Homologous recombination in plant cells is enhanced by *in vivo* induction of double strand breaks into DNA by a site-specific endonuclease

Holger Puchta, Bernard Dujon¹ and Barbara Hohn

Friedrich Miescher-Institut, PO Box 2543, CH-4002 Basel, Switzerland and ¹Unité de Génétique Moléculaire des Levures, Département de Biologie Moléculaire (URA 1149/CNRS), Institut Pasteur, 25 rue du Dr Roux, F-75724 Paris-Cedex 15, France

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ABSTRACT

Induction of double strand breaks (DSBs) is coupled to meiotic and mitotic recombination in yeast. We show that also in a higher eukaryote induction of DSBs is directly correlated with a strong enhancement of recombination frequencies. We cotransfected *Nicotiana plumbaginifolia* protoplasts with a plasmid carrying a synthetic *I-SceI* gene, coding for a highly sequence specific endonuclease, together with recombination substrates carrying an *I-SceI*-site adjacent to their homologous sequences. We measured efficiencies of extrachromosomal recombination, using a well established transient β -glucuronidase (GUS) assay. GUS enzyme activities were strongly increased when a plasmid carrying the *I-SceI* gene in sense but not in antisense orientation with respect to the promoter was included in the transfections. The *in vivo* induced DSBs were detected in the recombination substrates by Southern blotting, demonstrating that the yeast enzyme is functional in plant cells. At high ratios of transfected *I-SceI*-genes to *I-SceI*-sites the majority of the *I-SceI*-sites in the recombination substrates are cleaved, indicating that the induction of the DSBs is the rate limiting step in the described recombination reaction. These results imply that *in vivo* induction of transient breaks at specific sites in the plant genome could allow foreign DNA to be targeted to these sites via homologous recombination.

INTRODUCTION

Recombination between homologous DNA sequences plays a central role in the life of all eukaryotes. The current models of homologous recombination postulate the formation of single-strand (ss) nicks or double-strand breaks (DSBs) as primary events (1,2). In yeast DSBs have been detected at the initiation sites for both site-specific gene conversion and meiotic recombination. Physical detection of transient meiotic DSBs in the vicinity of the *ARG4* hot-spot of gene conversion allows a correlation between DSBs and meiotic recombination (3–6).

After the identification of several other sites for meiotic DSBs it now seems that, in a fraction of all cases, meiotic recombination events are initiated by localized DSBs. Yeast mating type switching is due to somatic site-specific gene conversion. The recombination reaction starts with the expression of HO nuclease, which induces a transient DSB within a non-symmetrical 24-bp sequence. The break is subsequently repaired by using an intact ectopic copy of the mating-type alleles (for review see 7). The HO nuclease recognition site has been used to enhance intra- and intermolecular recombination by induction of the HO nuclease expression in yeast cells (8–12).

In higher eukaryotes, to our knowledge, the direct influence of site-specific transient DSBs on recombination has not been studied yet. Indirect evidence indicates, however, that transient DSBs might enhance recombination. In *Drosophila* (13), in *Caenorhabditis elegans* (14) and in maize (15) excision of transposable elements, which is believed to be accompanied by a transient DSB, strongly enhances gap repair using homologous sequences. We wanted to study the influence of a DSB on recombination in plant cells. We chose *I-SceI* as the enzyme for site-specific introduction of DSBs. It is responsible for the mobility of the optional intron Sc LSU.1 within the 21S rRNA gene of yeast mitochondrial DNA (16). It initiates gene conversion events that resemble HO-dependent recombination (for review see 17). *I-SceI* introduces a DSB at the intron insertion site, an 18 bp non-symmetrical sequence, resulting in 4-bp staggered cuts with 3'-OH overhangs (18,19). Although some base substitutions are tolerated within the recognition sequence of *I-SceI*, they strongly reduce the efficiency of cleavage by the enzyme (20,21). Thus even larger genomes, like the nuclear DNA of yeast, are not cleaved by the enzyme (22,23)—a feature that avoids lethal effects on the host cell during *in vivo* expression of the enzyme. *I-SceI* expression, induced in yeast cells, strongly enhanced homologous recombination in a plasmid containing an *I-SceI*-site between direct repeats of the *lacZ* gene (24).

To analyze the influence of DSB induction on recombination in plant cells we performed experiments on extrachromosomal homologous DNA recombination (ECR) with model substrates (for review see 25). ECR is analyzed by transfecting plant

protoplasts with plasmids carrying overlapping, nonfunctional deletion mutants of specific genes—in our case the β -glucuronidase (*uidA*) gene. Recombination frequencies are measured as transient β -glucuronidase (GUS) expression which is due to restoration of the functional *uidA* gene. GUS activity was shown to be directly correlated with the appearance of the restored gene (26–28). Using the described procedure we transfected circular or linearized recombination substrates that contained an *I-SceI* recognition sequence adjacent to the homologous region, together with a plasmid containing the *I-SceI* open reading frame (ORF) under the control of plant expression signals. In comparison to cotransfections with a control plasmid carrying the *I-SceI* gene in antisense orientation, strong enhancement of GUS activity was detected. The induction of DSBs could be correlated to enhancement of recombination in plant cells, since we could detect specific DSBs in the recombination substrates at the *I-SceI* sites.

MATERIALS AND METHODS

Cloning procedures

Standard cloning procedures were used (29). The deletion mutants of the *uidA* gene were obtained from the chimeric functional *uidA* ORF of the plasmid pGUS 23 (26) and cloned into the plasmid pAF100, containing an *I-SceI* site in the middle of the polylinker (30). Using the two primers 5' pGGCAGCAGGG CATTCA-GTCT GGATCGCGAA AAC 3' and 5' pCAGCCGGGAA GC-TTATTCCG ATCTAGTAAC ATAGATGACA CC 3' a fragment from the *BsmI* site of the *uidA* ORF to the nopaline terminator of the gene was amplified by polymerase chain reaction (PCR). The fragment was cut by *HindIII* and *XbaI* and cloned into pUC18, which had been digested with *HindIII* and *XbaI*, to yield the plasmid pUCN1. After digesting this plasmid with *HindIII* and *BamHI* the *uidA* ORF containing fragment was isolated and cloned into pAF100 cut by *HindIII* and *BglII*. In the plasmid obtained, pAF100N1, the N-terminal deletion of the *uidA* gene was placed such that the overlapping region was located directly adjacent to the *I-SceI* site. To obtain a C-terminal deletion mutant of the GUS ORF, the *EcoRI* fragment of pGUS 23 containing the complete gene was inserted into the *EcoRI* site of pUC18. By cutting the respective clone with *HindIII* in the polylinker and *MscI* in the *uidA* ORF, a fragment carrying a C-terminal deletion mutant was obtained and inserted into pAF100 digested with *HindIII* and *EcoRV*. The clone obtained, pAF100C4, contained the 566 bp overlap directly adjacent to the *I-SceI* site. The functional state of the *I-SceI* sites was tested by digesting the plasmids with *I-SceI* meganuclease, as described in the appropriate kit (Boehringer, Mannheim, FRG).

To obtain a plasmid carrying the *I-SceI* gene under the control of plant expression signals, the plasmid pSCM525 carrying a synthetic gene encoding the *I-SceI* endonuclease (A.Thierry and B.Dujon, unpublished data) was used for PCR with the primers 5' pGGCGACGTGG TACCGATTCC AGTACTGTAC CTA-GAATACA AAGAAGAGGA AGAAGAAACC TCTACA-GAAG AAGTGATGAA AAACATCAAA AAAAACCAGG 3' and 5' pGCGCTGACGA GCTCGGATCC AGTACTCGAC TTATTATTTC AGGAAAGT 3'. In the resulting PCR fragment, the *I-SceI* gene is fused to the eukaryotic translational initiation site of cauliflower mosaic virus (CaMV) gene V, to enhance translation in plant cells. The fragment was cut with *BamHI* and cloned into the *BamHI* site between the CaMV 35S promoter and the CaMV terminator of the plasmid pDH51 (31).

The plasmids obtained, p35SISceI+ and p35SISceI–, carried the *I-SceI* gene either in expression or antisense orientation.

Plasmids were propagated and purified by CsCl density gradient centrifugation, as described (26). In some cases defined amounts of plasmid DNA were linearized by digestion with the appropriate restriction enzymes and the digestion pattern was checked by agarose gel electrophoresis. After digestion DNA was extracted with phenol/chloroform (1:1 v/v) and precipitated with ethanol. The DNA concentration of the samples was determined spectrophotometrically.

Isolation and transfection of protoplasts for the analysis of recombination

Preparation of mesophyll protoplasts of *N.plumbaginifolia* from leaves of sterile shoot cultures, and transfection of DNA were done as previously described (26). For the MUG (Methyl-umbelliferyl-D-glucuronide, Sigma) assays 0.3 ml aliquots of freshly isolated protoplasts at a density of 2×10^6 /ml were used per transfection. The protoplasts were incubated 15 hours in the dark before harvesting. Every experimental series included 2 μ g of pGUS 23 as a 'positive control', to assure that the recombination assays were performed under conditions of nonsaturation. As 'background' control for a batch of protoplasts, plasmid DNA was omitted in the transfection procedure.

β -Glucuronidase assays

Soluble extracts of protoplasts were prepared as described (26). GUS activities were assayed fluorometrically with MUG as substrate in the Spectrometer Titertek Fluoroskan II. Typically, 100 μ l of protoplast extract was added to 200 μ l solution of 1 mM MUG in 100 mM Sodiumphosphate pH 7.0, 10 mM EDTA, 0.1 % (v/v) Triton X-100, 0.1 % (v/v) Sarcosyl and 10 mM 2-Mercaptoethanol. At four time points, mostly after 2, 4, 8 and 22 h, 30 μ l aliquots were removed and the reaction was stopped by adding 200 μ l 2 M Na_2CO_3 in Dynatech Microfluor microtiter plates. Average and normalized β -glucuronidase activity were calculated as previously described (26).

Isolation of total DNA from protoplasts and detection of the transient DSB by Southern blotting

Protoplasts (0.6×10^6 per sample) were transfected with plasmid DNA (10 μ g of p35SISceI+ or p35SISceI– and 0.5 μ g pAF100N1 or 0.5 μ g pAF100C4). Total DNA was isolated from the protoplasts 8 hours after transfection, as previously described (27). After restriction digestion with *EcoO109I* and gel electrophoresis, Southern blotting was performed, as described (29), using the hybridization membrane 'Hybond N' (DuPont, Boston, USA). The 0.75 kb fragment, obtained from pGUS 23 by cleavage with *Acc65I* and *MscI* (see 26), was labeled with α - ^{32}P -ATP using the random primed labeling kit of Boehringer (Mannheim, FRG) and was used as hybridization probe.

RESULTS

Experimental setup

Most ECR events in plants can be described best by the single strand annealing (SSA) model of recombination (32–34; for review see 25). SSA events have also been shown to take place in mammalian cells (35), in yeast cells (8), and in frog oocytes (36). The SSA model predicts that a DSB is introduced in each of the substrate molecules (Figure 1; the figure shows the general model except that it includes specific sites for induced DSB

cleavage). DSBs are the entry sites for ss exonucleases which preferentially digest the 5' end. The resulting overhanging 3' ss ends of the two recombination partners are complementary and are thus able to anneal—provided the DSB was within or close to the overlapping sequences of the two recombination partners. After basepairing of the two strands the intermediate structure is repaired using the corresponding 3' ends of the ds DNAs as primers. As both recombination partners suffer degradation of the DNA ends not involved in the exchange, the recombination process is nonconservative. The recombination efficiency is expected to be highest if the location of the DSBs is adjacent to the overlapping regions (37).

In order to investigate whether a specific DSB introduced *in vivo* into a recombination substrate would enhance recombination in plant cells we constructed plasmids that contained nonfunctional parts of a marker gene and the recognition site for a highly sequence specific endonuclease—in our case *I-SceI*. The endonuclease recognition sites were placed adjacent to the overlapping sequence in such a way that the two extra-chromosomal recombination partners would recombine efficiently after *in vivo* DSB induction.

ECR can be quantitatively measured after cotransfection of protoplasts with deletion mutants of the *uidA* gene, by assaying the β -glucuronidase activity (26,28). A non-functional N-terminal and a non-functional C-terminal deletion mutant were each cloned adjacent to an *I-SceI*-site resulting in plasmids pAF100N1 and pAF100C4, as depicted in Figure 2. The overlapping sequence within the *uidA* gene consists of 566 bp (26). To express *I-SceI*

in plant cells, its ORF was fused to the translational start site of cauliflower mosaic virus ORF V to enhance translation in plants. The modified gene was then inserted in both orientations between the CaMV 35S promoter and the CaMV terminator, as depicted in Figure 2. This resulted in the plasmids p35SISceI+ and p35SISceI−.

Recombination is enhanced in cotransfections of the supercoiled recombination substrates with the *I-SceI* gene

When supercoiled circular plasmid DNA of pAF100C4 and pAF100N1 was transfected independently into *Nicotiana plumbaginifolia* protoplasts, no GUS activity could be detected. However, when the plasmids were cotransfected GUS activity was detected. This activity is due to extrachromosomal homologous DNA recombination within the overlapping region of the *uidA* gene, as shown before with similar constructs (26,27).

To test whether expression of *I-SceI* could enhance ECR, the recombination substrates were cotransfected with plasmids carrying the *I-SceI* gene in + and − orientation with respect to the promoter. We found that the GUS activities of extracts of protoplasts cotransfected with the functional *I-SceI* gene were higher than with the control plasmid. The enhancement of recombination was dependent on the ratio of endonuclease genes to recombination substrates. As shown in Table 1, the strongest increase in recombination could be obtained when the molar ratio of p35SISceI+ to the *I-SceI* sites was 10 (all transfected plasmids are about the same size). Higher molar ratios could not be tested, as a further reduction of the amount of transfected recombination substrates would lead to GUS expression in the range of the background. Nevertheless our data clearly indicate that under the experimental conditions used the level of *I-SceI* expression is one of the rate limiting steps in this reaction.

DSBs are induced at the *I-SceI* sites of the recombination substrates

Cotransfection of the recombination substrates with the plasmid p35SISceI+, carrying the *I-SceI* gene in sense orientation, but not of p35SISceI−, carrying the gene in the antisense orientation, showed strongly enhanced recombination dependent GUS activities. This finding suggested that *I-SceI* was enhancing recombination *via* its characterized site-specific endonuclease activity. To directly provide the missing link between the cotransfection of the plasmid and the enhanced recombination efficiencies we searched for the site-specific breaks in the recombination substrates.

Protoplasts were transfected with either 0.5 μ g of pAF100N1 or pAF100C4 DNA, together with 10 μ g of either p35SISceI+ or p35SISceI− DNA. After incubation for 8 hours in the dark, the protoplasts were washed and total DNA was extracted. To detect a transient *I-SceI*-induced DSB in the recombination substrates the DNA was digested with *EcoO109I*. Digestion of pAF100N1 with *EcoO109I* alone should linearize the 4.8 kb plasmid, pAF100N1 already digested with *I-SceI* should result in a 2.3 and a 2.5 kb band, the larger one carrying all the *uidA* specific sequences. Digestion of pAF100C4 with *EcoO109I* alone should result in a 0.4 and a 3.6 kb band, pAF100C4 already digested with *I-SceI* should therefore result in, besides the 0.4 kb band, a 1.3 and a 2.3 kb band, the smaller one carrying all *uidA* specific sequences. Thus the presence of an *uidA*-specific fragment of 2.5 kb in the case of pAF100N1 and of 1.3 kb in the case of pAF100C4 would be indicative for site-specific *in vivo* cutting of the recombination substrates by *I-SceI*.

Figure 1. Scheme of homologous recombination initiated by site specific DSBs. Both recombination substrates contain a homologous region adjacent to which an endonuclease restriction site is located. The recognition sites for the endonuclease (in our case *I-SceI*) are located opposite of the respective 5' or 3' part of the marker gene (in our case the *uidA* gene, symbolized by +). Cotransfection of the substrates with a plasmid carrying the gene of restriction endonuclease leads to the introduction of DSBs at the specific restriction sites *in vivo*. After ss exonuclease digestion complementary single strands are produced, which can anneal to each other in a way that respective parts of the marker gene (+ and +) are combined. Repair of the intermediate leads to the restoration of a functional marker gene, the expression of which therefore is correlated with the recombination efficiency.

Table 1. Relative GUS activity of protoplast extracts after cotransfection of specific amounts of supercoiled circular pAF100C4 and pAF100N1 together with the supercoiled circular plasmid p35SISceI+ or p35SISceI-

DNA amount (in μ g) p35SISceI/pAF100N1/pAF100C4	approximate molar ratio <i>I-SceI</i> genes/ <i>I-SceI</i> sites	normalized GUS activity p35SISceI-	p35SISceI+
5/5/5	0.5	1	2.3 ± 0.5 (4) (Bg < 0.03)
10/2/2	2.5	1	3.2 ± 0.2 (3) (Bg < 0.08)
10/0.5/0.5	10	1	4.5 ± 0.7 (4) (Bg < 0.35)

The average values of the indicated number of different cotransformation experiments (in brackets) are given with standard deviations normalized to the value for the cotransfection with p35SISceI-. Note that due to the different DNA amounts transfected, every line has a different standard.

Bg: Background

Figure 2. Plasmids used for the transfection experiments (individual parts not to scale). The construction of the clones is described in detail in Materials and Methods. In the upper part, below the positive control plasmid (pGUS 23) the two recombination substrates pAF100N1 and pAF100C4 are depicted. The bold arrows indicate the *I-SceI* sites in the constructs that were used *in vivo* induction of DSBs. The *HindIII* and *Sall* sites in the construct were used for *in vitro* introduction of DSBs. The *EcoO109I*-sites used for the detection of *I-SceI*-specific cleavage in the recombination substrates are also depicted. In the lower part the expression vectors for the sense (p35SISceI+) and the antisense (p35SISceI-) *I-SceI* gene are shown (35S: cauliflower mosaic virus 35S promoter; Nos T: nopaline synthetase terminator; CaMV T: cauliflower mosaic virus terminator).

Figure 3 shows the results of the described assay. After hybridization with the *uidA* gene specific probe the diagnostic band of 2.5 kb of pAF100N1 cut *in vivo* by *I-SceI* was only seen in the cotransfection with sense (lane 2) but not with the antisense construct of *I-SceI* gene (lane 1). The corresponding result was found for pAF100C4; the *I-SceI*-digest specific 1.3 kb band is visible in lane 3 (cotransfection with p35SISceI+) but not in lane 4 (cotransfection with p35SISceI-). Thus both recombination substrates were cleaved specifically at their *I-SceI*-sites, *in vivo*. DSBs can be introduced into the major part of the active recombination substrates

DSBs can be introduced into the major part of the active recombination substrates

The previous experiment demonstrated in a qualitative fashion that DSBs were indeed introduced at the *I-SceI* recognition sites of the recombination substrates. However, no quantitative information about the total number of introduced DSBs could be obtained with this experiment, for two reasons: 1) In the Southern blot all plasmid DNA associated somehow with the protoplasts is visible. However, only a fraction of it residing inside the cell is likely to be accessible to newly expressed *I-SceI*; 2) The life time of a DSB introduced by *I-SceI* in plant

cells is not known. Only a fraction of all molecules that are cleaved throughout the time span of the experiment might be in a broken configuration at any given moment.

In order to get an estimate of the percentage of molecules that were cut and underwent recombination, a reconstruction experiment was performed. Molecules resembling *in vivo* cut recombination substrates were cotransfected, and their recombination efficiency was compared to the one of the molecules site-specifically cleaved *in vivo* in the protoplasts. To allow for a true comparison, all recombination substrates had to be linearized first by cleavage outside the regions involved in site-specific cutting. *HindIII* was used for this purpose. It cuts the plasmids within a polylinker before the 35S promoter of pAF100C4 and the nopaline terminator of pAF100N1 (see Figure 2). As larger amounts of *I-SceI* endonuclease were not available to us, we substituted the enzyme by *Sall*, the recognition site of which is immediately adjacent to the *I-SceI* site. The use of *Sall* for the *in vitro* cutting was justified by the fact that we were not able to detect significant differences in the recombination behavior of plasmids cut with different enzymes within the same polylinker (32). Since a second *Sall* site is present next to the *HindIII* site in both pAF100N1 and pAF100C4, a digestion of *Sall* cut substrates with *HindIII* was not necessary. Thus a

Figure 3. Detection of DSBs introduced into the recombination substrates by expression of *I-SceI* in plant protoplasts. Southern blot of a gel in which *EcoO109I*-digested DNA from protoplasts transfected with the respective recombination substrate and p35SISceI+ or p35SISceI- was separated. As hybridization probe the *Acc65I-MscI* fragment of the *uidA* ORF was used. Lane 1: *EcoO109I*-digested DNA extracted from protoplasts transfected with pAF100N1 and p35SISceI-; lane 2: *EcoO109I*-digested DNA extracted from protoplasts transfected with pAF100N1 and p35SISceI+; lane 3: *EcoO109I*-digested DNA extracted from protoplasts transfected with pAF100C4 and p35SISceI-; lane 4: *EcoO109I*-digested DNA extracted from protoplasts transfected with pAF100C4 and p35SISceI+. The arrows mark the bands in lanes 2 and 4 that are indicative for *in vivo* induction of a DSB in the respective recombination substrate. The strong upper bands reflect *EcoO109I*-digested recombination substrates, the two weak bands present in all lanes are due to weak cross hybridization of the *uidA*-probe with a large excess of *EcoO109I*-digested p35SISceI+ or p35SISceI- plasmid DNA.

Table 2. Efficiency of homologous recombination of plasmids with DSBs introduced *in vivo* or *in vitro* adjacent to the homologous region. Relative GUS activity of protoplast extracts after cotransfection of 1 μ g each of cut plasmids pAF100C4 and pAF100N1 with 10 μ g of the supercoiled circular plasmid p35SISceI+ or p35SISceI-

recombination substrates cut by	cotransfected with	normalized GUS activity
<i>HindIII</i>	p35SISceI-	0.06 $\pm$ 0.02 (6)
<i>HindIII</i>	p35SISceI+	0.58 $\pm$ 0.05 (6)
<i>Sall</i>	p35SISceI-	1

The average values of the indicated number of different cotransformation experiments (in brackets) are given with standard deviations normalized to the value for the cotransfection of pAF100C4 *HindIII*/pAF100N1 *HindIII* with p35SISceI+ (Background < 0.03).

recombination substrate that is linearized *in vitro* by *HindIII* and that is further cut *in vivo* by *I-SceI* should functionally be equivalent to an *in vitro* *Sall* digested recombination substrate (see Figure 2). Cotransfections of the *Sall* cut recombination substrates should therefore reflect the maximum of recombination after complete DSB induction and cotransfection of the *HindIII* digested recombination substrate should represent the normal rate of non-*I-SceI*-induced recombination.

Cotransfections were performed using 1 μ g of each recombination substrate and 10 μ g of the respective p35SISceI plasmid which represents a molar ratio of about 5/1 between *I-SceI* genes and *I-SceI* sites. The results of six experiments are

represented in Table 2. Surprisingly, the linearized recombination substrates exhibited a more pronounced effect of *I-SceI* expression on recombination (enhancement by a factor of 10) than the circular substrates (enhancement by a factor of 4.5). The reason for this is not known but one possible explanation is that linear substrates are more easily cut by *I-SceI*. The recombination efficiency detected in the cotransfections of the *HindIII* digested recombination substrates with the *I-SceI* sense construct was more than half that of the *Sall* cut plasmids. This indicates that a large fraction of the functional recombination substrates are cleaved *in vivo* by *I-SceI*. Thus, under appropriate conditions, *in vivo* cleavage by *I-SceI* can lead to an optimal enhancement of recombination.

DISCUSSION

DSBs and recombination

DSBs are known to trigger homologous recombination. Two recombination models, the single strand annealing (SSA) model (35) and the double strand break repair (DSBR) model (2) have been proposed to explain this phenomenon. We wanted to test the effect of an *in vivo* introduced DSB at a given site on extrachromosomal homologous DNA recombination in plant cells. As ECR is described best by the SSA model, sites which are specifically cut *in vivo* were placed directly adjacent to the homologous sequences (see Figure 1). As the site for the introduction of specific DSBs, we chose the 18 bp long target sequence of the enzyme *I-SceI*. We purposely used this enzyme, which cuts only very rarely, because of later experiments involving recombination with plant genomic DNA (see below). DNA substrates containing *I-SceI* sites adjacent to their overlapping sequences of the *uidA* gene were cotransfected into *N.plumbaginifolia* protoplasts, along with a plasmid containing the gene for the *I-SceI* endonuclease under the control of a strong plant promoter.

We show that indeed *I-SceI* is inducing specific DSBs in plant cells. We show further that these DSBs enhance homologous recombination. These conclusions are based on the following observations: 1) The expression of the *I-SceI* gene resulted in a DSB in both recombination partners, at the expected positions (Figure 3). 2) Cotransfection of plasmids containing an *I-SceI* restriction site adjacent to their homologous sequences, together with a plasmid carrying the *I-SceI* gene in sense but not in antisense orientation, resulted in elevated recombination frequencies. The increase ranged from a factor of 4.5 for circular (Table 1) to 10 for linear ones (Table 2). 3) The efficiency of homologous recombination increased in parallel with increasing molar ratios of the plasmid expressing *I-SceI* to the *I-SceI* sites (Table 1). These results imply that the rate limiting step in the recombination reaction is, at least under the employed assay conditions, the production of DSBs close to the recombining sequences.

The efficiency of DSB induction in plant cells

An *a priori* unknown factor in these experiments was the efficiency of the *in vivo* induction of a DSB. A low efficiency could be due to poor expression or stability of the enzyme that normally is active in the environment of yeast mitochondria. The enzymatic machinery for ECR is expected to reside in the plant cell nucleus, as has been shown for mammalian cells (38). Therefore the *I-SceI* protein has to be targeted to the plant nucleus. However, it does not contain any known nuclear targeting signals.

Nevertheless, our experiments indicate that it does reach the plant cell nucleus, as it has been shown for the yeast nucleus (24). However, cleavage of substrate DNA by cytoplasmatically located restriction endonuclease, followed by entry of the cleaved DNA molecules in the nucleus cannot be excluded. The restriction enzyme has to be produced first in the transfected protoplasts before it can introduce a DSB in the recombination partners. In an earlier report we described that extrachromosomal recombination between DNA molecules takes place during a limited time period soon after transfection (27). As *I-SceI* expression is most probably rate limiting, *I-SceI*-induced recombination probably takes place later than the noninduced one.

The Southern experiment (Figure 3) demonstrated site specific cleavage in both recombination substrates qualitatively, but not quantitatively. A reconstruction experiment comparing *in vitro* and *in vivo* introduced site specific cleavage allowed the conclusion that under the employed condition most probably more than half of the substrate molecules that are eventually engaged in homologous recombination were cleaved *in vivo*. Since GUS enzyme accumulates with time in the protoplasts and since parts of the *in vivo* cut molecules might have recombined later than the *in vitro* cut material (see above), we might even underestimate the number of restored *uidA* molecules. The DSB-induced recombination is—under appropriate conditions—highly efficient. This is important information for the setting up of a site-specific integration system based on the *in vivo* induction of recombinogenic DSBs in the plant genome.

Induction of DSBs in the genomes of higher eukaryotes—a general strategy for homology dependent site-specific integration?

Transient expression was used before to test the functionality of site-specific recombination systems in plant cells. In this way the *cre* recombinase (39), *gin* recombinase (40), the *R* recombinase of *Zygosaccharomyces rouxii* (41), and the *FLP* recombinase (42) were shown to be functional and applicable in plant cells. The *cre-lox* system has been used to remove selectable marker genes from (43,44) or to integrate a segment of DNA at a specific location into the plant genome (D.Ow, personal communication). In the latter approach plants are transformed first with a specific DNA fragment carrying the recognition site of the recombinase. In a second step an extrachromosomal piece of DNA carrying the same recognition site is integrated in this position via expression of the recombinase. A problem that is not totally solved yet is the reversibility of the reaction. As long as recombinase is expressed, an equilibrium between integration and removal of the specific DNA fragment is established. Stable integration products have been observed, and some of these appear to have achieved stability through the loss of one of the flanking *lox* sites (D.Ow, personal communication). Site-specific recombinases perform in principle reversible reactions. In contrast to this, intron-homing, the natural activity of *I-SceI* in yeast mitochondria, is irreversible. Thus DSB induced homologous recombination would combine site-specificity with irreversibility. Transformation of plants with a construct containing an *I-SceI*-site has been done. Expression of *I-SceI* in any plant transgenic for the recognition sequence should lead to activation of the target site by the induction of a DSB. The DSB can then—as described by the DSB model—be repaired by an extrachromosomal segment of DNA homologous to the sequences adjacent to the *I-SceI*-site. During the repair process the *I-SceI*-site in the genome will be removed, thereby rendering the integration reaction

irreversible. The expression of *I-SceI* is not expected to have a lethal effect on the plant cell, as the 18 bp recognition site is found statistically only once in 6.9×10^{10} bp, which is the equivalent of about 70 *Arabidopsis* and four tobacco genomes. This system will allow further studies on the integration of any foreign segment of DNA into specific positions of the plant genome.

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