-DNA recombination substrate by specific endonuclease (Haber, 1995; Puchta *et al.,* 1996; Salomon and Puchta, 1998; Puchta, 1999; Malkova *et al.*, 2000).

To date the mechanistical understanding of the recombination process in plants is just starting to emerge and little is known about the molecular basis of DSB repair process.

The aim of the current study was to set up systems for study of the DSB repair by illegitimate and homologous DNA recombination in *Arabidopsis thaliana* and to compare recombination behaviour during illegitimate DSB repair between two plant species *Arabidopsis* and tobacco.

Analysing of the molecular changes after DSB repair will provide new data about the recombination process in higher plants and its role in the genome evolution.

2. MATERIAL

2.1. Plant material

The *Arabidopsis thaliana* ecotype Colambia and ecotype C-24 were used for root transformation. In the vacuum transformation experiments Columbia ecotype was used.

2.2. Bacterial strains

Escherichia coli strains:

DH5□	recA1, endA1, gyrA96, thi-1,hsdR17, (r_K-m_K+), relA1, supE44, [u80ΔlacZΔM15, Tn10, (Tet^r)]^c ;Gibco BRL, Eggenstein (Sambrook *et al.*, 1989)
TOP 10	One shot cells; Invitrogen, Leek, NL

Agrobacterium tumefaciens strains:

C58 Cl Rf	pGV 2260 in C58C1 (Deblaere *et al.*,1985)
GV 3101 Km	PTiAch5 derivative pPM6000 (Bonnard *et al.*, 1989)

2.3. Plasmids

pGEM®-T	Amp^r (Promega)
pBNE3I	Salomon & Puchta, 1998
pCNE3I	Salomon & Puchta, 1998

pCISceI	Puchta *et al.*, 1996
$pCHN_1DC_4BI$	Puchta, unpublished
pZpp-ISceI	Puchta, unpublished
pPM6000K	Rossi *et al.*, 1993
pCIB200	Rothstein *et al.*, 1987
pGUS23	Puchta and Hohn, 1991
pNE3I	Salomon & Puchta, 1998

2.4. Primers and oligonucleotides

PCR primers

S0	5`-CCAATCCCACAAAAATCTGAGC-3`
S1	5`-GGCTCTAGAGCTTAACAGCACAGTTGC-3`
S10	5`-GCGGGAAGCTTCAGCTGACGCGTACACAAC-3`
S11	5`-GCGGGAAGCTTGAACTCGCCGTGAAGACTGG-3`
A4	5` -CGCTCTAGAGCCCCTCTTCGCCTGGTTAAC-3`
A11	5` -GACTGATTCCAGTTCGGTTG-3`
A10	5` -GCGTCTAGACATTTTCAGCCGGCAGGATA-3`
A1	5` -TTGACTCTAGAGGATCAACG-3`

Oligonucleotides for Southern hybridisation probes

4620	5`-GCAAGCTTGATGGTATCGGTGTGAGCGTCGC-3`
4619	5`-GCTCTAGAGTCCTGTAGAAACCCCAACCCGTG-3`
Npt-1	5`-ATGATTGAACAAGATGGATTG-3`
Ntp-2	5`-GAAGAACTCGTCAAGAAGCCGA-3`

DMC1-F	5`-GATTTGTCACGGTCTACTCG-3`
DMC1-R	5`-CACTACCCCCACCAAACG-3`
S13	5`-AAGTTCATTTCATTTGGAGAGG-3`

Sequencing primers

M13 uni	5`-GTAAAACGACGGCCAGT-3` (Boehringer/Mannheim)
M13 rev	5`-AACAGCTATGACCATG-3` (Boehringer/Mannheim)

2.6. DNA, enzymes and kits

Fichsperma DNA	Serva, Heidelberg
λ/Hind III - marker	Stratagene, Heidelberg
λ/Hind III/EcoR I - marker	Stratagene, Heidelberg
Smart - ladder	Eurogentec, Seraing, Belgium
Taq-DNA-Polymerase	Biomaster, Köln
ISce-I meganuclease kit	Boehringer, Mannheim
Restriction enzymes	New England Biolabs, Schwalbach; Amersham, Braunschweig; Boehringer, Mannheim; Sigma, München
T4 DNA-ligase	Amersham, Braunschweig
pGEM®-T Cloning Kit	Promega, Madison WI, USA
QIAEX Gel Extraction kit	Qiagen, Hilden
Qiagen Plasmid Isolation Kit	Qiagen, Hilden
Ribonuclease A (RNase A)	Sigma, München
Plant DNA Mini Kit	Peqlab, Erlangen

Plant DNA Midi Kit	Peqlab, Erlangen
ABI Prism ™ DNA Sequencing Kit	Applied Biosystems, Weiterstadt
EASY Pure DNA Purification Kit	Biozym, Olbendorf
DIG Wash and Block Buffer Set	Boehringer, Mannheim
TOPO TA Cloning Kit	Invitrogene, Carlsbad, USA

2.7. Chemicals

Nylon-Membrane „Hybond-N^+“, Nitro-cellulose-Membrane, X-ray film	*Amersham, Braunschweig*
Chloroform, Phenol, Phenol-Chloroform, Ammoniumperoxydisulfat (APS), Bor-acid, Cetyltrimethylammonium bromide (CTAB), Deoxynucleosidtriphosphat (dNTPs), Phenol-Chloroform-Isoamylalcohol (25:24:1), Sorbitol, Sterile filters (nylon), Formaldehyde, Glycerine, Iso-propanol, β-mercaptoethanol, Sodium chloride, Sodium-hypochlorit	*Roth, Karlsruhe*
Isopropyl-β-D-thiogalactopyranosid (IPTG)	Boehringer, Mannheim
Bacto®-Agar, Bacto®-Trypton, Yeast extract, Beef-extract, Pepton	Difco, Detroit MI, USA
5-Brom-4-chlor-3-indolyl-β-D-galactopyranosid (X-Gal), 5-Brom-4-chlor-3-indolyl-β-D-glucuronacid (X-Gluc), Indoleacetic acid (IAA), 2-isopentenyl adenin (2iP), Na-cefotaxim, Chloramphenicol,	Duchefa, Haarlem, NL

Murashige-Skoog whole medium solid substance, Rifampicin, Carbenicillin, Kanamycin, Agar, 6-Benzylaminopurin (BAP), Gentamicin sulphate	
Casein hydrolysed, 3,5-Dimethoxy-4-hydroxyaceto-phenon, N,N-Dimethyl formamid (DMF), Glucose, Potassium hydroxid, Cobalt(II) sulphate, Magnesium (II) sulphate, Antrim aside, Natrium citrate, Natrium hydroxid	Fluka, Deisenhofen
Saccharose, Tris-(hydroxymethyl)-aminomethan (Tris), Tris-(hydroxymethyl)-aminomethan-HCl (Tris-HCl)	Gibco-BRL, Eggenstein
Agarose	FMC, Rockland, USA
Whatman 3MM- filter paper	Bender&Hobein, Karlsruhe
Polaroid-Film Type 667	Polaroid, Herfordshire, GB
Calcium chloride, Zinc chloride, Zinc sulphate, Potassium jodid, Potassium nitrate, Potassium chloride, Potassium-hydrogenphosfat, Magnesium chloride, Magnesium sulphate, Na-dehydrogen phosphate	Merck, Darmstadt
Sephadex	Pharmacia, Uppsala, Sweden
Ethylenediamine tetraacetic acid (EDTA), 3-Morpholinopropansulfon acid (MOPS)	AppliChem, Gatersleben
Fe-sulphate, Ethanol, Natriummolybdat	Riedel-de Haen, Seelze
Natriumacetat, Natrium-EDTA	USB, Cleveland, OH, USA

Pyridoxin-HCl, Spermidin, Tiamin-HCl, Ethidiumbromid, Dimethylsulfoxid (DMSO), myo-Inositol, Nicotine acid	Serva, Heidelberg
Parafilm, Polyoxyethylensorbitanmonolaurat (Tween 20), Sarcosyl, Streptomycin, Triton X-100, 5-Fluorocytosin, 1-Naphtylasid acid (NAA), Ampicillin	Sigma, München
Silwet L-77	Lehle seeds, Round Rock, USA
Timentin (ticarcillin + clavulanic acid, 30:1)	SmithKline Beecham Pharmaceuticals, Philadelphia

2.8. Laboratory tools

DNA Gel-electrophoresis tanks	AGS, Heidelberg, BioRad, München
Spectrophotometer Spectronic 1201	Milton Roy, Rochester, NY, USA
Gene-Pulser™	BioRad, München
GeneAmp®PCR System 2400	Perklin Elmer, Langen
Sorvall Centrifuge RC5C	Du Pont, Bad Homburg
Thermomixer 5436 and 5437, Cold centrifuge 5402	Eppendorf, Hamburg
Hybridisation oven	Biometra, Goetingen
Shaker (Vortex Genie 2™), centrifuge	Heraeus, Osterode
Sequence apparatus	Bio-Rad, München

pH-meter Hanna-Hi9321	Schütt, Goettingen
MP-4 Camera	Polaroid, Offenbach
Weight	Sartorius, Goetingen
UV-Stratalinker® 1800	Stratagene, Heidelberg
Stereo microscope Stemi 200C	Zeiss, Jena
Microscope Photometer MPM-200	Zeiss, Oberkochen
Water bath	Haake, Karlsruhe
Sterilisator Varioclav 400	H+P Labortechnik, Oberschleißheim

2.9. Media

Bacterial media:

LB	10 g NaCl, 5 g Tryptone, 5 g Yeast extract for 1L (pH 7.4)
SOC	0.58 g NaCl, 0.186 g KCl, 20 g tryptone, 5 g yeast extract, 2 ml of 2M glucose (add after autoclaving) for 1L (pH 7.4)
YEB	0.5 g $MgSO_4 \cdot 7H_2O$, 5 g Beef extract, 5 g Peptone, 5 g Saccharose, 1 g yeast extract for 1L (pH 7.0)
NZY	5 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$, 10 g Caseine-Hydrolysate, 5 g yeast extract for 1L (pH 7.4)

All solidified bacteria media contain 1.5 % Difco-agar.

Plant growth media:

GM	0.5xMS salts, 1xB5 vitamins, 1g/l MES, 10g/l sucrose, Fe-EDTA (1862 mg EDTA + 1392 mg Fe-sulphate / 200 ml), 8 g/l agar (pH

	5.7).
CIM	B5 salts, 1xB5 vitamins, 0.05% MES, 2% glucose,1 mg/L 2.4-D, 0.2 mg/L kinetin, biotin 1 mg/l, NAA 0.1 mg/l, BAP 1 mg/l, 8g/l agar (pH 5.7)
B5-medium	B5 salts, 1xB5 vitamins, 0.05% MES, 2% glucose, 8 g/l agar (pH 5,7)
SIM-1	B5-medium + phytohormones (2iP-0.15 mg/l; IAA- 0.15 mg/l), Timentin-400 mg/l, antibiotics*
SIM-2	B5-medium + phytohormones (2iP-5 mg/l, IAA-0.15 mg/l), Timentin -375 mg/l, antibiotics
SIM-3	B5-medium + phytohormones (2iP-7.5 mg/l, IAA-0.15 mg/l), Timentin -350 mg/l, antibiotics
SIM-4	B5-medium + phytohormones (2iP-7.5 mg/l, IAA-0.45 mg/l), Timentin –300 mg/l, antibiotics
SIM-5	B5-medium + phytohormones (2iP-7.5 mg/l, IAA-0.45 mg/l, GA_3-0.5 mg/l), Timentin -275 mg/l, antibiotics
SIM-6	B5-medium + phytohormones (2iP-5 mg/l, IAA-0.45 mg/l, zeatin-1.0 mg/l), Timentin -250 mg/l, antibiotics
SIM-7	B5-medium + phytohormones (2iP-10 mg/l, IAA-0.5 mg/l, ABA-0.5 mg/l) Timentin - 250 mg/l, antibiotic
SIM-8	B5-medium + phytohormones (2iP-5 mg/l, IAA-0.5 mg/l, thidiazuron-5 mg/l), Timentin -250 mg/l, antibiotics
SIM-9	B5-medium + phytohormones (2iP-5 mg/l, IAA-0.5 mg/l, thidiazuron-10 mg/l), Timentin -125 mg/l, antibiotics
SIM-10	B5-medium + phytohormones (2iP-5 mg/l, IAA-0.5 mg/l, GA_3-1 mg/l, zeatin-2 mg/l), Timentin -125 mg/l, antibiotics

SM	0.5xMS salts, 1xB5 vitamins, 8 g/l agar, antibiotics (pH 5.7)
LGM	0.5xMS salts, 1xB5 vitamins, 1 g/l MES, 30 g/l sucrose (pH 5.7)
IF	0.5xMS salts, 1xB5 vitamins, 0,44 μM BAP, 50 g/l sucrose, 0.5% Silwet L-77, 100 mg/l 3,5-Dimethoxy-4-hydroxyacetophenon (pH 5.7)

*Antibiotics: in correspondent experiments following antibiotics and other agents were used:

Kanamycin	25-50 mg/l
Gentamycin	100-200 mg/l
FC (5-Fluorocytosin)	50-200 mg/l
Hygromycin	25-40 mg/l
Streptomycin	10 mg/l
Ampicillin	50 mg/l
ppt (phosphyno-thricin)	3-8 mg/l
Rifampicin	50 mg/l
Timentin (ticarcillin + clavulanic acid, 30:1)	125-400 mg/l

2.10. Software

DNA and protein sequence data were processed using the programs “DNA-star” and ”BLAST” (Basic Local Alignment Search Tool (Altschul *et al.*, 1990).

3. METHODS

3.1. Plant growth

After planting, *Arabidopsis* plants were put in dark cold room for at least 2 days of vernalisation period. Plants were grown in a growth room at 22°C with 6000 lux of white light for 8 hours (short day) during 3 weeks. Then they were transfered at the long day condition (16 hours of white light / 8 hours dark). Seeds were collected from yellow coloured siliques (~22 DAP) before their opening. For transformation in planta 6 weeks old *Arabidopsis* plants were used. Crosses were carried out with 5-6 weeks old plants.

3.2. Seeds sterilisation

The seeds were put in an eppendorf tubes. 1 ml of 70% Ethanol was added for 1-2 minutes incubation. Then ethanol was taken out and 1 ml 4% Na-hypochlorid solution was added. After 10 minutes incubation the eppendorf tubes with *Arabidopsis* seeds were centrifuged (6000 rpm, 7-10 seconds) and the solution was poured out. Washing (4 times) contain three steps: add 1 ml sterile distillate water, incubation during 5 minutes and centrifugation (6000 rpm, 5 seconds). For last step, the seeds were re-suspended in 0.5 ml sterile, but normal water-pipe water. Sterilised seeds were germinated and cultured in SM or GM.

3.3. Crosses

All crosses were made as described in Koornneef *et al.*, 1992.

3.4. Segregation analysis

The method of the genetic segregation analysis allows to determinate the number of genes that segregate and to identify homozygous plants for future experiments (Koornneef *et al.*, 1992). The T_1 (first progeny from transgenic plants) and F_1 (first progeny plants after crosses) generation seeds were planted and their progeny (T_2 or F_2) were harvested individually. The seeds were sterilised and put onto petry dishes with selection medium. Segregating lines that showed monogenic inheritance (3:1) were selected. Homozygous individuals (T_3 or F_3) who did not segregate on selection medium were chosen for the molecular analysis.

3.4. PCR analysis

Genomic DNA was analysed by PCR using the primers SO and A1 (Uni) in a first amplification and later, nested primers pairs S10, S11, A4, A10 were used as described (Salomon and Puchta, 1998).

3.5. Basic cloning methods and sequencing

The standard molecular cloning methods (restriction digestion, ligation, DNA gel electrophoresis) were performed according to Sambrook *et al.*, 1989.

The transformation of *E. coli* was performed using the heat-shock procedure (Cohen *et al.*, 1972) or by electroporation (Inoue *et al.*, 1990). Plasmid DNA extraction and

purification was done using the Qiagene Plasmid Kit or according to the fast preparation method described by Holmes and Quigley (1981). PCR products were cloned either into pGem®-T Vector System (Promega) or the PCR-2.1-TOPO vector using TOPO TA Cloning Kit (Introgen, Carlsbad, USA) and propagated in DH 5α or TOP 10 One Shot Cells (Introgene, Carlsbad, USA) according to manufacturer's instructions. DNA sequences were determined by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). DNA was detected using fluorescent-labelled primers (standard M13 – 20 Forward, M13 Reverse primers) by the A. L. F. Sequencer (Pharmacia LKB) and the Autoread Sequencing Kit (Pharmacia).

3.6. Transformation of Agrobacterium tumefaciens

Transformation of *Agrobacterium tumefaciens* was performed using the electroporation procedure. For electroporation, competent cells of the Agrobacterium (C58 strain) were incubated in 20 ml YEB medium (50 mg/l rifampicin) 18 h at 28°C. Bacterial suspension was centrifuged 20 min, 6000 rpm, 4°C and the pellet was re-suspended in 10% glycerine. The centrifugation and re-suspension procedures were repeated two times. After all the pellet was dissolved in 400 ml of 10% glycerine. The 50 µl of competent cells suspension was poured out in to a 1.5 ml eppendorf tubes each and placed in -80°C refrigerator.

5 µl DNA (about 10 ng) and 50 µl Agrobacterium competent cells were mixed on the ice in the electroporation cuvete. The electroporation was carried out using 2500 V, 25 µF, 200 Ω, 5 m/sec impulse conditions. Then 1 ml SOC medium was added to the cuvete, mixed and placed onto petry dishes with YEB medium + selective agent. The dishes were put in the 28°C thermostat. After 24 h incubation the growing colonies

were transfered onto the new medium. Later the colonies were put in 4°C conditions for storage.

3.7. Agrobacterium tumefaciens-mediated transformation of Arabidopsis thaliana via root explants

Seeds of *Arabidopsis thaliana* were sterilised and germinated and cultured in germination medium (GM). The roots were excised from the plants, placed onto sterile glass petry dish and cut into 1-2 cm pieces with a scalpel. The explants were placed on callus induction medium (CIM) for four days. The *Arabidopsis* transformation procedure was performed as described by Valvekens *et al.* (1992) with modifications. Roots in Agrobacterium solution were exposed to a soft vacuum infiltration for 5 minutes. After this roots were placed onto CIM-medium without selective antibiotics. Two days later the roots were shifted to SIM-1 medium. Callus induced on SIM-1, was subcultured on the SIM-2 – SIM-15 for each 10-14 days. Regenerated from the green callus shoots were transferred to the GM medium for root induction in the presence of corresponding selective agents. The seeds were collected and T2 progeny was used for PCR, Southern analysis and for selecting plants homozygous for the transgene.

3.8. In planta transformation of Arabidopsis thaliana by vacuum infiltration

Transformation of *Arabidopsis* was performed based on the protocol of Bechtold *et al.* (1993). Plants of *Arabidopsis thaliana* (ecotype Columbia) were grown for three weeks under the short day conditions (8 hours light, 16 hours dark) and transferred to

long day (16 hours light, 8 hours dark). After three weeks the emerging bolts were cut to induce growth of multiple secondary bolts. Vacuum infiltration of plants with the solution of *A. tumefaciens* was done one week after clipping. Bacteria were grown till OD_{600}>2.0, harvested by centrifugation and re-suspended in three volumes of infiltration medium (IFM) (OD_{600} approx. 0.8). Entire plants were submerged into the *A. tumefaciens* suspension in beaker. Vacuum was applied by oil pump for 5 min and then rapidly released. Plants were removed from the beaker, placed on their side and kept at high humidity under plastic wrap for 24 hours, after that they were uncovered and set upright. Seeds were harvested from the dry siliques, sterilised (as described in 3.3) and plated onto SM (selection medium) plates with corresponding selective agent. After two - three weeks resistant plants were transferred in soil, grown up and their seeds were collected for genetic and molecular analysis.

3.9. Plant DNA extraction

DNA extraction from flowers, leaf tissues and calli was carried out as described by Thompson *et al.* (1983) or performed according to Plant DNA mini and Plant DNA midi isolation kits (Peqlab, Erlangen) instructions.

3.10. Southern analysis

DNA was digested with restriction enzymes, separated on a 0,8% gel in Tris-borate buffer (Sambrook *et al.*, 1989) and transferred onto a Hybond-N+ membrane (Amerscham, Braunschweig). Southern DIG non-radioactive system blot analysis was performed using Wash and Block buffer set, PCR Dig probe synthesis kit and DIG

Nucleic acid detection kit from Boehringer, Mannheim according to the manufacturer's instructions.

The digoxigenin-labeled probes were prepared using digoxigenin-11-dUTP (DIG-dUTP), which was incorporated by Taq DNA polymerase during the polymerase chain reactions. The following primers were used for different probes synthesis and labelling:

Probe for hybridisation	Template for amplification	Primers
Km-specific	pCIB 200	Ntp-1, Ntp-2
GUS-specific	pGUS 23	4619, 4620
codA-specific	pNE3I	S13, A10
DMC1-specific	pZpp-ISceI	DMC1-F, DMC1-R

3.11. Destructive GUS assay

10 days old *Arabidopsis thaliana* seedlings raised in sterile conditions were used for destructive GUS-assay. This method was carried out as described by Rossi *et al.* (1993). The blue sectors present on the plantlets were counted using a binocular.

3.12. Non-destructive GUS-assay

In order to detect and to analyse recombination events in vivo non-destructive GUS assay was used.

The Arabidopsis plantlets grew for 7-10 days onto petry dishes with GM. 4 ml 2.5 % X-Glu-potassium buffer (pH 7) was added in every dish. After overnight incubation under normal growth conditions (20° C - 22° C, 6000 Lux of white light for 16h) the plantlets with blue roots were screened. This method was performed according to Martin *et al.* (1992).

3.13. Restriction analysis with I-SceI enzyme

The restriction analysis with meganuclease I-SceI (Boehringer, Mannheim) was carried out on genomic DNA and on PCR-fragments. The I-SceI recognises the 18 bp sequence 5´-TAG GGA TAA / GAG GGT AAT-3´. For analysis 2-5 µg DNA were taken up in 1-2 µl incubation buffer (10x concentration) (I-SceI meganuclease kit, Boehringer, Mannheim). The procedure was performed according to manufacturer‘s instructions. The digestion was analysed by gel electrophoresis (0.8% agarose gel).

4. RESULTS

4.1. Study of double-strand break repair by illegitimate recombination in *Arabidopsis thaliana*

4.1.1. Set up of a system for induction and detection of the DSB repair by illegitimate recombination

To characterise illegitimate DSB repair in *A. thaliana* and compare whether there are differences in recombination behaviour between plants with smaller (*Arabidopsis*) and larger (tobacco) genomes the experimental system was set up. *Arabidopsis* plants were transformed with either binary vector pBNE3I or pCNE3I (Salomon and Puchta, 1998). The constructs contain an I-SceI site between the 35S promoter and coding region of the negative selectable marker gene cytosine deaminase (*codA*; Stougaard, 1993), (Salomon and Puchta, 1998).

Transgenic plants with single copy insertions were identified by segregation analysis on kanamycin selection medium and by Southern blotting. For DSBs induction the second (transient) transformation of transgenic plants (roots) with I-SceI open reading frame (ORF) was performed. The cutting of genomic I-SceI site would induce repair process (Fig.2). The cells in which repair does not result in restoration of the functional *codA* gene can be selected after addition of 5-FC in the cultivation medium. The cells expressing the functional gene convert 5-FC into the highly toxic compound 5-fluorouracil. Only cells lacking enzyme activity will grow and can be propagated (Stougaard, 1993). Kanamycin selection was applied simultaneously.

After DNA extraction from resistant calli, recombination junctions were amplified by PCR. Obtained PCR products with reduced size were cloned and sequenced.

4.1.1.1. Production of transgenic plants for BNE3I and CNE3I and their genetic and molecular characterisation

Arabidopsis plants of Columbia and C-24 ecotypes were used for *in planta* transformation by the pBNE3I and pCNE3I vector constructs.

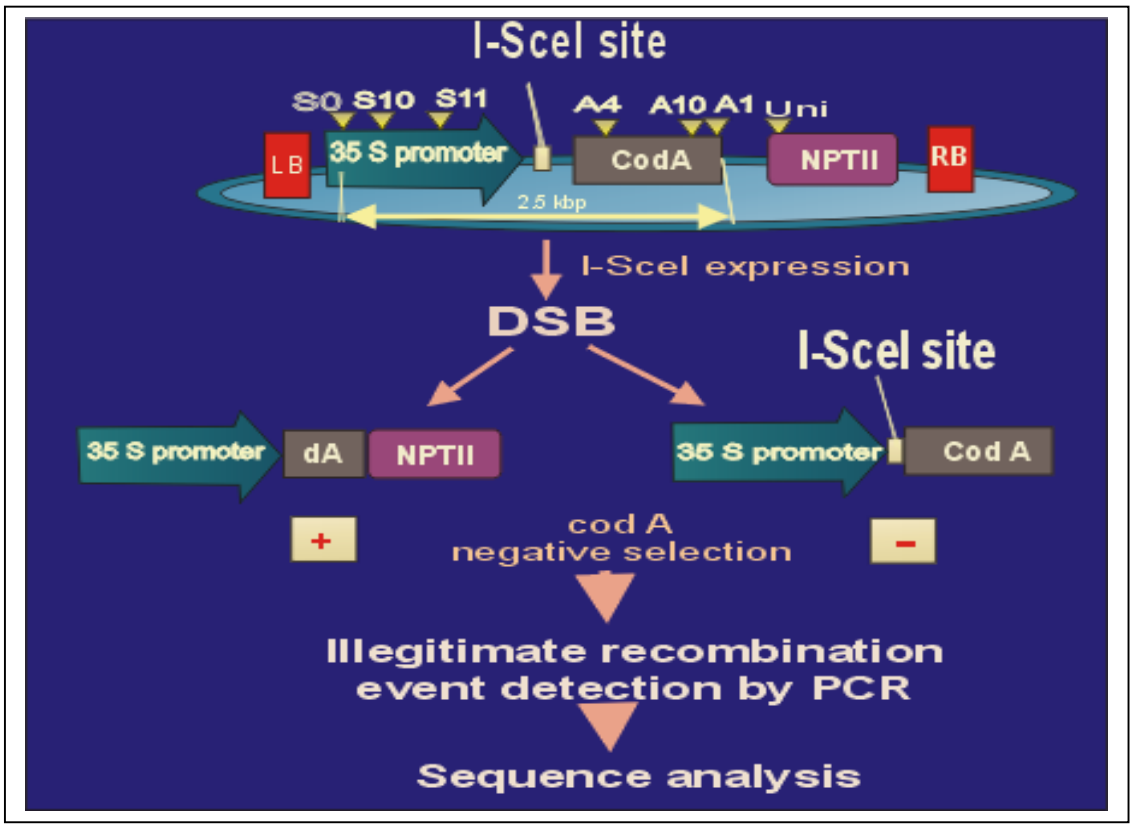


Fig. 2. Set up of the system for induction and detection of the DSBs repair by illegitimate recombination. On the top of the figure is a schematic map of the T-DNA from the binary plasmid pBNE3I inserted into the plant genome. An I-SceI site is integrated between the *codA* ORF and 35S promoter. T-DNA contains a kanamycin resistance gene (npt). S0, S1, S10, S11, A1, A4 and A10 indicate primer-binding sites for PCR amplification of recombined junctions. The arrangement of genes on the T-DNA of the binary vector pCNE3I is identical to pBNE3I; RB, right border; LB, left border.

Both binary vectors carry an identical arrangement, *codA* and a kanamycin-resistant gene (Fig. 2). The pBNE3I was constructed on the basis of binary vector pBin19

(Bevan, 1984) and pCNE3I – on the basis of the binary vector pCIB200 (Rothstein *et al.*, 1987).

115 plants were transformed: 81 by the pCNE3I and 34 by the pBNE3I constructs. The seeds were collected from transformed plants, sterilised and put on GM containing petry dishes with kanamycin (50mg/l) as a selectable marker (positive selection). The transformation efficiency was between 0.2% and 0.37%. Transformation results are presented in Table 1.

35 BNE3I and 207 CNE3I transgenic lines were obtained after *in planta* transformation. Transgenic plants with single copy insertions were identified by segregation analysis and Southern blotting. Five BNE3I and twenty-three CNE3I transgenic lines showed 3:1 (one copy T-DNA) segregation.

Table 1. Transformation efficiency for CNE3I and BNE3I lines

Transformed plants 1 1 5	Seeds tested	Transgenic lines	Efficiency of transfor-mation, %	Lines with 3:1 kanamycin segregation
BNE3I (Col-0) 34	17600	35	0,2	5
CNE3I (C-24) 81	69400	257	0,37	23

Genomic DNA of transgenic lines that showed 3:1 segregation was isolated for Southern blot analysis. Digestion of DNA from BNE3I and CNE3I transgenic lines

was performed with EcoRI.-restriction endonuclease. Hybridisation with the kanamycin-specific probe was performed.

Three BNE3I transgenic lines and 19 CNE3I transgenic lines containing one copy of T-DNA were detected by Southern blot analysis. To avoid misinterpretations that might be due to potential genomic position effects several transgenic lines were included in following experiments: B1, B2 lines for BNE3I and C1, C2, C3, C4, C5 and C6 lines for CNE3I (Kirik *et al.*, 2000) (Fig. 3).

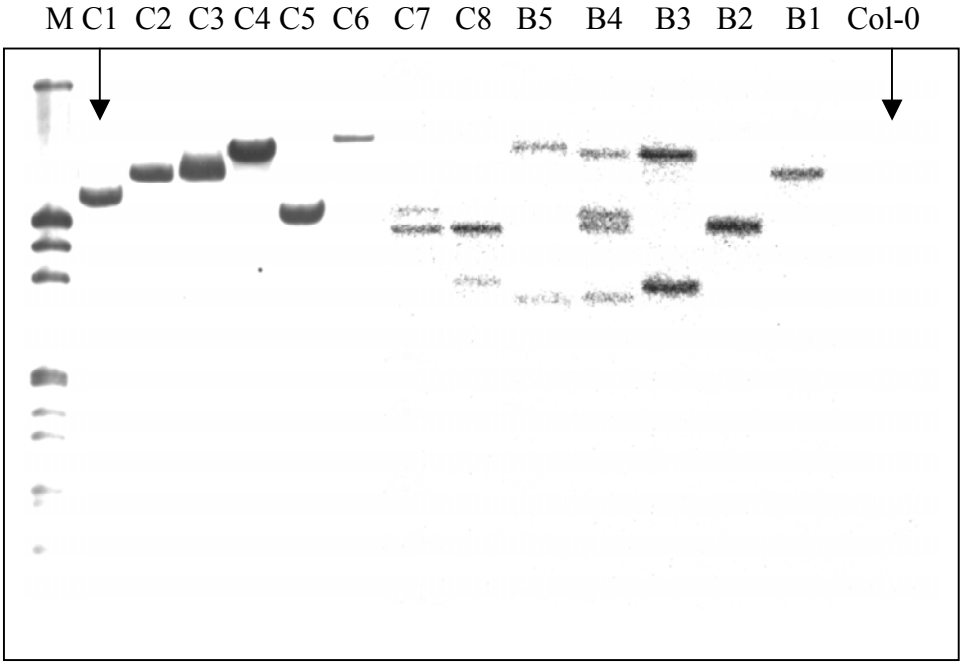


Fig. 3. Southern blot analysis of transgenic lines containing CNE3I and BNE3I for detection of lines with one copy of T-DNA. DNA was digested by EcoRI and hybridised with a kanamycin-specific probe. Lines C1, C2, C3, C4, C5, C6 and B1, B2 were selected. M – λ DNA digested with HindIII and EcoRI.

4.1.1.2. In vivo induction of DNA DSBs and selection of recombination events

Eight different *Arabidopsis* lines containing a single T-DNA copy of either the pBNE3I binary vector (lines B1, B2) or pCNE3I binary vector (lines C1, C2, C3, C4, C5, C6) were chosen for further investigatios.

Before performing the *in vivo* induction of DSBs, the functionality of I-SceI site in transgenic lines was checked. For this test Southern blot analysis was performed. Genomic DNA from C1, C2, C3, C4, C5, C6 and B1, B2 transgenic plants were cut with HindIII/I-SceI and with HindIII enzymes respectively and hybridised with *codA*-specific probe. Southern analysis demonstrated, that all eight transgenic lines had functional I-SceI sites, which were cut by exogenic I-SceI enzyme (Fig. 4). After this test, root transformation with the I-SceI expression cassette was carried out for the *in vivo* induction of DNA double-strand breaks.

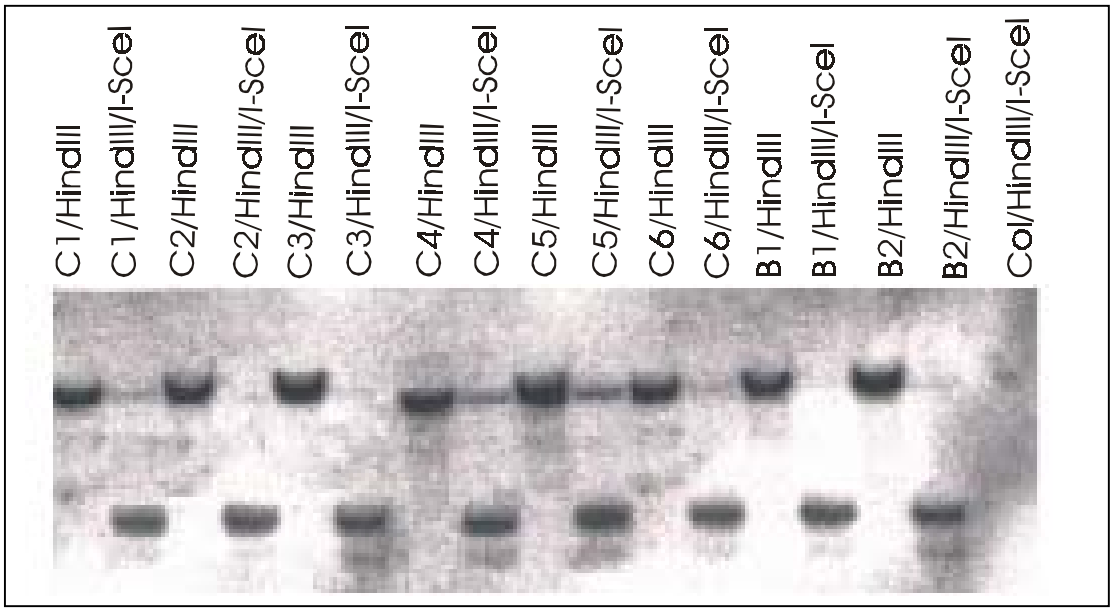


Fig. 4. Southern blot analysis to test the functionality of the I-SceI site in the selected transgenic lines. Plant DNA was digested with HindIII and HindIII/I-SceI and probed with a *codA*-specific probe.

Roots of the homozygous transgenic plants were inoculated with *Agrobacterium* strain containing the binary pCISceI vector. This vector carries a plant expression cassette for the *I-SceI* gene (Puchta *et al.*, 1996) but no selectable marker gene. Thus, no selection for the integration of this T-DNA can be applied and the T-DNA may integrate or be lost in transformed cells after DSB induction.

After three days of co-cultivation the roots were transferred from calli induction medium (CIM) to shoot induction medium (SIM) plates containing 200 mg/l 5-FC and 50 mg/l kanamycin. Every 10-14 days tissue culture were transferred to fresh SIM medium. Double-resistant calli in which function of the *codA* gene was lost could be detected after 5-6 weeks of cultivation (Fig. 5).

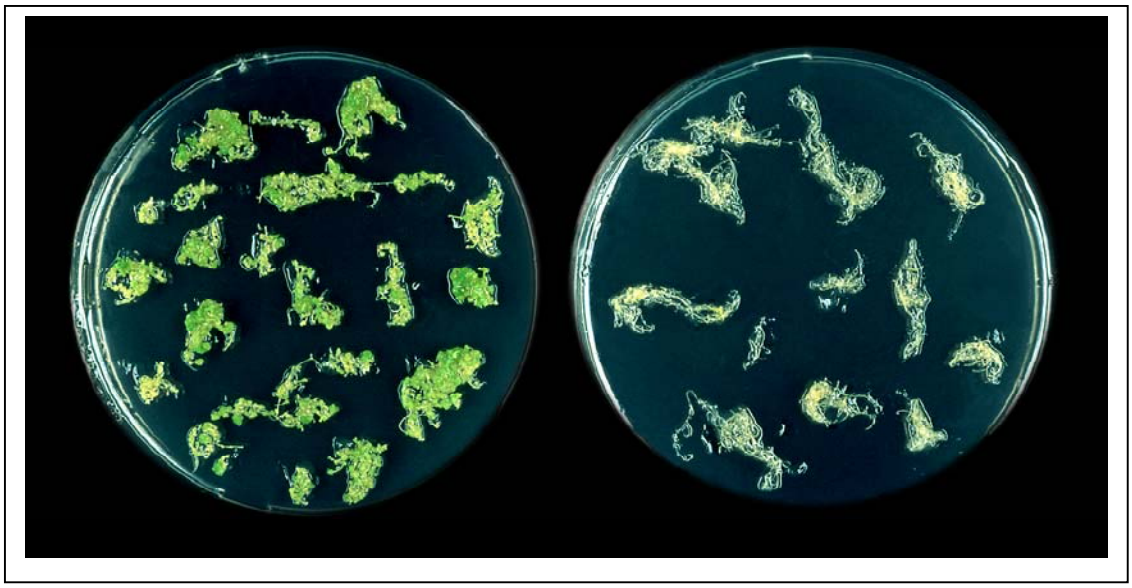


Fig. 5. codA negative selection. 1. Double-resistant calli which lost the function of the *codA* were propagated on medium with 150 mg/L 5-FC and 50 mg/L Km; 2. Control plate contains transgenic plant material in which no DSBs induction was performed.

Small *Arabidopsis* calli size (1-3mm) and their high concentration on the roots hampered individual calli detection. Therefore only regenerated calli were taken for analysis.

4.1.2. Detection of recombination products after DSB repair

4.1.2.1. PCR-analysis of DNA from kanamycin and fluorocytosin resistant calli

After of *in vivo* induction DSBs and repair process 1432 kanamycin and fluorocytosin resistant calli were selected. DNA was extracted from 300 regenerating calli. PCR amplification was performed using two or three consecutive nested PCR. The first PCR reaction (50 cycles) was carried out with S0-A1 primer pair. This amplification resulted in ´´fragment-library´´. To detect repair events in which the I-SceI site was destroyed I-SceI restriction analysis of PCR fragments was performed. DNA that was not cut was taken for second PCR amplification. The S10-A10 or S11-A4 primer pairs were used for the second PCR (30 cycles). Deletions in range from 300 bp to 2200 bp can be detected by second PCR.

Not every DNA sample isolated from double resistant calli resulted in PCR fragments containing deletions or insertions. Since the oligonucleotides used as PCR primers bind within a 2.3 kb region of the T-DNA (Fig. 2), deletions involving at least one primer binding site, major genomic rearrangements or so small deletions might account for this fact. 173 DNA samples from 321 analysed calli (46%) did not show any deletions or insertions. This can be explained by not sufficient 5-FC concentration for selection when some calli survived in spite of an intact *codA* gene or by low stability of 5-FC in medium.

Table 2. Compilation of deleted transgenic junctions in *Arabidopsis thaliana*

N	Callus	Insertion	Deletion (total)	Region of deletion 35S promoter	codA	Homology at junction (bp)
1	C2-607	-	212	-	212	2
2	C6-179	-	233	233	-	1
3	C5-241	-	240	-	240	-
4	B1-28	-	247	247	-	1
5	C2-181	-	248	-	248	1
6	C6-210	-	249	-	249	-
7	C6-916	-	860	860	-	2
8	B1-230	-	929	144	785	-
9	C5-150	-	967	277	690	2
10	C6-09	-	1110	1048	62	1
11	C1-181	-	1122	39	1083	2
12	B1-150	-	1164	217	927	2
13	B2-150	-	1207	279	928	3
14	C2-10	-	1235	273	926	-
15	B1-99	-	1256	40	1216	1
16	C4-179	-	1281	-	1281	1
17	C3-179	-	1493	840	653	-
18	B1-50	-	1497	1057	440	3
19	C3-17	-	1498	840	658	1
20	C6-111	-	1499	1055	446	2
21	B2-7	-	1501	1055	448	3
22	B1-286	-	1503	1056	447	3
23	C2-108	-	1525	929	596	1
24	C1-30	-	1539	1129	410	2
25	C2-15	-	1545	888	657	3
26	C6-215	-	1553	1027	526	2
27	C2-2	-	1556	407	1149	2
28	C2-29	-	1588	979	609	-
29	C3-10	-	1637	1194	443	2
30	C1-20	-	1647	651	996	1
31	C3-12	-	1658	659	999	2
32	C1-21	-	1740	654	1086	-
33	C3-13	-	1889	893	996	2
34	C5-241	-	1915	934	981	3
35	C2-5	-	1916	922	988	3
36	C2-812	-	1958	945	1013	3
37	C2-18	-	2025	1088	937	2
38	C1-18	-	2093	1064	1029	3
39	C1-185	-	2095	1070	1025	6
40	B2-1	-	2207	1195	1012	-

4.1.2.2. Sequence analysis of PCR amplificates

Forty PCR fragments that showed negative I-SceI restriction results and were reduced in size, were cloned in pGEM-T or TOPO plasmid vector and sequenced. As shown in the Table 2 forty deletions between 200 and 2200 bp were determined by sequence analysis.

A class of deletions between 1400 to 2300 bp was detected in the most cases. No deletions were associated with insertion sequences. Three lines: C6-179, B1-28, C6-916 had deletions only within 35S promoter region and five lines: C2-607, C5-241, C2-181, C6-210, C4-179 had deletions only within the *codA* gene region.

Most of recombination junctions (57.5%) showed small patches of homologous nucleotides (two or more) between the rejoined strands of DNA.

4.1.2.3. Classification of recombination junctions

All detected recombination junctions can be divided into three classes:

1. junctions with deletion only within the 35S promoter region;
2. junctions with deletion only within the *codA* gene region;
3. junctions with deletions within both the 35S promoter and *codA* gene regions.

Junctions with deletion in the 35S promoter region were obtained in three cases. All this junctions had deletions, which were accompanied by disruption of the I-SceI site (Fig. 6).

Junctions with deletion in the *codA* gene region. Five such junctions were obtained. In two cases the I-SceI site was completely lost (Fig. 7). In three cases only 5 nucleotides of the I-SceI site were deleted together with a part of *codA* gene(Fig. 8).

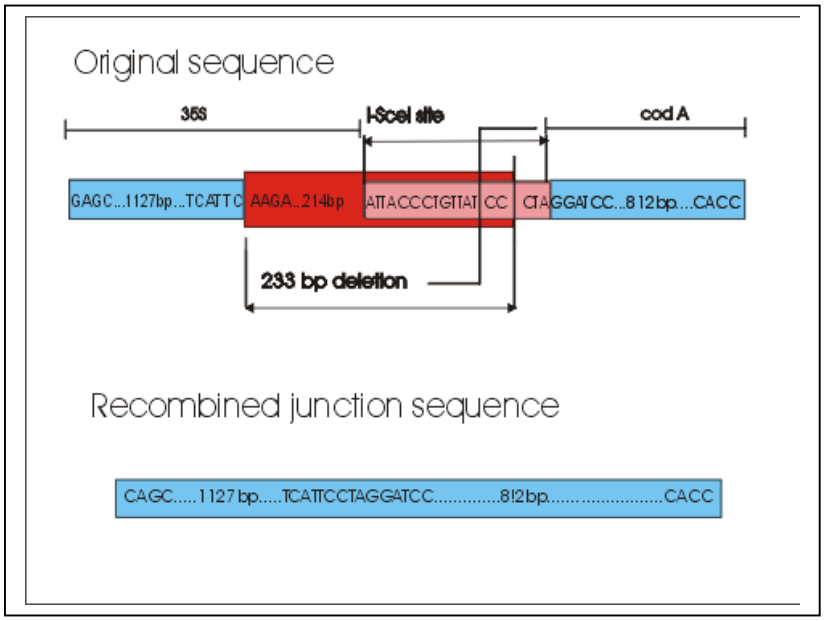


Fig. 6. C6-179 recombination junction. Deletion is only within the 35S promoter region. The deletion (233 bp) is shown in red colour. I-SceI site (18 bp) is rose; staggered vertical line indicates the site of I-SceI digestion.

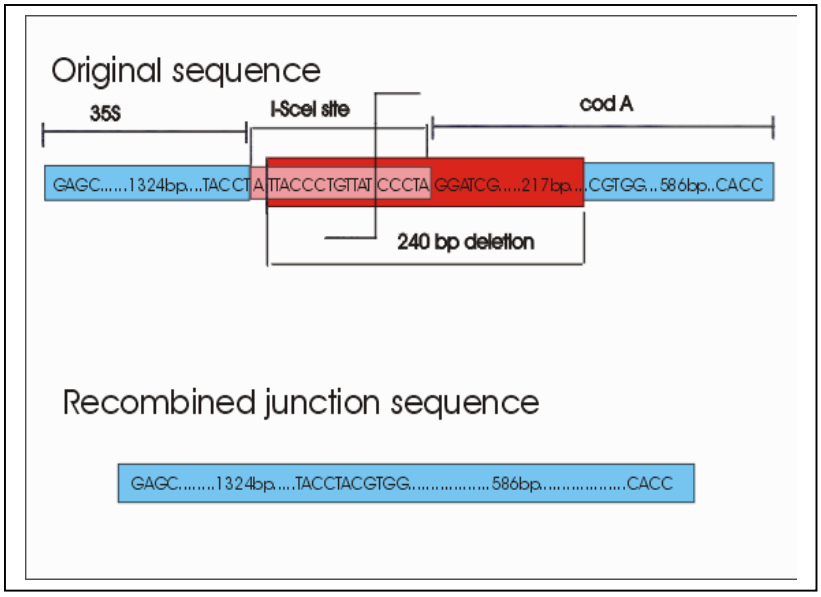


Fig. 7. C5-241 recombination junction. Deletion is within the *codA* gene. Red colour marks deletion (240 bp); the I-SceI site (18 bp) is shown in rose. Due to DSB repair process the I-SceI site is completely lost.

Junctions with deletions within both the 35S promoter and *codA* gene regions. The absolutely majority (80%) of recombination junctions had deletions within both the 35S promoter and *codA* regions (Fig. 9).

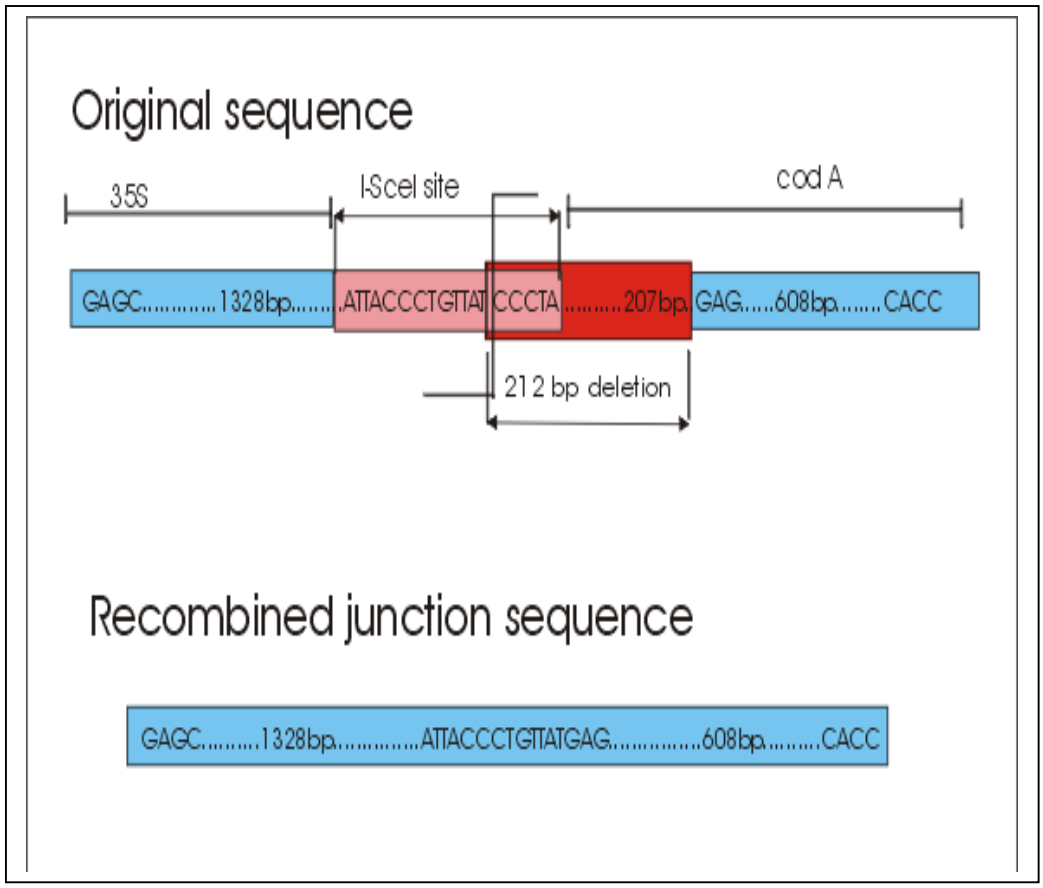


Fig. 8. C2-607 recombination junction. In the upper part the I-SceI site (rose) is shown. The staggered line indicates the site of I-SceI digestion. The I-SceI site was rejoined in place of digestion.

Junctions with small homologies between recombined DNA strands were obtained in 1,35 times more often that without (Table 2).

For eukaryotes two pathways of illegitimate recombination were postulated (Gorbunova and Levy, 1997). Junctions without homologies were explained by simple

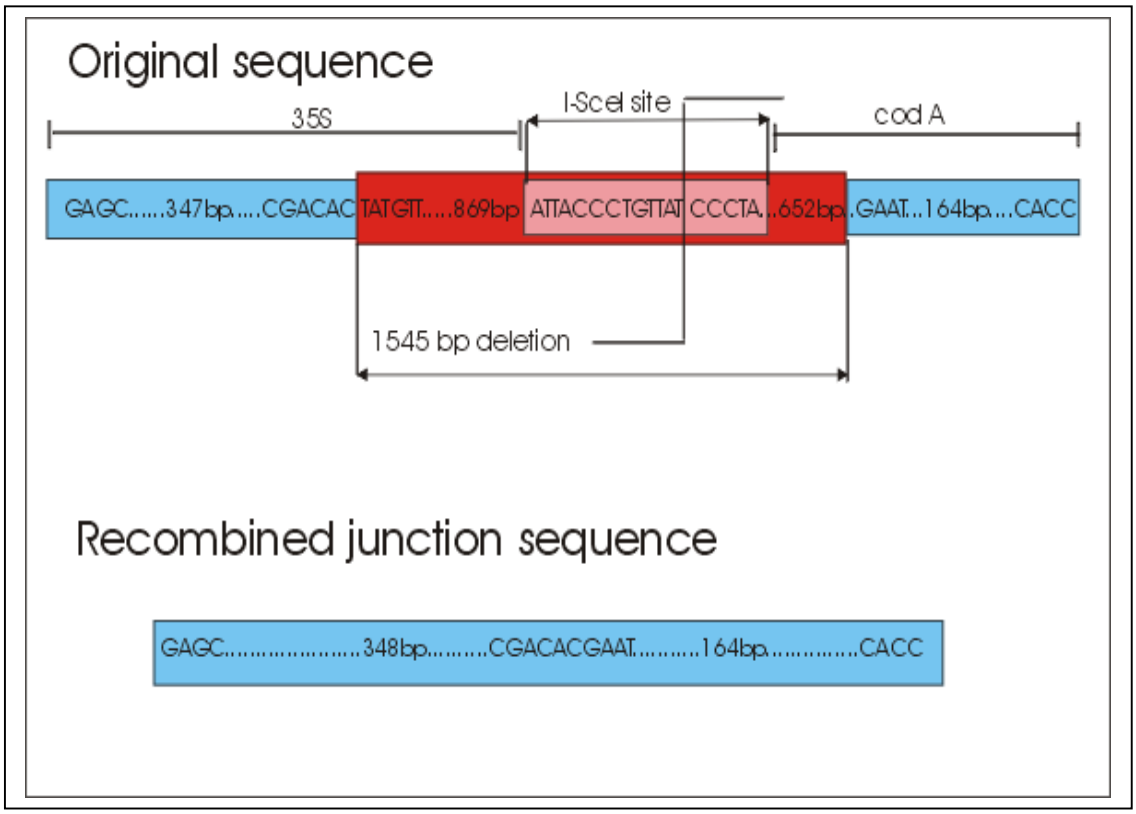


Fig. 9. C2-15 recombination junction. Deletion (1545 bp) was detected within both the 35S promoter and *codA* gene region (red colour). 18 bp of the I-SceI site is shown in rose.

ligations, whereas small patches of homologous nucleotides (two or more) within junctions were considered to be a prerequisite for the operation of a single-strand annealing mechanism (Nicolas *et al.*, 1995).

4.1.3. Comparison of double-strand break repair in *Arabidopsis* and tobacco

The deletion formation via DSB repair in somatic cells was compared between tobacco and *Arabidopsis*, two dicotyledonous plants species with more than 20-fold difference in genome size (Bennett and Leitch, 1997).

As shown in Tables 2 and 3 (Table 3 shows data from Salomon and Puchta, unpublished, and 10 junctions of tobacco described before in Salomon and Puchta, 1998), 40 deletions between 200 and 2300 bp were detected for each plant species. For tobacco 1,5 times more junctions were found with small homologies than without. The similar ratio was determined for *Arabidopsis* (Fig. 10). Both species do not differ significantly (p>0,8 in a goodness of fit test) as to the occurrence of the two junction classes.

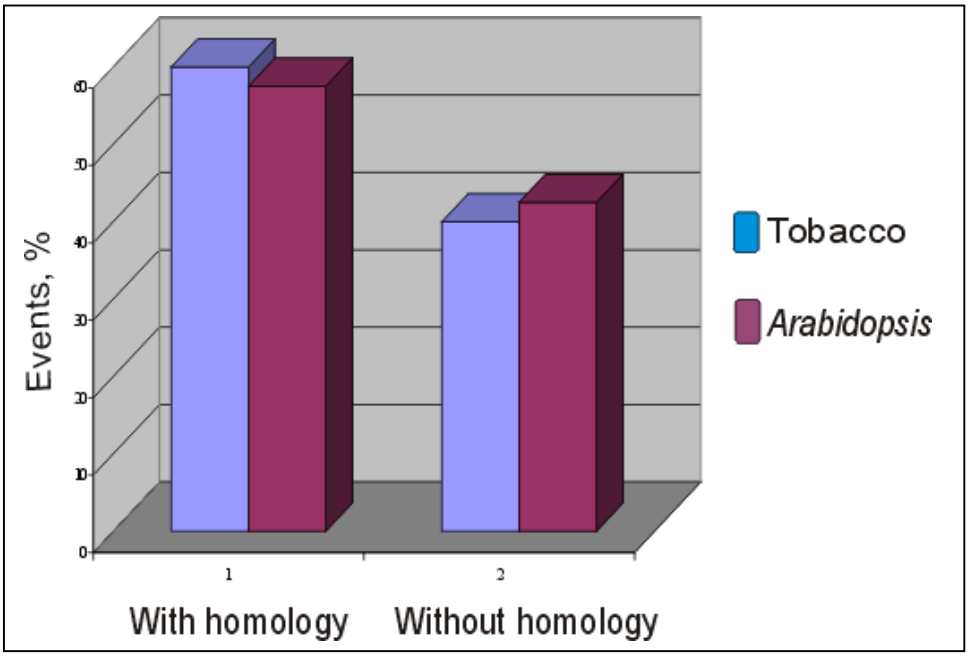


Fig. 10. Comparison of the molecular properties of sequenced recombination junctions in tobacco and *Arabidopsis*. Involvement of homology in junction formation during the process of DSB repair.

However, in two other aspects significant differences were detected. In 40% of the cases the linkage of DNA ends in tobacco was associated with insertion of filler sequences, whereas in *Arabidopsis* such events were not detected (Fig. 11). This represent a dramatic difference between the two species (p>0,0005).

Table 3. Compilation of deleted transgenic junctions in tobacco

N	Callus	Insertion	Deletion (total)	Region of deletion 35S promoter	codA	Homology at junction
1	B9-73	-	222	1	221	1
2	C15-71	-	236	236	-	-
3	B9-48	-	236	236	-	-
4	B9-842	-	257	170	87	1
5	C15-51	19	262	227	35	n.t.
6	B9-74	-	290	2	288	1
7	C15-67	-	305	260	45	3
8	B9-67	58	309	-	309	n.t.
9	B9-50	32	395	290	105	n.t.
10	B9-46	121	458	439	19	n.t.
11	C15-471	98	549	539	10	n.t.
12	C15-18	-	549	533	16	5
13	C15-39	-	609	13	596	5
14	B9-300	-	661	659	2	1
15	B9-87	-	736	-	736	1
16	B9-80	-	783	781	2	3
17	C15-59	-	822	518	304	8
18	C15-19	-	862	14	848	4
19	B9-69	25	901	877	24	n.t.
20	B9-76	-	984	984	-	-
21	C19-11	-	1055	1047	8	2
22	C15-44	-	1055	1047	8	2
23	B9-300	-	1060	1057	3	2
24	B9-16	-	1097	-	1097	3
25	B9-842	-	1122	1100	22	2
26	B9-561	6	1144	1144	6	n.t.
27	C15-29	13	1152	-	1152	n.t.
28	B9-21	-	1173	1136	37	4
29	B9-8	-	1175	1142	33	1
30	C19-5	-	1218	390	828	4
31	B9-33	11	1223	1223	-	n.t.
32	C15-12	63	1236	1223	13	n.t.
33	B9-17	76	1255	1.239	16	n.t.
34	B9-841	-	1275	1231	44	1
35	B9-88	5	1287	1281	6	n.t.
36	C15-193	4	1294	1282	12	n.t.
37	C15-22	13	1322	1294	30	n.t.
38	C15-191	-	1912	921	991	3
39	C15-192	-	2023	1319	704	4
40	B9-58	88	2294	1197	1097	n.t.

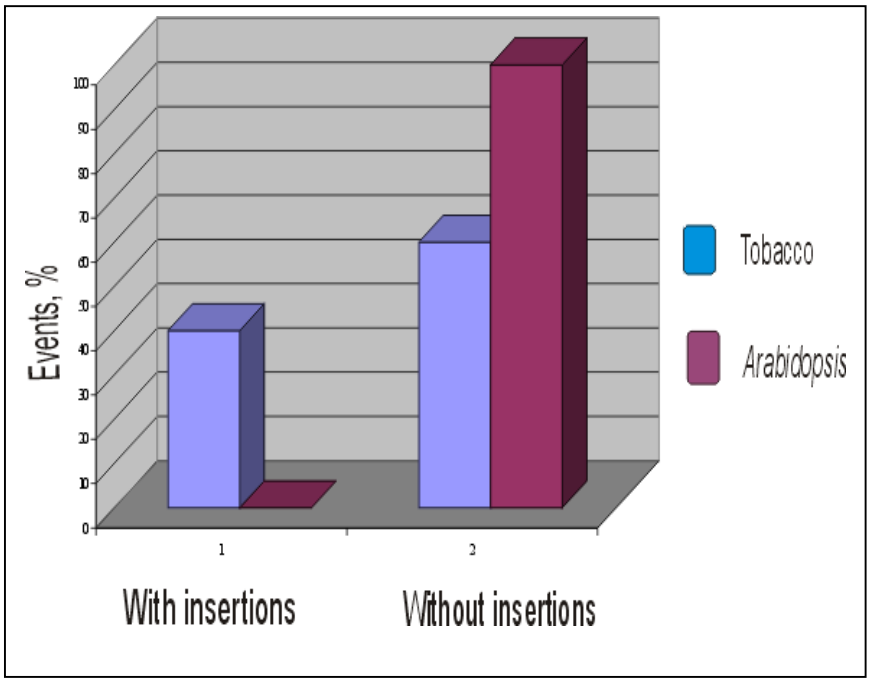


Fig. 11. Comparison of the molecular properties of sequenced recombination junctions in tobacco and *Arabidopsis*. Inclusion of filler sequences into newly formed junctions.

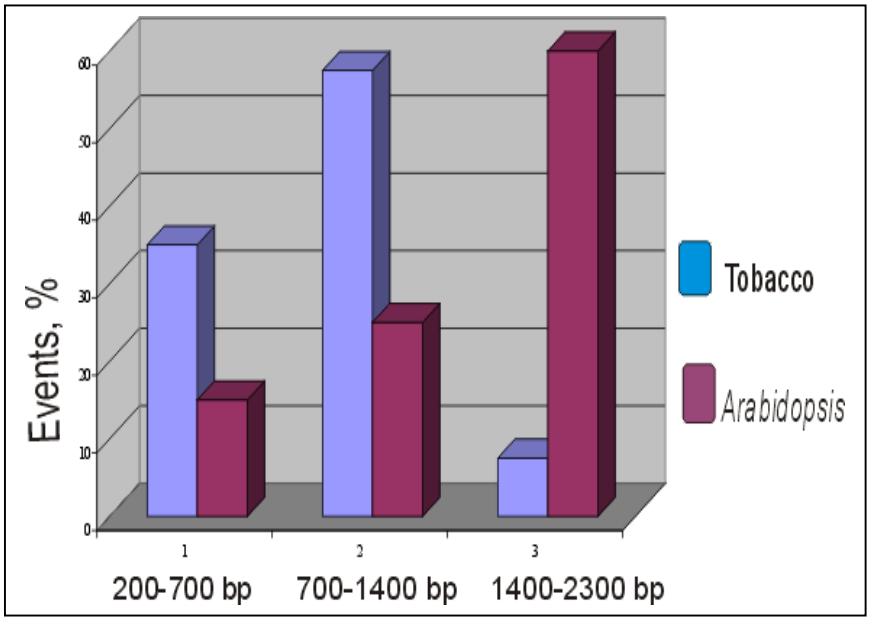


Fig. 12. Comparison of the molecular properties of sequenced recombination junctions in tobacco and *Arabidopsis*. Length distribution of the deletion events obtained.

In addition, the average size of detectable deletions was different between the two species. The average deletion size in tobacco was 920 bp (without consideration of the inserted sequences of 4-188 bp) whereas in *Arabidopsis* it was 1341 bp. The distribution of individual sizes of the deletions differs strongly in a direct comparison (Fig. 12). If the data were pooled in two different size classes (above and below 1400 bp) the difference was highly significant ($p<0,0005$ in a goodness of fit test).

4.2. Study of double-strand break repair by homologous recombination in Arabidopsis thaliana

4.2.1. Set up of a system to study DSB repair by intrachromosomal homologous recombination in *Arabidopsis thaliana*

To study DSB repair by intrachromosomal homologous recombination an assay system based on binary vector that contained I-SceI site between overlapping non-functional parts of the marker gene β-glucuronidase (Swoboda *et al.*, 1994) (Fig. 13) was used as a recombination substrate. The marker gene can be restored either by intrachromatid recombination via single-strand annealing or via allelic conversion process using the homology from the sister chromatid.

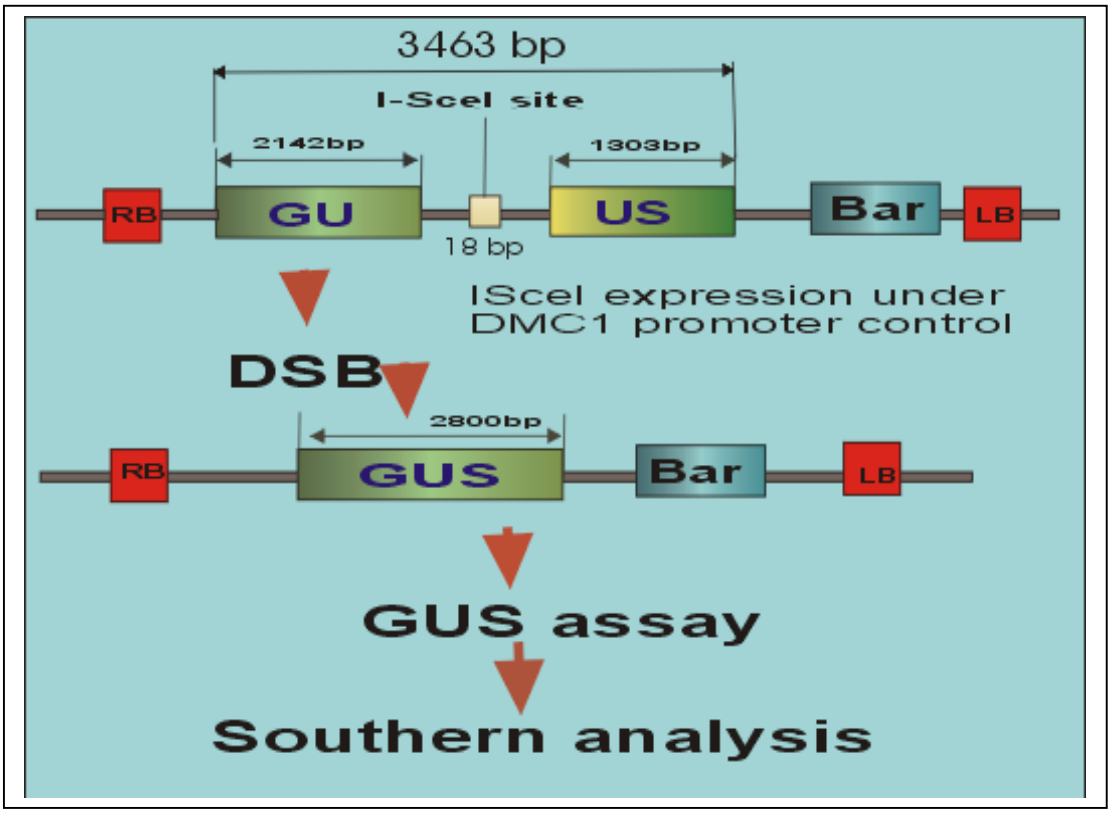


Fig. 13. Set up of the system for induction and detection of DSB repair by homologous recombination.

To induce recombination the *I-SceI* open reading frame (Puchta *et al.*, 1993) was expressed under the DMC1 promoter control. The promoter of the *AtDMC1* gene, which is a homolog of the *RecA* bacterial gene, should enable meiosis-associated expression and is active also in meristematic and embryonic tissue (Klimyuk and Jones, 1997). The recombination substrate construct as well as *I-SceI* expression cassette was transformed in *Arabidopsis* by *in planta* transformation. The recombination substrate transgenic lines GUIUS-1 and GUIUS-2, which contained one copy of the transgene in genome, were crossed with DI-SceI-1 and DI-Sce-2 transgenic plants, respectively.

From the F2 progeny transgenic plants homozygous for expression cassette and either homo- or hemizygous for GUIUS-1 and GUIUS-2 were isolated. In the progeny of these plants (F3) recombination events were detected by histochemical GUS assays and analysed by Southern hybridisation. Hemi- and homozygous lines of GUIUS-1 and GUIUS-2 were used as controls.

4.2.1.1. Genetic and molecular characterisation of plants containing recombination substrate and I-SceI expression cassette

Arabidopsis thaliana plants of Columbia ecotype were used for *in planta* transformation by pGUIUS, which contained the disrupted chimeric β-glucuronidase gene with the I-SceI site between two non-functional parts that have 618 bp sequence overlap. Phosphinothricin was used as selective marker for transgenic plants (Fig. 13). The transformation of *Arabidopsis* was also carried out with the pDI-SceI expression cassette that contains the *I-SceI* gene under the DMC1 promoter control and gentamycin as selective marker (Puchta, unpublished). Forty-two 6 weeks old

Arabidopsis plants were used for *Agrobacterium*-mediated transformation with the recombination substrate and 21 plants were transformed with pDI-SceI. As a transformation result 53 GUIUS and 41 DI-SceI transgenic lines were selected. The transformation effectivity was 0,22 % and 0,315 % respectively (Table 4). To check T-DNA copy number 2 DI-SceI transgenic lines and 5 GUIUS lines with 3:1 segregation revealed on a phosphinothricin-containing medium were used in Southern analysis.

Table 4. Transformation efficiency by pGUIUS and pDI-SceI

Transformed plants (Col-0)	Seeds tested	Transgenic lines	Efficiency of transformation, %	Lines with 3:1 segregation
GUIUS 42	24100	53	0.22	5.0
DI-SceI 21	12810	41	0.32	-

Southern hybridisation to test of T-DNA copy number in transgenic GUIUS plant lines. Genomic DNA of five different GUIUS containing plant lines was extracted, digested with HindIII and hybridised with a GUS-specific probe. As showed Southern analysis, two from five GUIUS transgenic lines contained one copy of insert in genome: GUIUS-1 and GUIUS-2 lines (Fig.14).

Southern hybridisation to test of the T-DNA copy number in transgenic DI-SceI plants. PCR amplification fragment of the DMC1 promoter of *A. thaliana* was used as a hybridisation probe. Since *Arabidopsis* contains endogenous *AtDMC1* gene, wild

type (Columbia) should give one band after hybridisation with the DMC1-specific probe.

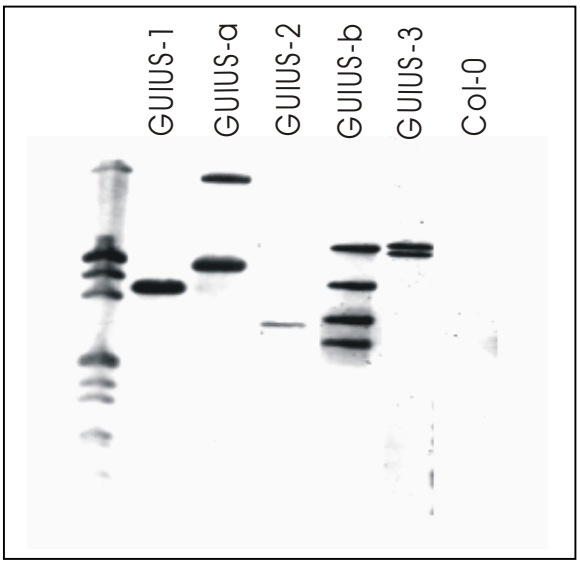


Fig. 14. Southern analysis of five GUIUS containing plant lines. Genomic DNA was digested with HindIII and hybridised with GUS-specific probe.

Two and more bands will indicate presence of the DI-SceI construct in *Arabidopsis* genome and copy number.

Southern analysis showed that DI-SceI-1 transgenic line had three and DI-SceI-2 had two copies of T-DNA insert in genome (data not shown).

4.2.1.2. Effect of the DMC1 controlled I-SceI expression on the recombination frequency of the GUIUS locus

To check whether DSBs caused by *I-SceI* expression under the control of the DMC1 promoter can induce recombination frequency the following experiments were carried out.

Transgenic plant lines with one copy of T-DNA in genome – GUIUS-1 and GUIUS-2 were exposed to seedling transformation by *Agrobacterium tumefaciens,* which contained the pDI-SceI plasmid. GUS activity after the transient

Table 5. Effect of the DMC1 controlled I-SceI expression on the recombination frequency of the GUIUS locus

Line	Number of blue sectors in 30 seedlings	Enhancement of intrachromosomal homologous recombination
1. GUIUS-1	2	-
2. GUIUS-2	1	-
3. GUIUS-1 ←(tr-on) pDI-SceI	250	125
4: GUIUS-1 ←(tr-on) pDI-SceI	192	96
5. GUIUS-1 ←(tr-on) pDI-SceI	147	73,5
6. GUIUS-1 ←(tr-on) pDI-SceI	247	123,5
7. GUIUS-2 ←(tr-on) pDI-SceI	144	144
8. GUIUS-2 ←(tr-on) pDI-SceI	104	104
9. GUIUS-2 ←(tr-on) pDI-SceI	95	95
10. GUIUS-2 ←(tr-on) pDI-SceI	107	107
11. GUIUS-1 ←(tr-on) pDI-SceI/ / p6000K (1/10)	104	52
12. GUIUS-2 ←(tr-on) pDI-SceI/ / p6000K (1/10)	60	60
13. GUIUS-1 ←(tr-on) pDI-SceI/ / p6000K(1/100)	60	30
14. GUIUS-2 ←(tr-on) pDI-SceI/ /p6000K(1/100)	42	42
15. GUIUS-1 ←(tr-on) pDI-SceI/ / p6000K (1/1000)	24	12
16. GUIUS-2 ←(tr-on) pDI-SceI/ / p6000K (1/1000)	15	15
17. GUIUS-2 ←(tr-on) pDI-SceI/ / p6000K (1/1000)	12	12

transformation was detected three days later by a destructive GUS assay (Swoboda *et al.*, 1994; Rossi *et al.*, 1993). The recombination events were detected as blue sectors on the seedlings. For transformation control 6000K T-DNA transfer-defective strain with deletion in Ti-plasmid (Rossi *et al.*, 1993) was used. A suspension of *A. tumefaciens* containing the pDI-SceI plasmid (optical density of bacterial solution OD = 1) was diluted with 6000K strain (OD = 1: 1:10, 1:100, 1:1000). GUIUS-1 and GUIUS-2 plants were used as the control.

A strong enhancement of recombination frequency after transformation of GUIUS-1 and GUIUS-2 plants with the *I-SceI* expression cassette was detected (Table 5). Taken together, intrachromosomal homologous recombination in somatic cells could be enhanced in two orders of magnitude by DSB induction that was due to the *I-SceI* expression under the DMC1 promoter control.

4.2.1.3. Induction of intrachromosomal homologous recombination via DSB in vivo initiation

The recombination substrate transgenic lines GUIUS-1 and GUIUS-2 which contain one copy transgene in genome were crossed with DI-SceI-1 and DI-SceI-2 transgenic plant lines respectively. The cross-product lines GUIUS-1 x DI-SceI-1 and GUIUS-2 x DI-SceI-2 were detected via double selection on the petry dishes which contained SM with gentamycin 100 mg/l (for DI-SceI) and phosphinotricin (BAR) 3 mg/l (for GUIUS). From the F2 progeny transgenic plants homozygous for expression cassette (selection on gentamycin 150 mg/l) and either homo- or hemizygous for GUIUS-1 and GUIUS-2 (selection on phosphinotricin (BAR) 8 mg/l) were isolated. The

progeny (F3) of these plants were grown together with hemi- and homozygous lines of GUIUS-1 and GUIUS-2 as controls.

Table 6. Compilation of transgenic lines after induction of homologous recombination via DSB initiation

Lines	Gentamicin segregation	BAR segregation
GUIUS-1-1	-	4:0 homo
GUIUS-1	-	3:1 hemi
GUIUS-1 x DI-SceI-1	4:0 homo	3:1 hemi
GUIUS-1 x DI-SceI-1	4:0 homo	4:0 homo
GUIUS-2-1	-	4:0 homo
GUIUS-2	-	3:1 hemi
GUIUS-2 x DI-SceI-2	4:0 homo	3:1 hemi
GUIUS-2 x DI-SceI-2	4:0 homo	4:0 homo

Lines that are shown in the Table 6 were used in the further study.

4.2.1.4. Detection of recombination events by histochemical GUS-assay.

For detection of somatic recombination events histochemical GUS assay was performed on 10 days old *Arabidopsis* seedlings of the F3 generation (Fig. 15) (Swoboda *et al.*, 1994; Rossi *et al.*, 1993). The AtDMC1 promoter driven expression of the *I-SceI* resulted in an increase of recombination events in more that two orders of magnitude for both lines (Fig. 16).

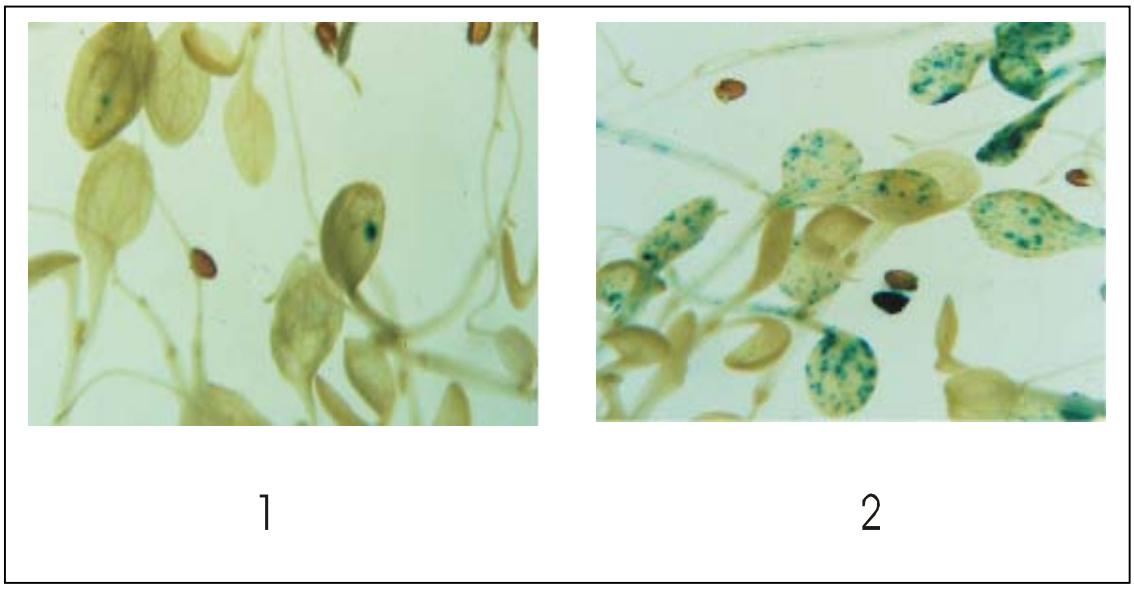
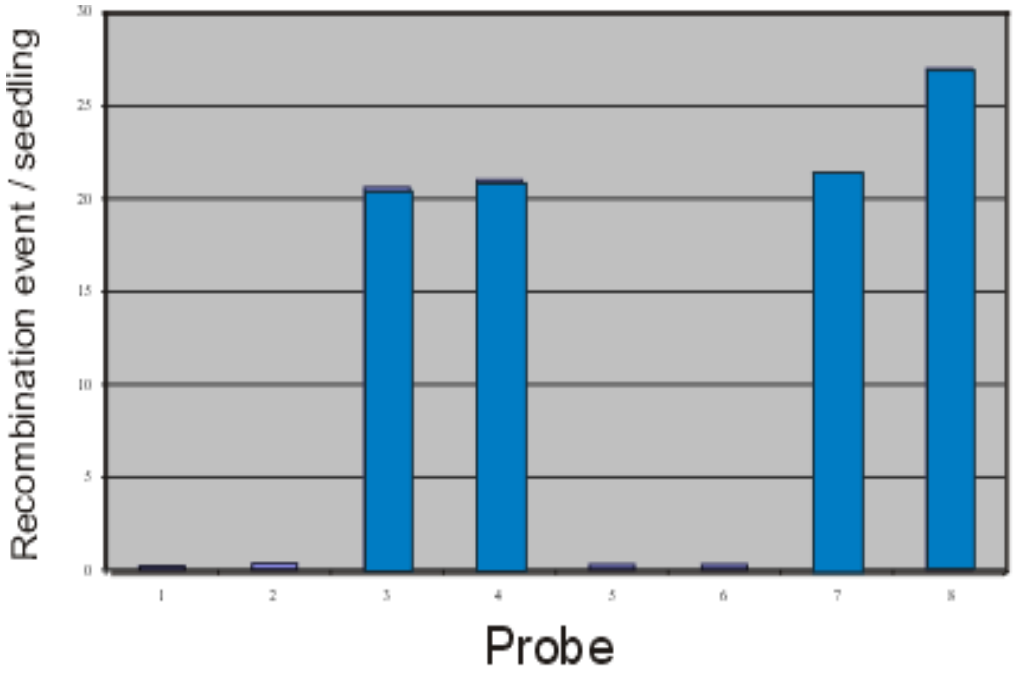


Fig. 15. Detection of somatic recombination events by histochemical GUS-assay. 1. Control: transgenic plants that contain only recombination substrate (the non-functional ß-glucuronidase gene); 2. Strong enhancement of the intrachromosomal homologous recombination via DSB induction by the AtDMC1 promoter driven expression of *I-SceI* was detected.

This result is in agreement with pervious studies (Xiao and Peterson, 2000; Chiurazzi *et al.*, 1996). Plants homozygous for the recombination substrate showed increase in blue sectors in comparison to the hemizygous lines in 1,27-1,6 times (Fig. 16).

Seedlings of the next generation (F4) were again histochemically stained. Due to the experimental set up the expression of *I-SceI* during meiosis in F3 plants should have taken place. As a result of recombination during meiosis or germinal haploid phase, the plantlets should become totally blue after GUS-staining.



Lines (Probe)	Sumblue spots/30 seedl. after destruct GUS-assay	Recombinat. event/seedl.	Enhancement - /DSB	Enhancement hemi/homo
1. GUIUS N1, homo	6	0,2	-	-
2. GUIUS N1, hemi	10	0,33	-	-
3. GUIUS N1 x DI-SceI N1 hemi / homo	618	20,6	62,4	-
4. GUIUS N1 x DI-Sce N1 homo / homo	640	21	105	1,6
5. GUIUS N2, homo	8	0,27	-	-
6. GUIUS N2, hemi	8	0,27	-	-
7. GUIUS N2 x DI-SceI N2 hemi / homo	636	21,2	78,5	-
8. GUIUS N2 x DI-SceI N2 homo / homo	811	27	100	1,27

Fig. 16. Enhancement of the somatic intrachromosomal recombination frequency by DSB induction.

About 30% of totally blue seedlings were detected (Fig. 17).

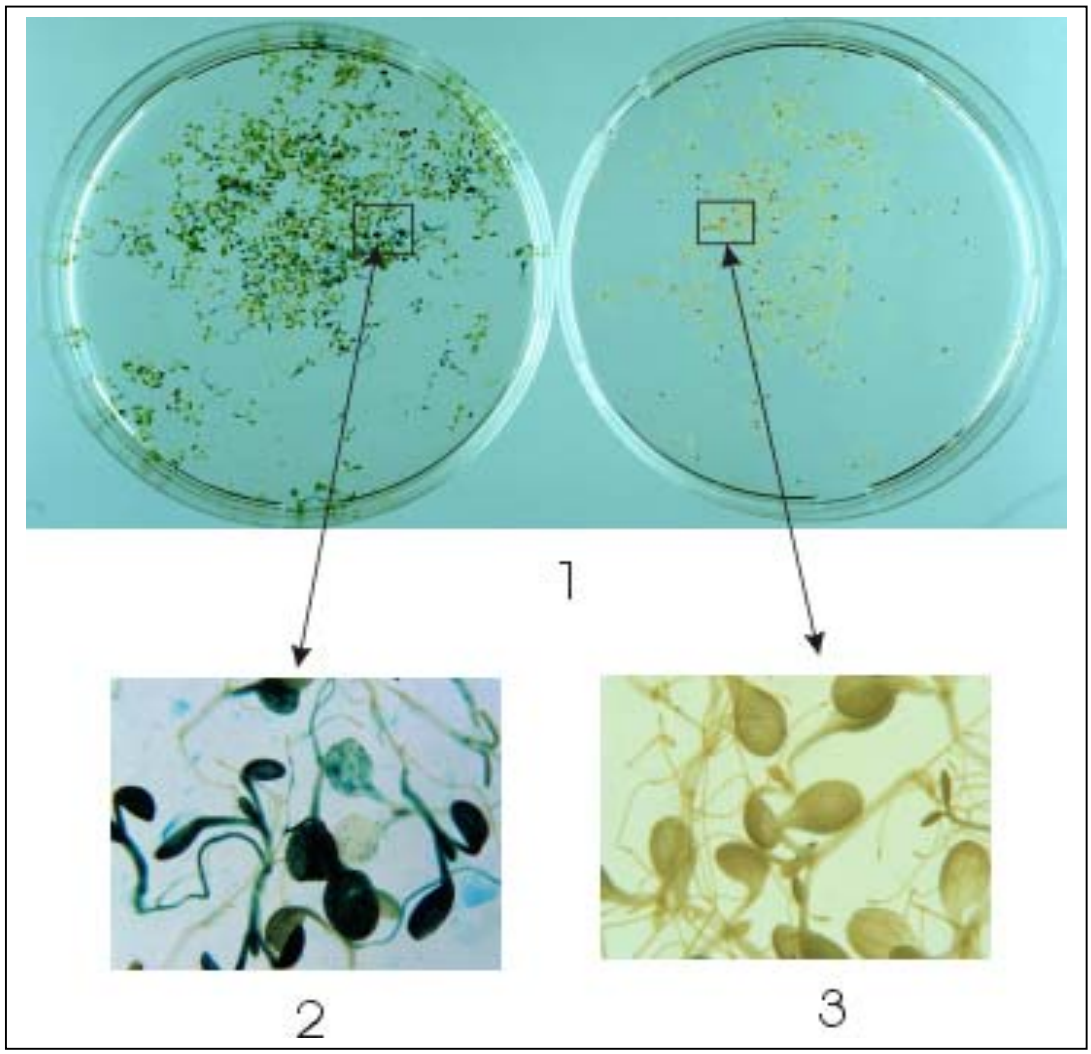
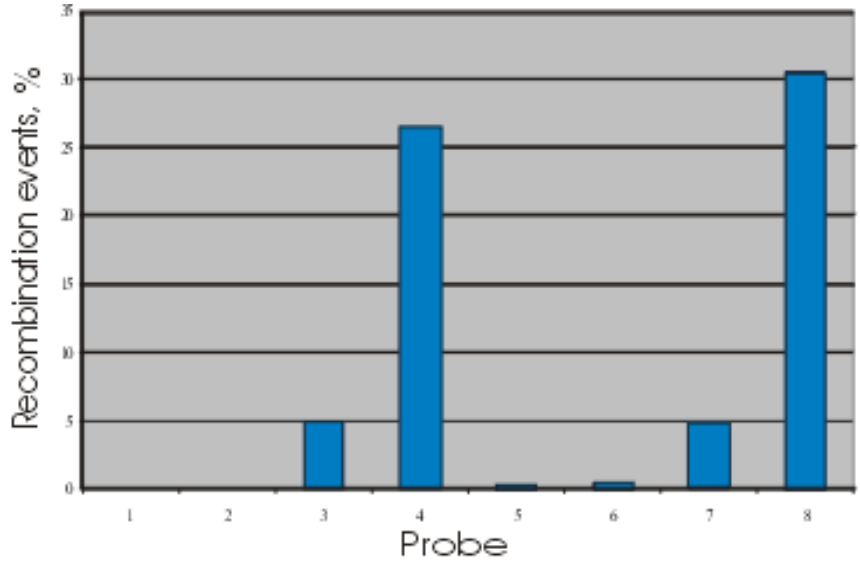


Fig. 17. Detection of recombination during meiosis or germinal haploid phase by histochemical GUS assay. As a result of such recombination plantlets become totally blue (**1**, left dish and **2**); Control plants contain only recombination substrate - non-functional marker gene β-glucuronidase - did not show the enhancement of recombination in the absence of DSBs initiation (**1**, right dish and **3**).

Moreover, the homozygous for GUIUS and DI-SceI lines showed strongly enhanced recombination frequency. Five to six fold more blue seedlings were observed in the progeny of homozygous lines in comparison with hemizygous state (Fig. 18).

In the somatic tissue the observed differences between hemi- and homozygous lines were 1,27-1,6 fold (Fig. 16). This can indicate, that most of the recombination events

in homozygous plants took place during meiosis and did not occur in somatic tissue or during the germinal haploid phase after meiosis.



Lines (Probe)	analysed seedlings	totally blue seedlings	% of recombination	Enhancement homo / hemi
1. GUIUS N1, homo	245	0	0	-
2. GUIUS N1, hemi	573	0	0	-
3. GUIUS N1 x DI-SceI N1 hemi / homo	637	31	4,9	-
4. GUIUS N1 x DI-Sce N1 homo / homo	388	103	26,5	5.4
5. GUIUS N2, homo	437	1	0,23	-
6. GUIUS N2, hemi	559	2	0,36	-
7. GUIUS N2 x DI-SceI N2 hemi / homo	660	32	4,8	-
8. GUIUS N2 x DI-SceI N2 homo / homo	556	170	30,6	6.3

Fig. 18. Enhancement of meiotic and germinal haploid phase recombination frequency by DSB induction.

4.2.1.5. Analysis of the homologous recombination reaction on the molecular level

To analyse the molecular changes in the *GUS* gene, plants containing a recombined transgene were first identified by a non-destructive ß-glucuronidase assay where only roots *in vivo* culture were stained (Martin *et al.*, 1992). Positive plants (Fig. 19) were transferred to soil and extracted DNA was used for Southern analysis.

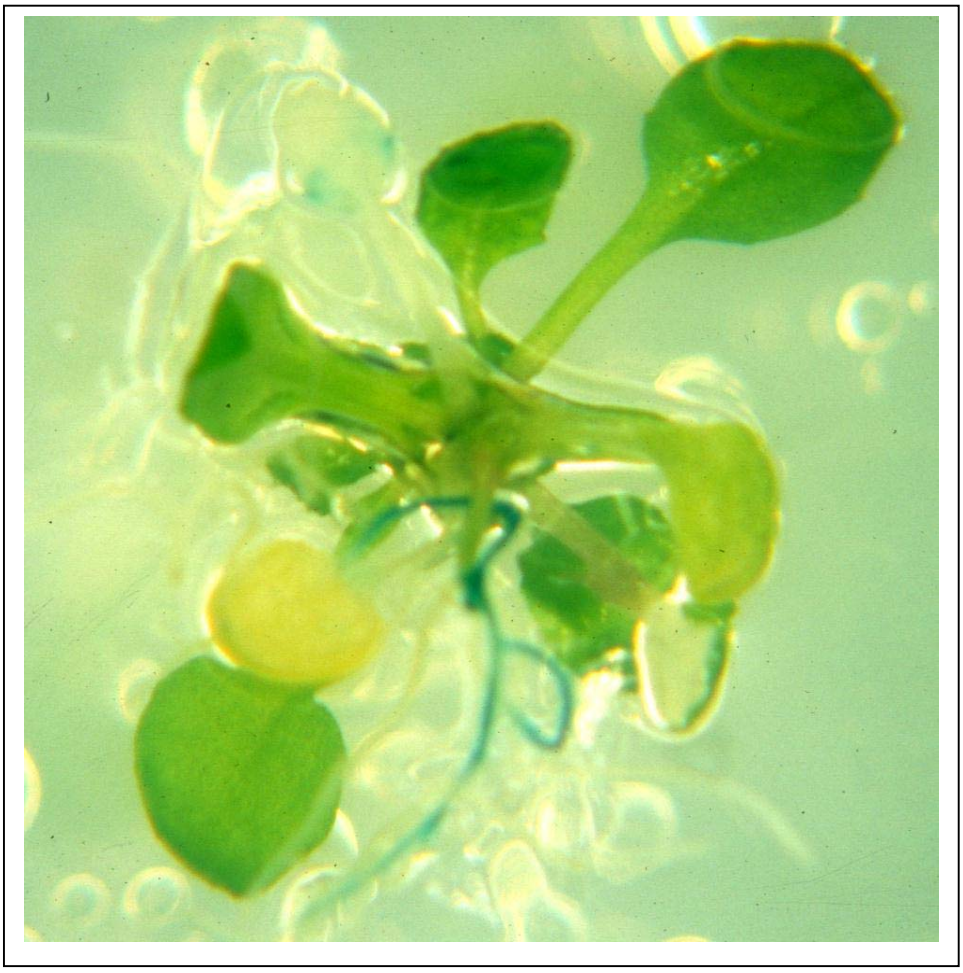


Fig. 19. Non-destructive GUS-assay detects plants containing a recombined functional GUS gene.

Genomic DNA was digested with HindIII and EcoRI. Hybridisation with the digoxygenin-labelled β-glucuronidase probe was performed.

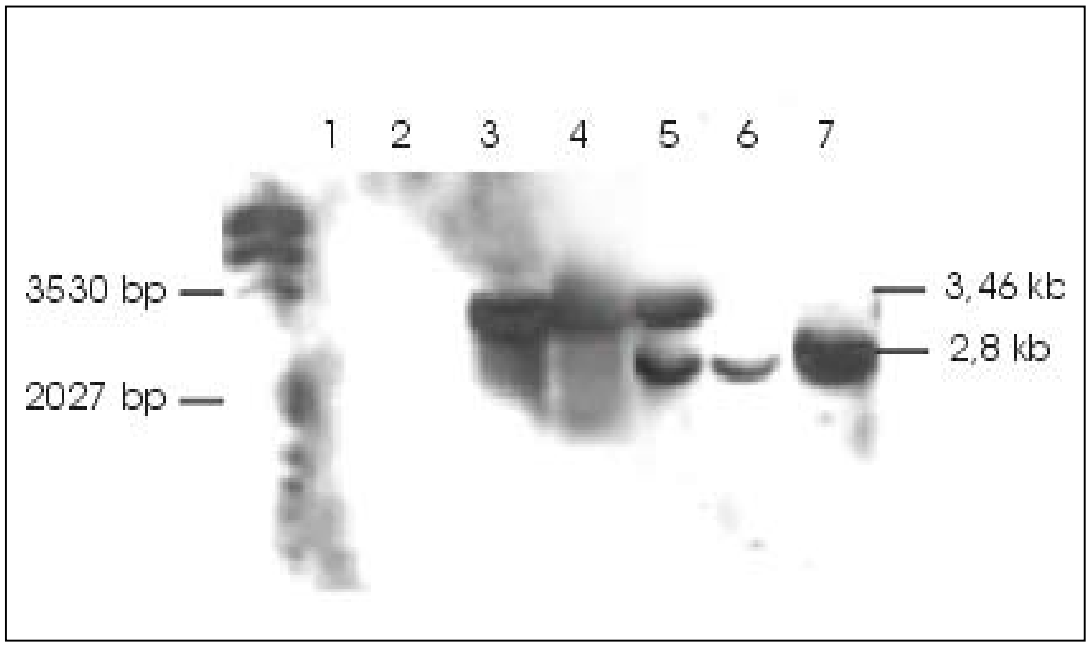


Fig. 20. Southern analysis for detection of the restored *GUS* gene. DNA (5μg) was digested with HindIII/EcoRI and hybridised with β-glucuronidase-specific probe. As negative controls DNA from Col w. t. (1) and expression cassette contains line (2) was digested with HindIII/EcoRI. DNA from transgenic lines contains recombination substrate in homo- and hemizygous state is shown in 3 and 4 respectively. 5 and 6 show lines that are homo- or hemizygous (respectively) for recombination substrate and homozygous for expression cassette and hemizigous for restored *GUS* gene. Line 7 is homozygous for recombination substrate and expression cassette and homozygous for restored *GUS* gene.

Bands corresponding in size to the restored GUS gene (2.8 kb) were detected (Fig. 20) demonstrating that restoration of the functional GUS gene after the recombination process took place. The 3.46 kb β-glucuronidase-specific band corresponds to the original construct (Fig. 20, 3 and 4).

For lines 5 and 6 that contain recombination substrate (non-functional *GUS* gene) in hemizygous and homozygous state correspondently and expression cassette (*I-SceI* gene expression controlled by DMC1 promoter) in homozygous state two bands were detected. The first one –3.45 kb – corresponds to non-restored *GUS* gene and the second one – 2.8 kb – corresponds to functional *GUS* gene. Both these lines are hemizygous for *GUS* restored gene. The line that is homozygous for restored *GUS*

gene (Fig. 20, 7) was selected from line 6 by non-destructive GUS-assay (had 100% blue roots) and carries only the 2.8 kb band.

5. DISCUSSION

5.1. Illegitimate DSB repair in plants

Eight different transgenic lines of *Arabidopsis thaliana* that harboured a single artificial I-SceI site between the 35S promoter and ORF of a cytosine deaminase gene (Salomon and Puchta, 1998; Stougaard, 1993) were used to study illegitimate DSB repair in plants. Recombination events were detected via loss of function of the cytosine deaminase by selection on medium containing 5-FC (Salomon and Puchta, 1998). Not every DNA sample isolated from 5-FC resistant calli contained changes in transgene. 46% of analysed PCR products calli represented original transgene. This can be explained by either low stability of 5-FC in the medium or non-sufficient 5-FC concentration for selection. These observations are in agreement with previously published results (Salomon and Puchta, 1998; Risseeuw *et al.*, 1997).

To address the molecular basis of somatic DSB repair sequence analysis of the modified junctions was performed. All obtained junctions contained simple deletions from 200 bp to 2200 bp. No insertions of filler sequences were detected (Table 2).

Three classes of junctions were obtained and characterised: junctions with deletions only within the 35S promoter region, junctions with deletions only within codA region and junctions with deletions within both the 35S promoter and *codA* gene regions. Latter class represent the majority of recombination junctions (80%) (Table 2).

Whereas some of the recombination junctions had small homologous patches (two or more nucleotides) between the rejoined DNA strands others did not utilised homologous sequences for rejoining of DNA ends. In 23 out of 40 cases 2-6 homologous base pairs present in both recombination partners could be detected at the junctions borders (Table 2). This is strongly reminiscent of data found in other

eukaryotic systems (Mason *et al.*, 1996; Lehman *et al.*, 1994) and for T-DNAs in plants (Bakkeren *et al.*, 1989; Gheysen *et al.*, 1991; Mayerhofer *et al.*, 1991), and confirms data reported for DSB repair in plants (Gorbunova and Levy, 1997; Salomon and Puchta, 1998).

For cells of higher eukaryotes, junctions without homologies were explained by simple ligation, whereas small patches of homologous nucleotides were considered to be a pre-requisite for the operation of a single-strand annealing (SSA) mechanism (Fig. 1, II) (Lehman *et al.*, 1994; Nicolas *et al.*, 1995; Mason *et al.*, 1996; Lieber *et al.*, 1997).

5.2. Species–specific double-strand break repair and genome evolution in plants

The formation of the plant body is a result of activity of the small group of cells of the meristem. Cell fate mapping revealed that the origin of the generative cells could be traced through plant development back to 3 to 6 cells in the embryonic apical meristem (Li and Redei, 1969). Thus, genomic alterations in meristematic cells can be transferred to the offspring and, consequently, genetic changes in somatic cells are relevant for evolutionary consideration (Das *et al.*, 1990; Walbot, 1996). Therefore, on the evolutionary scale somatic DSB repair might influence genome size and genome organisation.

The cause for the large differences in the nuclear DNA content of eukaryotes, particularly plants, known as the “C value paradox”, has been a matter of discussion for a long time (Cavalier-Smith, 1985; Dove and Flavell, 1988). Even closely related species with a similar phenotype may differ significantly as to their diploid genome size. One mechanism responsible for these size differences can be related 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 genome (SanMiguel *et al.*, 1996, 1998; Bennetzen and Kellog, 1997). Conversely, deletions might reduce genome size and counterbalance enlargements (Petrov, 1997).

By using suitable restriction endonucleases for induction of breaks at the specific loci in eukaryotic genomes it has been possible to characterise DSB-induced DNA repair. In this study, due to the selection on 5-FC and kanamycin and the position of the PCR primer binding sites, deletion size in represented experiments could only be analysed in the range 0.2-2.5 kb. Although other kinds of repair events, like changes that are not linked to the loss of the *codA* gene or bigger deletions, could not be addressed with the current experimental set up, the presented data reveal a surprisingly strong difference in DSB repair between tobacco and Arabidopsis, two dicotyledonous plant species with a >20-fold difference in genome size (Bennet and Leitch, 1997).

Whereas the mechanism of junction formation itself was not different between the two species (Fig. 10), the size classes of deletions in *Arabidopsis* and tobacco differed remarkably (Fig. 12). This is reminiscent of theoretical calculations for two insect species (Petrov *et al.*, 2000), in which an inverse correlation between the genome size and the average length of deletions was suggested. The authors demonstrated that deletions of significantly different extension within retroelements yielded species-specific genome size alterations in related insect species over evolutionary time periods. Investigation of the fate of non-LTR (long terminal repeat) retrotransposons in the cricket *Laupala* and fruit fly *Drosophila* elucidated differences in the average size of genomic deletions. *Laupala* has an approximately 11 times bigger genome than *Drosophila* and the overall rate of DNA loss is about 40 times slower (Petrov, *et al.*, 2000). But what kind of process might be responsible for these genomic changes? Deletions may occur by different mechanisms: by replication slippage (Capy, 2000), by unequal crossover (Smith, 1976) or by DSB repair. Although just two species –

Arabidopsis and tobacco - were compared in the presented work and a final conclusion on the matter is of course not justified yet, it is nevertheless tempting to speculate that species-specific differences in DSB repair pathways may indeed contribute significantly to the evolution of eukaryotic genome size. In *Arabidopsis* in comparison to the much larger soybean genome a number of segmental duplications or possibly complete genome duplication occurred during evolution (Grant *et al.*, 2000). Thus, deletion formation must have played a prominent role resulting in small size of the present day *Arabidopsis* genome.

In tobacco, beside deletions that resulted in a loss of function of the marker gene, it was possible to isolate insertions that led to an enlargement of the transgenic locus (Salomon and Puchta, 1998). It was demonstrated, that all these insertions were of nuclear origin. No insertions were detected in *Arabidopsis*.

In tobacco, almost every second deletion is associated with an insertion into the break site (Fig. 11, Table 3). The insertion of filler sequences has also been reported to accompany deletions in large maize genome (Wessler *et al.*, 1990). Earlier studies indicated that the frequency of insertion of filler sequences as well as their origin seems to differ strongly between lower and higher eukaryotes. In yeast, DSB repair rarely leads to the insertion of the filler sequences; if present, insertions were of non-nuclear origin [cDNAs of retrotransposons or mitochondrial DNA (Moore and Haber, 1996b; Teng *et al.*, 1996; Ricchetti *et al.*, 1999; Yu and Gabriel, 1999)]. In contrast, like in tobacco, in hamster cells insertions of filler sequences are common (Liang *et al.*, 1998). On the evolutionary scale, repeated insertion of filler sequences is expected to increase the complexity of genome organisation.

But what are the molecular causes for the differences in DSB repair between tobacco and *Arabidopsis*? The average length of deletions in tobacco might be smaller either

due to lower endo- or exonuclease activities attacking the break ends or, alternatively, to a better protection of the broken end against degradation.

Furthermore, it has been postulated that insertion of filler sequences occurs via a DNA synthesis-dependent strand annealing like mechanism that plays a prominent role in homologous DSB repair in somatic plant cells (Rubin and Levy, 1997; Puchta, 1998; Reiss *et al.*, 2000). Such a pathway would also require the protection of invading strands during the annealing and copying process. Thus, both the different sizes of deletions as well as different frequencies of insertions of filler sequences could eventually be due to a single difference in activity of the same regulatory process.

5.3. DSB repair by homologous DNA recombination in *Arabidopsis thaliana*

It was demonstrated in this study that artificially induced site-specific genomic DSBs in plant cells could be repaired by homologous recombination. Induction of the DSB by rarely cutting I-SceI endonuclease under control of the DMC1 promoter led to drastically enhanced recombination frequencies in somatic tissue, germinal haploid tissue and possibly during meiosis too.

5.3.1. Enhancement of intrachromosomal homologous recombination

In higher plants illegitimate recombination seems to be the main mode of DSB repair (Sargent *et al.*, 1997). However, as an alternative to illegitimate recombination, intrachromosomal homologous (Peterhans *et al.*, 1990), allelic and ectopic homologous recombination (Gisler *et al.*, 2001; Puchta, 1999a) and sister chromatid exchange (Natarajan *et al.*, 1985), are also possible mechanisms to repair DSBs using sequence homology.

Transgenic *Arabidopsis thaliana* plants containing partially overlapping sequences of a chimeric *uidA* (*GUS*) gene as recombination substrate were used to assay homologous recombination events. The restoration of the *GUS* gene either by intrachromosomal recombination via single-strand annealing or via a conversion process using the homology from the sister chromatid (Puchta and Hohn, 1996; Swoboda *et al.*, 1994) led to the functional gene product, which activity was detected by histochemical staining. This allows visualising and localising of recombination events.

Histochemical staining uncovered recombination events as blue sectors on plants (Fig. 15). To provide molecular evidence that blue sectors are the result of recombination events plants were propagated, stained by non-destructive GUS-assay and analysed by Southern blot (Fig. 20). Both genetic and molecular data (see results) confirmed that blue sectors on the plants represent recombination events at the DNA level. Southern blot analysis of plants positive for GUS-staining showed a 2.8 kb band (Fig. 20) that corresponds to the size of the *GUS* gene and confirms its restoration as a result of a homologous DSB repair process (Fig. 13). All previously described systems, with exception of one (Swoboda *et al.*, 1994), did not allow the localisation of independent recombination events in the whole organism (animals: Stoye *et al.*, 1988; Panthier and Condamine, 1991; Wilkie *et al.*, 1991; Gondo *et al.*, 1993; plants: Gal *et al.*, 1991; Assaad and Singer, 1992). Swoboda and colleagues (1994) set up a non-selective assay system that employs the hygromycin resistance gene located between the sequences overlaps of the *uidA* gene. The hygromycin resistance gene was lost during a recombination process and as a result of intrachromosomal recombination uidA gene was restored and visualised by histochemical staining. They detected frequency of intrachromosomal homologous recombination of 10^{-6}-10^{-7} events per genome (Swoboda *et al.*, 1994; see also: Puchta *et al.*, 1995; Assaad and Signer, 1992; Tovar and Lichtenstein, 1992).

In the current study DSB induction in the recombination substrate was used for the enhancement of homologous recombination. Double-strand breaks are known to initiate recombination in fungi (Szostak *et al.*, 1983) and plants (Gorbunova and Levy, 1999). Somatic crossover between homologous plant chromosomes can be induced by DNA-damaging agents such as gamma-radiation (Carlson, 1974). Low doses of X-rays, gamma rays and UV light are shown to increase the intrachromosomal homologous recombination frequency in plants with artificial substrates (Lebel *et al.*, 1993; Puchta *et al.*, 1995). Homologous recombination in plants can be enhanced by *in vivo* induction of DNA double-strand breaks by a site-specific endonuclease (Puchta *et al.*, 1993; Puchta, 1998). In *Arabidopsis*, generation of DSBs by HO endonuclease increased the frequency of somatic intrachromosomal homologous recombination about tenfold (Chiurazzi *et al.*, 1996). Results obtained in the present study demonstrate that intrachromosomal homologous recombination in somatic cells can be enhanced by more than two orders of magnitude by DSB induction with *I-SceI* expressed under control of the DMC1 promoter (Table 5, Fig. 16). Notably, plants homozygous for the recombination substrate did not show a significant increase in the frequency of blue sectors in comparison to hemizygous lines.

5.3.2. Can artificially induced double-strand break induce meiotic recombination in Arabidopsis thaliana?

To date meiotic recombination process is best studied in the yeast *Saccharomyces cerevisiae* (Roeder, 1997; Smith and Nicolas, 1998; Paques and Haber, 1999; Zickler and Kleckner, 1998). In yeast meiotic recombination is initiated by double-strand breaks. A specific protein, Spo11 was found to be covalently attached to the DSBs, strongly suggesting that it is involved in their formation. No data about the initiating

entity are available from other organisms, although the recent discovery of Spo11 homologous in genomes of various eukaryotes including plants (Hartung and Puchta, 2000) and mammals (Metzler-Guillemain and Massy, 2000) indicates that the initiation of meiotic recombination might be conserved and DSB formation might be a common mechanism in all eukaryotes.

It was demonstrated in yeast that meiotic recombination could be induced by artificial DSB at a specific genomic site via expression of a rarely cutting restriction endonuclease (Ray *et al.*, 1988; Malkova *et al.*, 2000).

In the present study the DMC1 promoter driving the expression of the I-SceI rarely cutting endonuclease was used. The *AtDMC1* gene shows homology to bacterial *RecA* gene and is expressed during meiosis in megaspore mother cells of ovules and pollen mother cells. However, AtDMC1 promoter GUS-fusion showed expression also in meristematic and embryonic tissue (Klimyuk and Jones, 1997; Klimyuk, unpublished). Therefore, the influence of DSB induction both in somatic and meiotic tissue was tested. Siblings from plants that were histochemically stained to detect somatic recombination events (Table 6) were again histochemically stained. Due to the experimental set up the expression of *I-SceI* during meiosis should have taken place. If recombination would have taken place during meiosis or during the consecutive germinal haploid phase progeny from such plants should have plantlets with the restored copy of the *GUS* gene in all cells resulting after GUS-staining in totally blue seedlings. Indeed, the totally blue plantlets were found (Fig. 17).

Previous studies on *Arabidopsis* indicated that the rate of meiotic and germinal haploid phase recombination between repeated sequences was in the order of 10^{-5} events (Jelesko *et al.*, 1999). Surprisingly high frequency of recombination – up to 30% were obtained in present study in plants that express *I-SceI* under control of the DMC1 promoter (Fig. 18). Noteworthy, lines homozygous for recombination substrate showed

drastically enhanced recombination frequency, between 5 and 6 times higher than in corresponding plants in hemizygous state (Fig. 18). The progeny from homozygous for recombination substrate lines (non-functional ß-glucuronidase gene) showed about 30% of totally blue seedlings whereas progeny from plants hemizygous for recombination substrate showed about 5%. This differs strongly from somatic tissue where only a minimal increase in 1.27 – 1.6 times was observed in lines homozygous for recombination substrate in comparison to hemizygous lines (Fig. 16).

Thus, the presence of a homologous sequence in allelic position on the homologue strongly enhances the recombination frequency. Preferred recombination between homologues was demonstrated for meiotic recombination in yeast (Petes and Pukkila, 1995; Schwacha and Kleckner, 1997). This can be taken as indirect indication that most recombination in homozygous plants took indeed place during meiosis and did not occur in somatic tissue or during the generative haploid phase after meiosis.

In summary, the obtained results demonstrate for the first time that via induction of DSB meiotic or germinal haploid phase recombination can be strongly enhanced in a higher eukaryote. This is surprising, because due to the DMC1 promoter control the break might not be induced at exactly the same time point as “natural” DSBs that appear at a specific stage in a highly complex pattern of the meiotic recombination reaction. Indeed, the double-strand break induced by I-SceI differs from the “natural” one, as for a certain time Spo11 is covalently attached to its ends (Roeder, 1997). Apparently independent of their origin the DSBs can be easily channelled into the homologous recombination pathway during meiosis (Goedecke *et al.*, 1999). In line with this finding is the fact that X-rays, which are supposed to induce DSBs randomly, can partly rescue the phenotype of a *Spo11* mutant of *C. elegans* (Derburg *et al.*, 1998). As the enzyme machinery involved in meiotic recombination is conserved between

yeast and other eukaryotes it is tempting to speculate that DSBs are generally initiators of meiotic recombination.

5.4. Outlook

The current study reveals new insights into double-strand break repair by illegitimate and homologous DNA recombination in *Arabidopsis thaliana*. Two different experimental systems were set up leading not only to new results but also to a row of new questions that can be followed up experimentally.

The first system, containing the I-SceI site between the 35S promoter and a negative selectable marker gene cytosine deaminase, was used to characterise illegitimate DSB repair in *Arabidopsis thaliana* and to compare recombination behaviour between plants with smaller (*Arabidopsis*) and larger (tobacco) genomes. DSBs were inducted by expression of the I-SceI endonuclease (ORF). This system allowed to select recombination events when marker gene function is lost and to identify the molecular structure of different genomic changes that have been induced by illegitimate DSB repair in *A. thaliana.* However, due to the experimental set up only deletions in the range of 0.2-2.5 kb could be isolated after DSB repair. Bigger deletions not associated with the loss of the marker gene or other genomic rearrangements were not detectible. It may therefore be interesting to analyse in future experiments also others classes of genomic changes.

In the current study the recombination behaviour between two plant species, which differ in genome size, was compared. It would be interesting also to compare the recombination behaviour of others plant species (e.g. monocotyledons), which have drastic differences in their genome size.

Moreover, it is interesting to check (e.g. by the application of plasmid molecules) whether the protection of the broken DNA ends against exo- and/or endonuclease degradation is different in tobacco and in *Arabidopsis*.

The second system, that was set up to study the DSB repair by homologous recombination was based on transgenic plants currying two components: a recombination substrate, that include the I-SceI site between overlapping non-functional parts of the marker gene β-glucuronidase and the expression cassette of the *I-SceI* gene under control of the DMC1 promoter. This system made it possible to localise and visualise recombination events and to test the influence of DSB induction on recombination in somatic and possibly meiotic tissue.

However, it is not possible to clearly differentiate intrachromosomal somatic recombination from meiotic recombination events because recombination in the present system could theoretically occur in all cases within the same chromatid, due to the 618 bp overlap between the interrupted *GUS* gene parts. In addition the DMC1 promoter has not only meiosis-associated expression but is also active in embryonic and meristematic tissue.

To sustain the putative enhancement of meiotic recombination via induction of DSBs by expression cassette of *I-SceI* gene under DMC1 promoter control it would be interesting to set up an alternative system, in which recombination between allelic sequences could be determined directly excluding intrachromatid events.

6.1. ABSTRACT.

DSB repair via illegitimate and homologous recombination was characterised in *Arabidopsis thaliana*.

To characterize illegitimate recombination DSBs in a negative selectable marker gene within the *Arabidopsis* genome were generated by expression of the rare cutting endonuclease I-SceI. After applying selection for the loss of the enzyme activity junctions of forty recombination events were isolated and sequenced. Most of the recombined junctions had deletions in the range between 1400 and 2300 bp. No inserted sequences were found associated with the deletions. Small patches of homologous nucleotides between rejoined DNA strands were detected in most recombination junctions.

The outcome of DSB repair by illegitimate recombination was compared between *Arabidopsis* and tobacco, two dicotyledonous plant species with more than 20 fold difference in genome size. Surprising species-specific differences were found. In *Arabidopsis* larger deletions than in tobacco occurred and these deletions were not accompanied by insertions of filler sequences as in tobacco. These differences in DSB repair might be on the one hand the basis for the evolution of the small genome size of *Arabidopsis* and on the other hand the basis for increased complexity of tobacco genome.

To study of the DSB repair by homologous recombination a two-component assay system was set up. The system consists of an intrachromosomal recombination

substrate based on a binary vector that contains an I-SceI site between overlapping non-functional parts of the marker gene β-glucuronidase and another binary vector containing the expression cassette of the *I-SceI* gene under the DMC1 promoter control for the induction of DSBs in plants. Since the DMC1 promoter is active during meiosis as well as in meristematic and embryonic tissue, homologous recombination in principle could be induced in somatic, germinal and meiotic tissue.

Strong enhancement (two order of magnitude) of homologous recombination in the GUIUS transgene was obtained via induction of DSBs by I-SceI expression under control of the DMC1 promoter in somatic tissue with differences between homo- and hemizygous lines between 1.27 – 1.6 times. This indicates that homologous recombination can be a prominent pathway of DSB repair in plants if homologies are available close to the DNA break.

Recombination events in meiotic or germinal haploid phase result in the next generation in progeny being genetically uniform for the recombined substrate. The presence of the I-SceI endonuclease drastically increased the recombination frequency. Moreover the frequency for homozygous GUIUS lines was 5 to 6 times higher than for the corresponding plants hemizygous for the recombination substrate. This can most easily be explained by the DSB-mediated induction of meiotic recombination as allelic sequences are the preferred target for this kind of recombination.

6.2. ZUSAMMENFASSUNG.

In der vorliegenden Arbeit wurde für die Modellpflanze *Arabidopsis thaliana* die Doppelstrangbruch (DSB) Reparatur durch illegitime und homologe Rekombination charakterisiert.

Zur Analyse der illegitimen Rekombination wurde in transgenen Arabidopsispflanzen mittels des selten schneidenden Restriktionsenzyms I-SceI DSBs in einem negativen Selektionsmarkergen induziert. Nach der Selektion auf den Verlust der Markergenfunktion wurden vierzig durch die DSB Reparatur neuverknüpfte Gensequenzen isoliert und sequenziert. Die meisten rekombinierten Moleküle wiesen Deletionen zwischen 1400 und 2300 Basenpaaren auf. Homologe Sequenzbereiche von wenigen Nukleotiden konnten in den meisten Fällen an den neu entstandenen Verknüpfungsstellen gefunden werden.

Das Ergebnis der DSB Reparatur zwischen den beiden dikotyledonen Pflanzen Arabidopsis und Tabak, die sich um mehr als zwanzigfach in ihrer Genomgröße unterscheiden, wurde verglichen. Dabei konnten überraschende Sequenzspezifische Unterschiede entdeckt werden. In Arabidopsis waren die Deletionen im Durchschnitt länger als im Tabak. In diese Deletionen hatten, nicht wie häufig beim Tabak, „Fillersequenzen“ insertiert. Die gefundenen Unterschiede in der DSB Reparatur mögen auf der einen Seite eine Ursache für die kleine Genomgröße von Arabidopsis darstellen, auf der anderen mögen sie auch für die größere Komplexität des Tabakgenoms mitverantwortlich sein.

Um die DSB Reparatur durch homologe Rekombination zu untersuchen wurden ein Zweikomponentensystem entwickelt. Auf der einen Seite wurden ein Rekombinationssubstrat hergestellt, dass zwischen überlappende nicht funktionelle Hälften eines ß-Glucuronidasegens eine I-SceI Schnittstelle insertiert hatte, auf der anderen Seite eine Expressionskasette, in der das I-SceI Gen unter der Kontrolle des DMC1 Promoters *in planta* exprimiert wurde. Da der DMC1 Promoter sowohl die Expression in der Meiose als auch in meristematischen und embryonalen Zellen bewirkt, war es so prinzipiell möglich die homologe Rekombination im somatischen, im germinalen und im meiotischen Gewebe zu induzieren.

Tatsächlich führte im somatischen Gewebe die Anwesenheit der I-SceI Expressionskasette zu einer starken Erhöhung der Rekombinationsfrequenz (um zwei Größenordnungen). Dies deutet darauf hin, dass homologe Rekombination ein wichtiger Weg der DSB Reparatur in Pflanzen sein kann, wenn sich homologe Sequenzen in der Nähe des Bruches befinden. Die für das Rekombinationssubstrat homozygote Linien wiesen eine 1,27 bis 1,6 mal höhere Frequenz auf als die heterozygote Linien.

Rekombinationsereignisse in der meiotischen oder generativen Phasen führen zu Nachkommen, die in all ihren Zellen ein rekombiniertes ß-Glucuronidasegen aufweisen. Die Anwesenheit der I-SceI Expressionskasette führte zu einem drastischen Anstieg von solchen Rekombinationsereignissen. Die Frequenz solcher Ereignisse war im Falle von homozygoten Elternpflanzen 5 bis 6 mal höher als bei Pflanzen, die für das Rekombinationssubstrat heterozygot waren. Dies deutet tatsächlich auf DSB-induzierte meiotische Rekombinationsereignisse hin, da nur bei dieser Art von Rekombination allelische Sequenzen bevorzugt rekombinieren.

7. REFERENCES.

Anderson, P., and Eliason, S. L. (1986) Recombination of homologous DNA fragments transfected into mammalian cells occurs predominantly by terminal pairing. Mol. Cell. Biol., 6: 3246-3252.

Anderson, P. (1987) Twenty years of illegitimate recombination. Genetics, 115: 581-584.

Assaad, F. F., and Signer, E. R. (1992) Somatic and germinal recombination of a direct repeat in Arabidopsis. Genetics, Oct., 132(2): 553-566.

Bakkeren, G., Koukolikova-Nicola, Z., Grimsley, N., and Hohn, B. (1989) Recovery of *Agrobacterium tumefaciens* T-DNA molecules from whole plants early after transfer. Cell, 57: 847-857.

Bascom-Slack, C. A., Ross L. O., Dowson D. S. (1997) Chiasmata, crossovers, and meiotic chromosome segregation. Adv. Genet., 35: 253-284.

Bechtold, N., Elleis, J., and Pelletier, G. (1993) In planta Agrobacterium mediated gene transfer by infiltration of adult Arabidopsis thaliana plants. CR Acad. Sci. Paris 316: 1194-1199.

Bechtold, N., and Pelletier, G.(1998) In planta Agrobacterium-mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration. (1998) Methods Mol. Biol., 82: 259-266.

Belmaaza, A., and Chartrand, P. (1994) One-sided invasion events in homologous recombination at double-strand breaks. Mutat. Res., 314: 199-208.

Bennett, M. D., and Smith, J. B. (1976) Nuclear DNA amounts in angiosperms. Phil. Trans. R. Soc. Lond., B274: 227-274.

Bennett, M. D., and Leitch, I. J. (1997) Nuclear DNA amounts in angiosperms – 583 new estimates. Ann. Bot., 80: 169-196.

Bennetzen, J. L., and Kellog, E. A. (1997) Do plants have a one-way ticket to the genomic obesity? Plant Cell, 9: 1509-1514.

Berg, P. (1991) Reverse genetics: its origins and prospects. Biotechnology, 9: 342-344.

Bevan, M (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res., 12: 8711-8721.

Bollag, R. J., Waldman, A. S., and Liskay, R. M. (1989) Homologous recombination in mammalian cells. Annu. Rev. Genet., 23: 199-225.

Bonnard, G., Tinland, B., Paulus, F., Szegedi, E., and Otten, L. (1989) Nucleotide sequence, evolutionary origin and biological role of a rearranged cytokinin gene isolated from a wide host range biotype III *Agrobacterium* strain. Mol. Gen. Genet., 216: 428-438.

Camerini-Otero, R. D., and Hsieh P. (1995) Homologous recombination proteins in prokaryotes and eukaryotes. Annu. Rev. Genet. 29: 509-552.

Capecchi, M. R. (1989) The new mouse genetics: altering the genome by gene targeting. Trends Genet. 5: 70-76.

Capy, P. (2000) Perspectives: evolution. Is bigger better in cricket? Science, 287: 985-986.

Carlson, P. S. (1974) Mitotic crossing-over in higher plant. Genet. Res. Camb., 24: 109-112. *Arabidopsis*

Cavalier-Smith, T. (1985) The Evolution of Genome Size. John Wiley and Sons, Chichester, UK.

Chiurazzi, M., Ray, A., Viret, J.-F., Perera, R., Wang, X. H., Lloyd, A., and Signer, E. R. (1996) Enhancement of somatic intrachromosomal homologous recombination in *Arabidopsis* by HO-endonuclease. Plant Cell, 8: 2057-2066.

Choulika, A., Perrin, A., Dujon, B., and Nicolas, J. F. (1995) Induction of homologous recombination in mammalian chromosomes by using the I-SceI system of *Saccharomyces cerevisiae*. Mol. Cell. Biol. 15: 1968-1973.

Clegg, M. T., Cummings, M. P., and Durbin, M. L. (1997) The evolution of plant nuclear genes. Proc. Natl. Acad. Sci. USA, 94: 7791-7798.

Cohen, S. N., Chang, A. C. Y., and Hsu, L. (1972) Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of *E. coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA, 69: 2110.

Colleaux, L., d'Auriol, L., Galibert, F., and Dujon, B. (1988) Recognition and cleavage site of the intron encoded omega transposase. Proc. Natl. Acad. Sci. USA, 85: 6022-6026.

Critchlow, S. E., and Jackson, S. P. (1998) DNA end-joining: from yeast to man. Trends Biochem. Sci., 23: 394-398.

Czako, M., and Marton, L. (1994) The herpes simplex virus thymidine kinase gene as a conditional negative-selection marker gene in *Arabidopsis thaliana*.

Das, O. P., Levi-Minzi, S., Koury, M., Benner, M., and Messing, J. (1990) A somatic gene rearrangement contributing to genetic diversity in maize. Proc. Natl. Acad. Sci. USA, 87: 7809-7813.

Deblaere, R., Bytebier, B., de Greve, H., Deboek, F., Schell, J., van Montagu, M., and Leemans, J. (1985) Efficient octopine Ti plasmid-derived vectors for Agrobacterium-mediated gene transfer in plants. Nucl. Acids Res., 13: 4777-4788.

De Groot, M. J., Offringa, R., Groet, J., Does, M. P., Hooykaas, P. J., and van den Elzen, P. J. (1994) Non-recombinant background in gene targeting: illegitimate recombination between a hpt gene and defective 5′deleted npt II gene can restore a Km^r phenotype in tobacco. Plant Mol. Biol. 25: 721-733.

Derburg, A. F., McDonald, K., Moudler, G., Barstead, R., Dresser, M., Villeneuve, A. M. (1998) Meiotic recombination in *C. elegans* initiates by a conserved mechanism and is dispensable for homologous chromosome synapsis. Cell, Aug., 7, 94(3): 387-398.

Doutriaux, M. P., Coutoon, F., Bergounioux, C., and White, C. (1998) Isolation and characterisation of the RAD51 and DMC1 homologs from *Arabidopsis thaliana*. Mol. Gen. Genet., 257: 283-291.

Dove, G. A., and Flavell, R. B. (1988) Genome Evolution. Academic Press, London, UK.

Gal, S., Pisan, B., Hohn, T., Grimsley, N., and Hohn, B. (1991) Genomic homologous recombination in planta. EMBO J., 10: 1571-1578.

Gheysen, G., Villarroel, R., and Van Montagu, M. (1991) Illegitimate recombination in plants: a model for DNA integration. Genes Dev., 5: 287-297.

Gisler, B., Salomon, S., and Puchta, H. (2001) The role of double-strand break-induced allelic homologous recombination in somatic plant cells. (Submitted).

Goedecke, W. E., Eijpe, M., Offenberg, H. H., van Aalderen, M., Heyting, C. (1999) Mre11 and Ku70 interact in somatic cells, but are differentially expressed in early meiosis. Nat. Genet., Oct., 23(2): 194-198.

Golub, E. I., Kovalenko, O. V., Gupta, R. C., Ward, D. C., and Radding, C. M. (1997) Interaction of human recombination proteins Rad51 and Rad54. Nucleic Acid Res. 25: 4106-4110.

Gondo, Y., Gardner, J. M., Nakatsu, Y., Durham-Pierre, D., Deveau, S. A., Kuper, C., and Brilliant, M. H. (1993) High-frequency genetic reversion mediated by a DNA duplication: the mouse pink-eyed unstable mutation. Proc. Natl. Acad. Sci., USA, 90: 297-301.

Gorbunova, V., and Levy, A. A. (1997) Non-homologous DNA end-joining in plant cells is associated with deletions and filler DNA insertions. Nucleic Acids Res. 25: 4650-4657.

Gorbunova, V., and Levy, A. A. (1999) How plants make ends meet: DNA double-strand break repair. Trends Plant Sci., 4: 263-269.

Grant, D., Cregan, P., and Shoemaker, R. C. (2000) Genome organization in dicots: genome duplication in *Arabidopsis* and synteny between soybean and *Arabidopsis*. Proc. Natl. Acad. Sci. USA, 97: 4168-4173.

Haber, J. E. (1992) Mating-type gene switching in *Saccharomyces cerevisiae*. Trends Genet., 8: 446-452.

Haber, J. E. (1995) In vivo biochemistry: Physical monitoring of recombination induced by site-specific endonucleases. Bioassays, 17: 609-620.

Haber, J. E. (1999) Gatekeepers of recombination. Nature, 398: 665-667.

Halfter, U., Morris, P.-C., and Wilmitzer, L. (1992) Gene targeting in Arabidopsis thaliana. Mol. Gen. Genet., 231: 186-193.

Hartung, F., and Puchta, H. (2000) Molecular characterisation of two paralogous Spo 11 homologues in *Arabidopsis thaliana*. Nucleic Acids Res., vol. 28, 7: 1548-1554.

Hays. S. L., Firmenich, A. A., and Berg, P. (1995) Complex formation in yeast double-strand break repair: participation of Rad51, Rad52, Rad55, and Rad57 proteins. Proc. Natl. Acad. Sci. USA, 92: 6925-6929.

Heyting, C. (1996) Synaptonemal complex: structure and function. Curr. Opin. Cell Biol., 8: 389-396.

Hiei, Y., Ohta, S., Komari, T., and Kumashiro, T. (1994) Efficient transformation of rice (Oriza Sativa L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. Plant J., 6: 271-282.

Hinnen, A., Hicks, J. B., Fink, G. R. (1978) Transformation of yeast. Proc. Natl. Acad. Sci. USA, 75: 1929-1933.

Hoffman, G. R. (1994) Induction of genetic recombination: consequences and model systems. Environ. Mol. Mutagen, 23: 59-66.

Holmes, A., and Haber, J. E. (1999) Double-strand break repair in yeast requires both leading and lagging strand DNA polymerases. Cell, 96: 415-424.

Holmes, D. S., and Quigley, M. (1981) A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem., 114: 193-197.

Iglesias, V. A., Moscone, E. A., Papp, I., Neuhuber, F., Michalowski, S., Phelan, T., Spiker, S., Matzke, M. A., and Matzke, A. J. (1997) Molecular and cytogenetic analyses of stably and unstably expressed transgene loci in tobacco. Plant Cell, 9: 1251-1264.

Inoue, H., Nojima, H., and Okayama, H. (1990) High efficiency transformation of *E. coli* with plasmids. Gene, 98: 23.

Irish, V. F., Sussex, I. M. (1990) Function of the apetala-1 gene during Arabidopsis floral development. Plant Cell, 2 (8): 741-753.

Irish, V. F. (1991) Cell lineage in plant development. Curr. Opin. Cell Biol., 3 (6): 983-987.

Ivanov, E. L., Sugawara, N., White, C. I., Fabre, F., and Haber, J. E. (1994) Mutations in XRS2 and RAD50 delay but do not prevent mating-type switching in *Saccaromyces cerevisiae*. Mol. Cell. Biol., 14: 3414-3425.

Ivanov, E. L., and Haber, J. E. (1995) RAD1 and RAD10, but not other excision repair genes, are required for double-strand break induced recombination in *Saccharomyces cerevisiae*. Mol. Cell. Biol., 15: 2245-2251.

Jablonovich, Z., Liefshitz, B., Steinlauf, R., Kupiec, M. (1999) Characterization of the role played by the Rad59 gene of *Saccharomyces serevisiae* in ectopic recombination. Curr. Genet., 36: 13-20.

Jasin, M. (2000) Chromosome breaks and genomic instability. Cancer Invest., 18: 78-86.

Jelesko, J. G., Harper, R., Furuya, M., Gruissem, W. (1999) Rare germinal unequal crossing-over leading to recombinant gene formation and gene duplication in *Arabidopsis thaliana.* Proc. Natl. Acad. Sci. USA, Aug., 31, 96(18): 10302-10307.

Jenik, P. D., and Irish, V. F. (2000) Regulation of cell proliferation patterns by homeotic genes during Arabidopsis floral development. Development, 127 (6) 1267-1276.

John, B., and Miklos, G. L. G. (1988) The Eukariotic Genome. Development and Evolution. Allen and Unwin, London.

Johnson, R. D., Liu, N., and Jasin, M. (1999) Mammalian XRCC2 promotes the repair of DNA double-strand breaks by homologous recombination. Nature, 402: 397-399.

Jonzuka, K., and Ogawa, H. (1995) Interaction of Mre11 and Rad50: two proteins required for DNA repair and meiosis-specific double-strand break formation in *Saccharomyces serevisiae*. Genetics, 139: 1521-1532.

Kathleen, N. Smith, and Alain Nicalas (1998) Recombination at work for meiosis. Curr. Opin. in Genetics & Development, 8: 200-211.

Keeney, S., Giroux, C. N., and Kleckner, N. (1997) Meiosis-spesific DNA double-strand breaks are catalysed by Spo11, a member of a widely conserved protein family. Cell, 88: 375-384.

Keeney, S., Baundat, F., Angeles, M., Copeland, N. C., Jenkins, N. A., Manova, K., and Jasin, M. (1999) A mouse homolog of the *Saccharomyces cerevisiae* meiotic recombination DNA transesterase Spo11p.Genomics, 61: 170-182.

Kirik, A., Salomon, S., and Puchta, H. (2000) Species-specific double-strand break repair and genome evolution in plants. EMBO J., 19: 5562-5566.

Kleckner, N. (1996) Meiosis: how could it work? Proc. Natl. Acad. Sci. USA, 93: 8167-8174.

Klimyuk, V., and I., Jones, J. D. (1997) AtDMC1, the Arabidopsis homologue of the yeast DMC1 gene: characterization, transposon-induced allelic variation and meiosis-associated expression. Plant J., 11(1): 1-14.

Koornneef, M., Alonso-Blanco, C., and Stam, P. (1992) Genetic Analysis. From: Methods in Molecular Biology, vol 82: Arabidopsis Protocols. Ed. By: J. Martinez-Zapater and J. Salinas. Numana Press Inc., Totowa, NJ: 105-117.

Kovalenko, O. V., Golub, E. I., Bray-Ward, P., Ward, D. C., and Radding, C. M. (1997) A novel nucleic acid-binding protein that interacts with human Rad51 recombinase. Nucleic Acids Res., 25: 4946-4953.

Kramer, K., Brock, J. A., Bloom, K., Moore, J. K., and Haber, J. E. (1994) Two different types of double-strand breaks in *Saccharomyces cerevisiae* are repaired by similar Rad52 – independent non-Homologous recombination events. Mol. Cell. Biol., 14: 1293-1301.

Lebel, E. G., Masson, J. Bogucki, A., and Paszkowski, J. (1993) Stress-induced intrachromosomal recombination in plant somatic cells. Proc. Natl. Acad. Sci. USA, 90: 422-426.

Lee, K. Y., Lund, P., Lowe, K., and Dunsmuir, P. (1990) Homologous recombination in plant cells after *Agrobacterium*-mediated transformation. Plant Cell, 2: 415-425.

Lehman, C. W., Trautman, J. K., and Carroll, D. (1994) Illegitimate recombination in *Xenopus*: characterization of end-joined junctions. Nucleic Acids Res., 22: 434-442.

Li, S. L., and Rédei, G. P. (1969) Thiamine mutants of the crucifer *Arabidopsis*. Bioch. Genetics, 3: 163-170.

Liang, F., Han, M., Romanenko, P., and Jasin, M. (1998) Homology-directed repair is a major double-strand break repair pathway in mammalian cells. Proc. Natl. Acad. Sci. USA, 95: 5172-5177.

Lieber, M. R., Grawunder, U., Wu, X., and Yaneva, M. (1997) Tying loose ends: roles of Ku and DNA-dependent protein kinase in the repair of double-strand breaks. Curr. Opin. Genet. Dev., 7: 99-104.

Lin, F. L., Sperle, K., and Sternberg, N. (1984) Model for homologous recombination during transfer of DNA into mouse L-cells: role for DNA ends in the recombination process. Mol. Cell. Biol., 4: 1020-1034.

Lin, F. L., Sperle, K., and Sternberg, N. (1990) Repair of double-stranded breaks by homologous fragments during transfer of DNA into mouse L-cells. Mol. Cell. Biol., 10: 113-119.

Low, K. B., and Porter, R. D. (1978) Modes of gene transfers and recombination in bacteria. Annu. Rev. Genet., 12: 259-287.

Malkova, A. Klein, F. Leung, W-Y., and Haber, J. E. (2000) HO endonuclease-induced recombination in yeast meiosis resembles Spo11-induced events. Proc. Natl. Acad. Sci. USA, 26: 14500-14505.

Martin, T., Wöhner, R. V., Hummel, S., Willmitzer, L., Frommer, W. B. (1992) The GUS reporter system as a tool to study plant gene expression. In GUS protocols: Using the GUS gene as a reporter of gene expression. Academic Press: 23-43.

Mason, R. M., Thacker, J., and Fairman, M. P. (1996) The joining of non-complementary DNA double-strand breaks by mammalian extracts. Nucleic Acids Res., 24: 4946-4953.

Mayerhofer, R., Koncz-Kalman, Z., Bakkeren, G., Crameri, A., Angelis, K. Redei, G., Schell, J., Hohn, B. and Koncz, C. (1991) T-DNA integration : a mode of illegitimate recombination in plants. EMBO J. 10: 271-280.

McKim, K. S., and Hayashi-Hagihara, A. (1998) mei-W68 in *Drosophila melanogaster* encodes a Spo11 homolog: evidence that the mechanism for initiating meiotic recombination is conserved. Genes Dev., 12: 2932-2942.

Mengiste, T., and Paszkowski, J. (1999) Prospects for the precise engineering of plant genomes by homologous recombination. Biol. Chem., 380: 749-758.

Metzler-Guillemain, C., and de Massy, B. (2000) Identification and characterization of an Spo11 homolog in the mouse. Chromosoma, 109: 133-138.

Moore, J. K., and Haber, J. E. (1996a) Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in *Saccharomyces cerevisiae.* Mol. Cell. Biol., 16: 2164-2173.

Moore, J. K., and Haber, J. E. (1996b) Capture of retrotransposen DNA at the sites of chromosomal double-strand breaks. Nature, 383: 644-646.

Mortensen, U. H., Bendixen, C., Sunjevaric, I., and Rothstein, R. (1996) DNA strand annealing is promoted by the yeast Rad52 protein. Proc. Natl. Acad. Sci. USA, 93: 10729-10734.

Nassif, N., Penny, J., Pal, S., Engels, W. R., and Gloor, G. B. (1994) Efficient copying of nonhomologous sequences from ectopic sites via P-element induced gap repair. Mol. Cell. Biol., 14: 1613-1625.

Natarajan, A. T., Mullenders, L. H., Meijers, M., Mukherjee, U. (1985) Induction of sister-chromatid exchanges by restriction endonucleases. Mutat. Res., Sep., 144(1): 33-39.

Nickoloff, J. A. (1990) Transcription stimulates homologous recombination in mammalian cells. Mol. Cell. Biol., 10: 4837-4845.

Nickoloff, J. A., and Hoekstra, M. F. (1998) in: DNA Damage and Repair, vol. I (eds. Nickoloff, J. A., and Hoekstra, M- F.), Humana, Totowa, 335-362.

Nicolas, A. L., Munz, P. L., and Young, C. S. (1995) A modified single-strand annealing model best explains the joining of DNA double-strand breaks in mammalian cells and cell extracts. Nucleic Acids Res., 23: 1036-1043.

Offringa, R., de Groot, M. J. A., Haagsman, H. J., Does, M. P., van den Elzen, P. J. M., and Hooykaas, P. J. J. (1990) Extrachromosomal homologous recombination and gene targeting in plant cells after *Agrobacterium*-mediated transformation. EMBO J., 9: 3077-3084.

Offringa, R. (1992) Gene targeting in plants using the Agrobacterium vector system. Ph.D. Thesis Leiden University, The Netherlands.

Offringa, R., Franke-van Dijk, M. E. I., de Groot, M. J. A., van den Elzen, P. J. M., and Hooykaas, P. J. J. (1993) Nonreciprocal homologous recombination between Agrobacterium transferred DNA and a plant chromosomal locus. Proc. Natl. Acad. Sci. USA, 90: 7346-7350.

Ohba, T., Yoshioka, Y., Machida, C., and Machida, Y. (1995) DNA rearrangement associated with the integration of T-DNA in tobacco: an example for multiple duplications of DNA around the integration target. Plant J., 7: 157-164.

Ohl, S., Offringa, R., van den Elsen, P. J. M., and Hooykaas, P. J. J. (1994) Gene replacement in plants. In: Homologous Recombination and Gene Silencing in Plants (ed. Paszkowski, J.). Kluwer Academic Publishers, 191-217.

Panthier, J. J., and Condamine, H. (1991) Mitotic recombination in mammals. BioEssays, 13: 351-356.

Papp, I., Iglesias, V. A., Moscone, E. A., Michalowski, S., Spiker, S., Park, Y. D., Matzke, M. A., and Matzke, A. J. (1996) Structural instability of a transgene locus in tobacco is associated with aneuploidy. Plant J., 10: 469-478.

Paques, F., and Haber, J. E. (1999) Multiple pathways of recombination induced by double-strand breaks in *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev., 63: 349-404.

Paszkowski, I., Baur, M., Bogucki, A., and Potrykus, I. (1988) Gene targeting in plants. EMBO J., 7: 4021-4026.

Perrin, A., Buckle, M., and Dujon, B. (1993) Assymetrical recognition and activity of the I-SceI endonuclease on its site and on intron-exon junctions. EMBO J., 12: 2939-2947.

Peterhans, A., Schlüpmann, H., Basse, C., and Paszkowski, J. (1990) Intrachromosomal recombination in plants. EMBO J., 9: 3437-3445.

Petes, T. D., and Pukkila, P. J. (1995) Meiotic sister chromatid recombination. Adv. Genet., 33: 41-62.

Petes, T. C., and Hill, C. W. (1988) Recombination between repeated genes in microorganisms. Annu. Rev. Genet., 22: 147-168.

Petrov, D. A. (1997) Slow but steady: Reduction of genome size through biased mutation. Plant Cell, 9: 1900-1901.

Petrov, D. A., Sangster, T. A., Johnston, J. S., Hartl, D. L., and Shaw, K. L. (2000) Evidence for DNA loss as a determinant of genome size. Science, 287: 1060-1062.

Plessis, A., Perrin, A., Haber, J. E., and Dujon, B. (1993) Site-specific recombination determined by I-SceI, a mitochondrial group I intron-encoded endonuclease expressed in the yeast nucleus. Genetics, 130: 451-460.

Puchta, H., and Hohn, B. (1991) A transient assay in plant cells reveals a positive correlation between extrachromosomal recombination rates and length of homologous overlap. Nucleic Acids Res., 19: 2693-2700.

Puchta, H., Dujon, B., and Hohn, B. (1993) Homologous recombination in plant cells is enhanced by in vivo induction of double-strand breaks into DNA by a site-specific endonuclease. Nucleic Acids Res., 21: 5034-5040.

Puchta, H., Swoboda, P., and Hohn, B. (1995) Induction of intrachromosomal homologous recombination in whole plants. Plant J., 7: 203-210.

Puchta, H., and Hohn, B. (1996) From centiMorgans to basepairs: Homologous recombination in plants. Trends in Plant. Sci., 1: 340-348.

Puchta, H., Dujon, B., and Hohn, B. (1996) Two different but related mechanisms are used in plants for the repair of genomic double-strand breaks by homologous recombination. Proc. Natl. Acad. Sci. USA, 93: 5055-5060

Puchta, H. (1998) Repair of genomic double-strand breaks in somatic plant cells by one-sided invasion of homologous sequences. Plant J., 13: 331-339.

Puchta, H. (1999a) Double-strand break-induced recombination between ectopic homologous sequences in somatic plant cells. Genetics, 152: 1173-1181.

Puchta, H. (1999b) Use of I-SceI to induce double-strand breaks in *Nicotiana.* Methods Mol. Biol., 113: 447-451.

Ray, A., Siddiqi, I., Kolodkin, A. L., Stahl, F. W. (1998) Intra-chromosomal gene conversion induced by a DNA double-strand break in *Saccharomyces cerevisiae.* J. Mol. Biol., May, 20; 201(2): 247-260.

Reiss, B., Schubert, I., Köpchen, K., Wendler, E., Schell, J., and Puchta, H. (2000) RecA stimulates sister chromatid exchange and the fidelity of double-strand break repair, but not gene targeting, in plants transformed by *Agrobacterium.* Proc. Natl. Acad. Sci., USA, 97: 3358-3363.

Ricchetti, M., Fairhead, C., and Dujon, B. (1999) Mitochondrial DNA repairs double-strand breaks in yeast chromosome. Nature, 402: 96-100.

Risseeuw, E., Offringa, R., Franke van Dijk, M. E. I., and Hooykaas, P. J. J. (1995) Targeted recombination in plants using *Agrobacterium* coincides with additional rearrangements at the target locus. Plant J., 7: 109-119.

Roeder, G. S. (1997) Meiotic chromosomes: it takes two to tango. Genes Dev., Oct., 11(20): 2600-2621.

Rossi, L., Escudero, J., Hohn, B., and Tinland, B. (1993) Efficient and sensitive assay for T-DNA-dependent transient gene expression. Plant Mol. Biol. Reporter, 11 (3): 220-229.

Roth, D., and Wilson, J. (1988) Illegitimate recombination in mammalian cells. In: Genetic recombination: 621-653. Kucherlapati, R., and Smith, G. R., Eds., American Society for Microbiology. Washington, DC.

Rothstein, R. J. (1983) One-step gene disruption in yeast. Methods Enzymol., 101: 202-211.

Rothstein, S. J., Lahners, K. N., Lotstein, R. J., Carozzi, N. B., Jayne, S. M., and Rice, D. (1987) Promoter cassettes, antibiotic resistance genes and vectors for plant transformation. Gene, 53: 153-161.

Rouet, P., Smith, F., and Jasin, M. (1994) Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. Mol. Cell. Biol., 14: 8096-8106.

Rubin, E., and Levy, A. A. (1997) Abortive gap repair: the underlying mechanism for Ds elements formation. Mol. Cell. Biol., 17: 6294-6302.

Salomon, S., and Puchta, H. (1998) Capture of genomic and T-DNA sequences during double-strand break repair in somatic plant cells. EMBO J., 17 (20): 6086-6095.

Sambrook, J., Fritsch, E. F., and Maniatis (1989) Molecular cloning: a laboratory manual, 2d. ed. Gold Spring Harbor Laboratory Press, N. Y.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977) DANN sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci., USA, 74: 5463-5467.

San-Miguel, P.,Tikhonov, A., Jin, Y. K., Motchoulskaya, N., Zakharov, D., Melake-Berhan, A., Springer, P. S., Edwards, K. J., Lee, M., Avramova, Z., and Bennetzen, J. L. (1996) Nested retrotransposons in the intergenic regions of the maize genome. Science, 274: 765-768.

San-Miguel, P., Gaut, B. S., Tikhonov, A., Nakajima, Y., and Bennetzen, J. L. (1998) The paleontology of intergene retrotransposons of maize. Nat. Genet., 20: 43-45.

Sargent, R. G., Brenneman, M. A., and Wilson, J. H. (1997) Repair of site-specific double-strand breaks in a mammalian chromosome by homologous and illegitimate recombination. Mol. Cell. Biol., 17: 267-277.

Schaefer, D. G., and Zryd, J. P. (1997) Efficient gene targeting in moss *Physcomitrella patens.* Plant J., 11: 1195-1206.

Schär, P., Herrmann, G., Daly, G., and Lindahl, T. (1997) A newly identified DNA ligase of *Saccharomyces cerevisiae* involved in RAD 52-independent repair of DNA double-strand breaks. Genes Dev., 11: 1912-1924.

Schwacha, A., and Kleckner, N. (1997) Interhomolog bias during meiotic recombination: meiotic functions promote a highly differentiated interhomolog-only pathwas. Cell, Sep., 19, 90(6): 1123-1135.

Segal, D. J., and Carrol, D. (1995) Endonuclease-indused, targeted homologous extrachromosomal recombination in *Xenopus oocytes.* Proc. Natl. Acad. Sci. USA, 92: 806-810.

Shinohara, A., and Ogawa, T. (1995) Homologous recombination and the roles of double-strand breaks. Trends Biol., 20: 387-391.

Simpson, G. G., Roe, A., and Lewontin, R. C. (1960) Quantitavive Zoology. Harcourt, Brace & World, New York.

Smith, G. P. (1976) Evolution of repeated DNA sequences by unequal crossover. Science, 191: 528-535.

Smith, G. P. (1989) Homologous recombination in *E. coli*: multiple pathways for multiple reasons. Cell, 58: 807-809.

Smith, K. L., and Nicolas, A. (1998) Recombination at work for meiosis. Curr. Opin. Genet. Dev., Apr., 8: 200-211.

Sonoda, E., Sasaki, M. S., Buerstedde, J., Bezzubova, O., Shinohara, A., Ogawa, H., Takata, M., Yamaguchi-Iwai, Y., and Takeda, S. (1998) Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death. EMBO J., 17: 598-608.

Stewart, S. E., and Roeder, G. S. (1989) Transcription by RNA polymerase I stimulates mitotic recombination in *Saccharomyces cerevisiae*. Mol. Cell. Biol., 9: 3464-3472.

Stougaard, J. (1993) Substrate-dependent negative selection in plants using a bacterial cytosine deaminase gene. Plant J., 3: 755-761.

Stoye, J. P., Fenner, S., Greenoak, G. E., Moran, C., and Coffin, J. M. (1988) Role of endogenous retroviruses as mutagens: the hairless mutation of mice. Cell, 54: 383-391.

Struhl, K. (1983) The new yeast genetics. Nature, 305: 391-397.

Sugawara, N., and Haber, J. E. (1992) Characterization of double-strand break-induced recombination: homology requirements and single-stranded DNA formation. Mol. Cell. Biol., 12: 563-575.

Sugawara, N., Paques, F., Colaiacovo, M., and Haber, J. E. (1997) Role of *Saccharomyces cerevisiae* Msh2 and Msh3 repair proteins in double-strand break-induced recombination. Proc. Natl. Acad. Sci. USA, 94: 9214-9219.

Sugiyama, T., Zaitseva, E. M., and Kowalczykowski, S. C. (1997) A single-strand DNA-binding protein is needed for efficient presynaptic complex formation by the *Saccharomyces cerevisiae* Rad51 protein., J. Biol. Chem., 272: 7940-7945.

Surosky, R. T., and Tye, B. K. (1985) Construction of telocentric chromosomes in *Saccharomyces cerevisiae.* Proc. Natl. Acad. Sci. USA, 82: 2106-2110.

Swoboda, P., Hohn, B., and Gal, S. (1993) Somatic homologous recombination in plants: The recombination frequency is dependent on the allelic state of recombining sequences and may be influenced by genomic position effects. Mol. Gen. Genet., 237: 33-40.

Swoboda, P., Gal, S., Hohn, B., and Puchta, H. (1994) Intrachromosomal homologous recombination in whole plants. EMBO J., 13: 484-489.

Szostak, J. N., Orr-Weaver, T. L., Rothstein, R. J., and Stahl, F. W. (1983) The double-strand break repair model for recombination. Cell, 33: 25-35.

Taghian, D. G., and Nickoloff, J. A. (1997) Chromosomal double-strand breaks induce gene conversion at high frequency in mammalian cells. Mol. Cell. Biol., 17 (11): 6386-6393.

Takano, M., Egawa, H. Ikeda, J. E., and Wakasa, K. (1997) The structures of integration sites in transgenic rice. Plant J., 11: 353-361.

Teng, S. C., Kim, B., and Gabriel, A. (1996) Retrotransposon reverse-transcriptase-mediated repair of chromosomal breaks. Nature, 383: 641-644.

Thykjaer, T., Finnemann, J., Schauser, L. (1997) Gene targeting approaches using positive – negative selection and large flanking regions. Plant Mol. Biol., 35: 523-530.

Tinland, B., Hohn, B., and Puchta, H. (1994) *Agrobacterium tumefaciens* transfers single-stranded transfer DNA (T-DNA) into the plant cell nucleus. Proc. Natl. Acad. Sci. USA, 91: 8000-8004.

Thompson, R. D., Barles, D., Harberd, N. P., and Flavell, R. B. (1983) Characterisation of the multigene family coding for HMW glutenin subunits in wheat using cDNA clones. Theor. Appl. Genet., 67: 87-96.

Tovar, J., and Lichtenstein, C. (1992) Somatic and meiotic chromosomal recombination between inverted duplications in transgenic tobacco plants. Plant Cell, 4: 319-332.

Tsukamoto, Y., Kato, J., and Ikeda, H. (1997) Budding yeast Rad50, Mre11, Xrs2., and Hdf1, but not Rad52, are involved in the formation of deletions on a dicentric plasmid. Mol. Gen. Genet., 255: 543-547.

Valvekens, D., van Lijsebettens, M., and van Montagu, M. (1992) Arabidopsis regeneration and transformation (root explant system). In: Plant Tissue Culture Manual: Fundamentals and Applications. Ed. Lindsey, K., Kluwer Academic Publishers, Dordrecht A8, 1-17.

Van Dyck, E., Stasiak, A. Z., Stasiak, A., and West, S. C. (1999) Binding of double-strand breaks in DNA by human Rad52 protein. Nature, 398: 728-731.

Vergunst, A. C., and Hooykaas, P. J. J. (1999) Recombination in the plant genome and ist application in biotechnology. Crit. Rev. Plant Sci., 18: 1-31.

Voelkel-Meiman, K., Keil, R. L., and Roeder, G. S. (1987) Recombination-stimulating sequences in yeast ribosomal DNA correspond to sequences regulating transcription by RNA polymerase I. Cell, 48: 1071-1079.

Walbot, V. (1996) Sources and consequences of phenotypic and genotypic plasticity in flowering plants. Trends Plant Sci., 1: 27-33.

Wessler, S., Tarley, A., Purugganan, M., Spell, M., and Okagaki, R. (1990) Filler DNA is associated with spontaneous deletions in maize. Pcoc. Natl. Acad. Sci. USA, 87: 8731-8735.

Wilkie, T. M., Braun, R. E., Ehrmann, W. J., Palmiter, R. D., and Hammer, R. (1991) Germ-line intrachromosomal recombination restores fertility in transgenic MyK-103 male mice. Genes Dev., 5: 38-48.

Wilson, T. E., Grawunder, U., and Lieber, M. R. (1997) Yeast DNA ligase IV mediates non-homologous DNA end-joining. Nature, 388: 495-498.

Wood, R. D. (1996) DNA repair in eukaryotes. Annu. Rev. Biochem., 65: 135-167

Wu, T.-C., and Lichten, M. (1994) Meiosis-induced double-strand break sites determined by yeast chromatin structure. Science, 263: 515-518.

Xiao, Y. L., and Peterson, T. (2000) Intrachromosomal homologous recombination in Arabidopsis induced by a maize transposon. Mol. Gen. Genet., Feb., 263(1): 22-29.

Yu, X., and Gabriel, A. (1999) Patching broken chromosomes with extranuclear cellular DNA. Mol. Cell, 4: 873-881.

Zickler, D., and Kleckner, N. (1998) The leptotene-zygotene transition of meiosis. Annu. Rev. Genet., 32: 619-697.

Zickler, D., and Zickler, N. (1999) Meiotic chromosomes: integrating structure and function. Annu. Rev. Genet., 33: 603-754.

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Declaration.

Hereby I declare that all the work presented in this manuscript is my own, carried out solely with help of the literature and aid cited.

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Kirik, A., Salomon, S., and Puchta, H. Species-specific double-strand break repair and genome evolution in plants. Frühjahrsakademie der Gesellschaft für Genetik: "*Development and Evolution: the Effect of Gene and Genome Duplication*", April 21-22, 2001, Wittenberg, Germany.