

## Site-specific recombination in *Arabidopsis* plants promoted by the Integrase protein of coliphage HK022

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Received 31 May 2004; accepted in revised form 31 December 2004

**Key words:** *Arabidopsis*, Integrase, nuclear localization, phage HK022, site-specific recombination

### Abstract

The gene encoding the wild type Integrase protein of coliphage HK022 was integrated chromosomally and expressed in *Arabidopsis thaliana* plants. Double-transgenic plants cloned with the *int* gene as well as with a T-DNA fragment carrying the proper *att* sites in a tandem orientation showed that Int catalyzed a site-specific integration reaction (*attP* × *attB*) as well as a site-specific excision reaction (*attL* × *attR*). The reactions took place without the need to provide any of the accessory proteins that are required by Int in the bacterial host. When expressed in tobacco plants a GFP-Int fusion exhibits a predominant nuclear localization.

**Abbreviations:** bp, base pairs; CaMV, cauliflower mosaic virus; GFP, green fluorescent protein; IHF, integration host factor; Int, Integrase; LB, Luria–Bertani medium; PCR, polymerase chain reaction; Xis, excisionase

### Introduction

In the absence of efficient homologous recombination, site-specific recombination systems of lower organisms have become important tools for gene manipulations and for the formation of transgenic plants and mammals. In these systems the recombinase catalyzes a recombination reaction between two DNA molecules that carry specific short sequences. In transgenic plants, these systems are used to excise genes that are no longer desired, such as genes for antibiotic resistance. The two most commonly used recombinases are Cre of phage P1 and FLP of yeast, whose recombination targets (*lox* and *frt*, respectively) are 34 base pairs (bp) long. Except for the recombinases and their

identical pairs of DNA targets no other proteins are required for the recombination reactions. The disadvantage of these systems is that the integration and excision reactions are reversible and because excision is thermodynamically favored, gene insertions by integration reactions are more difficult to obtain (reviewed in Nagy, 2000; Baer and Bode, 2001; Ow, 2002; Lyznik *et al.*, 2003). A prokaryotic site-specific recombination system that proved to be efficient for stable site-specific gene insertions in mammals is that of the *Streptomyces* phage  $\phi$ C31 (Thyagarajan *et al.*, 2001; Groth and Calos, 2004). That is because its recombinase catalyses an integration reaction between two sites that are not completely identical. As a result the integrated prophage is flanked by

recombinant sites that are no more recognized by the recombinase alone (Thorpe *et al.*, 2000).

By using site-specific recombination systems for gene manipulations the sites of each system, and sometimes also the recombinase gene itself, are left behind in the genome of the transgenic organism. Therefore, if several gene manipulations are necessary in a specific organism, the recruitment of new site-specific recombination systems may be useful (Puchta, 2003).

The well-studied site-specific recombination systems of coliphages  $\lambda$  and its close relative HK022 are much more complex. They are used by the phage for its integration and excision into and out of the host chromosome by site-specific recombination reactions that are catalyzed by the phage-encoded Integrase (Int) protein. The recombination site on the host chromosome (*attB*) is composed of a core of 21 bp, and its partner site on the phage chromosome (*attP*) is over 200 bp long. In addition to a similar core, *attP* is composed of two arms that carry tight binding sites for Int and for accessory proteins that are required for the recombination reactions. As a result of the integration reaction, the formed prophage is flanked by the two recombinant sites *attL* and *attR* that serve as sites of the reverse excision of the phage. Int catalyzes both integration and excision reactions. For the integration reaction it requires the assistance of the host-encoded DNA-bending protein Integration Host Factor (IHF) and for the excision reaction Int also requires Excisionase (Xis), a second DNA-bending protein that is encoded by the phage (reviewed in Nash, 1996; Weisberg *et al.*, 1999; reviewed in Azaro and Landy, 2001). The differences in the protein requirement and in the structure of the recombination sites between the integration and excision processes bestow on the labmdoid Int system a potential unidirectional nature. However, this potential advantage has not yet become apparent in eukaryotic cells because the HK022 and  $\lambda$  systems, that were both cloned and expressed in mammalian cells, were shown to be active in integration as well as in excision without the need to supply any of the accessory proteins that are required in the bacterial reaction (Corona *et al.*, 2003; Kolot *et al.*, 2003).

Thus far the activity of the  $\lambda$  or the HK022 systems has not been tested in the plant kingdom. Here we show that Int, when transiently expressed

in plant cells is localized in the nucleus. In the presence of proper *att* sites in stable transformed transgenic *Arabidopsis thaliana* plants Int can catalyze integrative and excisive site-specific recombination in the plant milieu.

## Materials and methods

### *Plasmids and oligomers*

These are listed in Table 1

### *Media and growth conditions*

*Escherichia coli* was grown in Luria–Bertani (LB)-rich medium and when necessary, supplemented with the proper antibiotic(s) as recommended (Sambrook *et al.*, 1989).

*Agrobacterium tumefaciens* GV3101 *pMP90* (Koncz and Schell, 1986) was grown at 28 °C in LB broth in the presence of 50  $\mu$ g/ml gentamycin sulfate. Transformants were selected in the presence of the proper antibiotic.

*Arabidopsis thaliana* (*ecotype columbia glabra*) was grown at 18–22 °C, under a 16 h light 8 h dark photoperiod in a growth room.

*Nicotiana benthamiana* (tobacco) plants were grown at 25 °C, under a 16 h light 8 h dark photoperiod in a growth room.

### *Plasmid construction*

pAR1 is the plasmid that expresses Int cloned on the hygromycin-resistant binary vector pCambia 1300. A  $\sim$ 1.1 kb *NcoI-HindIII* fragment that carries the *int* gene of phage HK022, replaced the *NcoI-SacI* GUS fragment of plasmid pJD330 with the help of a *HindIII-SacI* adaptor. The 35S-*int-nos* (terminator) *PstI-EcoRI* fragment of the resulting plasmid was cloned into the same sites of pCambia 1300.

pAR2 is the plasmid that carries the 35S-*attR-Stop-attL-GFP-nos* excision substrate on the T-DNA segment of the kanamycin-resistant binary vector pGA492. Stop is a transcription terminator extracted from plasmid pBS302 (Sauer, 1993). First, the single *EcoRI* site of plasmid pJD330 was inactivated by cutting, filling-in and religation. Then, its *NcoI-SacI* GUS fragment was replaced with an *NcoI-EcoRI-NotI-SacI* adaptor.

Table 1. List of plasmids and oligomers.

Name	Description	References
<i>Plasmids</i>		
pAR1	Int expression in pCambia 1300	This work
pAR2	Excision substrate in pGA492	This work
pBinGFP	GFP-expressing derivative of pBINPLUS	(Liarzi and Epel, 2004)
pBINPLUS	Kanamycin-resistant binary vector	(van Engelen <i>et al.</i> , 1995)
pCAMBIA 1300	Hygromycin-resistant binary vector	<a href="http://www.cambia.org/main/r_et_camvec.htm">http://www.cambia.org/main/r_et_camvec.htm</a>
pGA492	Kanamycin-resistan binary vector	(An, 1995)
pJD330	35S-GUS cloned on Bluescript	(Shalev <i>et al.</i> , 1999)
pPG279	Integration substrate in pBinGFP	This work
pPG290	Recombination product of pAR2	This work
pPG280	Recombination product of pPG279	This work
pVB254	GFP-Int fusion	This work
<i>Oligomers</i>		
oEY192	gctctagaattcatgggaagaaggcgaagtcgatg	<i>int</i> HK022
oEY193	gctctagatttgcgatttcaattttgtccc	<i>int</i> HK022
oEY206	cgctgcgctccagctcgaccag	GFP
oEY208	caagacccttcctctatataag	35S promoter
oEY209	ttatcctagtttgcgcgcta	<i>nos</i> terminator

Next, the *EcoRI-attR-Stop-attL-GFP-NotI* fragment of plasmid pMK189 (Kolot *et al.*, 2003) was inserted between the *EcoRI* and *NotI* sites of the modified pJD330 vector. The cloning of the 35S-*attR-Stop-attL-GFP-nos* cassette onto the binary vector pGA492 was performed in two steps. First, the upstream *XbaI-Bg/III* fragment of the cassette (cloned on the pJD330 vector) was cloned into the same sites of pGA492 and then the remaining downstream *Bg/III* fragment of the cassette was cloned into the *Bg/III* site of the modified pGA492 vector, next to the upstream portion.

pPG279 is the plasmid that carries the 35S-*attP-Stop-attB-GFP-nos* sequence in the kanamycin-resistant binary vector pBINPLUS. A ~1.8 kb *EcoRI* fragment that carries the *attP-Stop-attB-GFP* sequence was extracted from plasmid pMK218 (Kolot *et al.*, 2003) and was inserted into the *SalI* site of pBinGFP with the help of a *XhoI-EcoRI* adaptor.

pVB254 is the plasmid that expresses the GFP-Int fusion. The *XbaI-PstI* fragment of GFP from plasmid pBinGFP was replaced with GFP-Int fusion from pMK181 (Kolot and Yagil, 2003) using the same restriction sites.

#### DNA extraction

Plasmid DNA preparations from *E. coli* were done using a Nucleospin kit (Macherey-Nagel).

Genomic DNA was extracted from plants by the CTAB method (Bernatzky and Tanksley, 1986)

#### Transformations

*Escherichia coli* and *Agrobacterium* were transformed by electroporation. Transformation of *Arabidopsis* was done by the floral dip method (Clough and Bent, 1998).

#### Transient expression of GFP

*Agrobacterium* cells transformed with plasmids pBinGFP or pVB254 were grown in a *vir*-Induction medium for 8 h and injected into *Tobacco benthiana* leaves (Ron and Avni, 2004). Protein localization was observed 72 h later under a fluorescent microscope.

#### Northern blot

Total RNA was isolated from rosette leaves of *Arabidopsis* using the SV total RNA isolation kit (Promega, Madison, USA). About 12 µg of RNA were separated on a 1.2% Agarose-MOPS gel, transferred to a positively charged nylon membrane (Roche Diagnostics GmbH, Germany) and hybridized to a DIG-labeled probe according to the manufacturer's instructions.

### Immunoblot

Plant tissue samples were frozen in liquid nitrogen, pulverized and suspended in lysis buffer [1 M sorbitol, 10 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 5 mM NaF, 0.1 M KCl, 0.2% Triton X-100 and a 1:20 dilution of a complete protease inhibitor mixture (Roche)]. The samples were homogenized, centrifuged and the supernatants were treated with diafiltration in Vivaspin 20 (Vivascience) against dialysis buffer (10 mM HEPES, 1 mM EDTA, 1 mM EGTA). The samples were stored at  $-70^{\circ}\text{C}$  until analysis. 10 and 20  $\mu\text{l}$  of each sample were separated by SDS polyacrylamide gel electrophoresis and underwent an immunoblot as previously described (Kolot *et al.*, 1999). The Int-antiserum was purified by protein A adsorption.

### Southern blots

One to two  $\mu\text{g}$  of *Arabidopsis* genomic DNA were cut with *EcoRI* and separated on 1% agarose gel. Alkaline transfer and hybridization were done according to the 'GeenScreen' manual (DuPont). The probe used was a 150 bp DNA fragment that carried the *attB* site that was  $^{32}\text{P}$ -labeled by the random-priming method.

### In vitro site-specific recombination reactions

The integration and excision reactions were essentially performed as previously described for the excision reaction (Gottfried *et al.*, 2001). In the integration reaction Xis was omitted. The products were transformed to *E. coli* cells and plasmid DNA was prepared from a transformant that carried the recombination product and the correct sequence was confirmed.

## Results

### Expression of HK022 Integrase in *Arabidopsis* plants

The *int* gene of HK022 was cloned under the constitutive cauliflower mosaic virus (CaMV) 35S promoter onto the T-DNA segment of the hygromycin-resistant binary vector pCambia 1300 (plasmid pAR1). *Arabidopsis* plants were trans-

formed with the T-DNA segment and a northern blot of five transformed plants (Figure 1) showed different quantities of an *int* transcript that was absent in the untransformed wild type control (Figure 1, lane WT). Leaf extracts of two of the transgenic plants whose transcription signal was the strongest (numbers 29, 33) were used for an immunoblot to test the expression of Int, using antiserum raised against Int-HK022 (Figure 2). Both plants clearly expressed the protein that was absent in the untransformed wild type control. All transgenic plants (about 50) grew normally and did not show any phenotypic aberrations.

### *Int* is located in the nucleus

It has been previously shown that Int, when expressed in human cells, is localized exclusively in the cell nucleus (Kolot and Yagil, 2003). To test if this is also the case in plants we transiently expressed the protein in tobacco leaves (Ron and Avni, 2004). The GFP-Int gene fusion previously constructed for mammalian cells was cloned downstream to the 35S promoter on the pBINPLUS binary vector. *Agrobacterium* cells transformed with this plasmid were injected into leaves of tobacco plants and 2 days later the leaves were observed under a UV microscope (Figure 3A and B). As a control, leaves were also injected with a plasmid (pBinGFP) that expresses GFP alone (Figure 3C). It is evident that in the plant, as well, the GFP-Int fusion is predominantly localized in the plant nucleus whereas the GFP alone is distributed in the cortical cytoplasm and the nucleus.

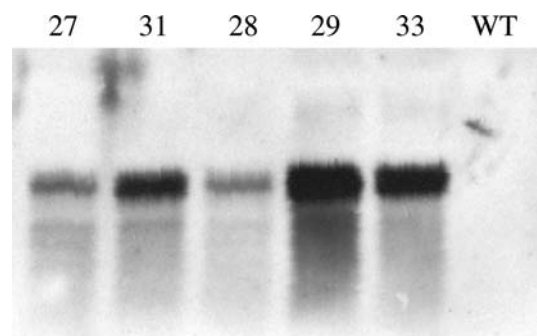


Figure 1. Northern blots of five plants transformed with the *int* gene. The probe was a PCR *int*-DNA fragment generated with oligomers oEY192 and oEY193. WT shows an untransformed control.

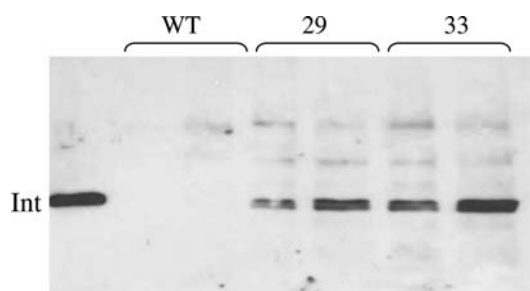


Figure 2. Immunoblot of *int*-transgenic plants. Extracts of plant numbers 29 and 33 were tested for Int-expression by a western blot analysis using polyclonal Int-antiserum. Each sample was tested with 10 and 20  $\mu$ l of cell extract. WT is an untransformed control. The left lane shows purified Int.

#### Excision activity in *Arabidopsis*

To test whether Int is active in the *Arabidopsis* plants the kanamycin-resistant binary vector pGA492 was constructed with a T-DNA segment that carries tandem *attR* and *attL* excision sites (top sites in Figure 4) that flank a transcription terminator (Stop, Figure 4A). The *attR*-Stop-*attL* sequence is flanked on its proximal end by the constitutive CaMV 35S promoter and on its distal end by the open reading frame of the reporter gene that encodes the green fluorescent protein (GFP), followed by the nopaline synthetase (*nos*) transcription terminator (plasmid pAR2). An excisive recombination reaction between *attR* and *attL* evicts the  $\sim$ 1.8 kb long Stop and *attP* circular sequence, leaving the 21 bp *attB* sequence between the 35S promoter and the GFP gene with the intention to allow the 35S-promoted expression of the GFP (Figure 4B).

Progenay of the transgenic *Arabidopsis* plant (number 33) that expressed the Int protein were transformed with the excision substrate (plasmid pAR2) described above. By selecting progeny resistant to hygromycin and kanamycin double transformants were generated. Leaves of three double-transgenic individual plants that were obtained did not show any fluorescence. However, to test for possible excision activity we performed PCR analyses using as templates DNA extracted from these three double-transgenic plants and control plants that carry only the excision substrate. One of the primers represented the sequence of the 35S promoter (arrow 'a' in Figure 4) and the second primer carried a sequence of the *nos* terminator (arrow 'c'). Figure 5A shows the results of PCR reactions using control plasmids and genomic DNA as templates. Lanes a–c under 'plasmids' show, respectively, the PCR products using the Int-expressing plasmid (pAR1, lane a), the plasmid that carried the *attR*  $\times$  *attL* substrate (pAR2, lane b) and a plasmid (pPG290, lane c) that was a product of an *in vitro* site-specific recombination reaction using plasmid pAR2 as substrate. The latter reveals the size of the expected product. Lanes d–f in Figure 5A (under 'plant') represent the PCR products obtained from genomic DNA of an untransformed control plant (lane d), of a plant that carried the substrate only (lane e) and from one of the double-transgenic plants that expressed Int and carried the excision plasmid (lane f). The size of the PCR product of the substrate plant agrees with that of the substrate control (compare lanes b and e). All three double-transgenic plants that carried both the *int*

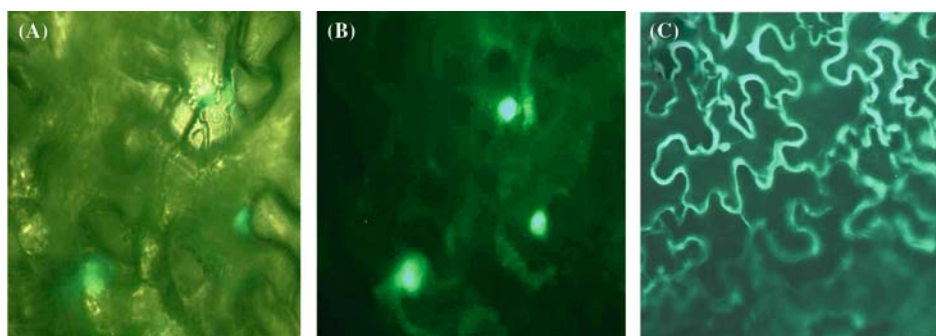
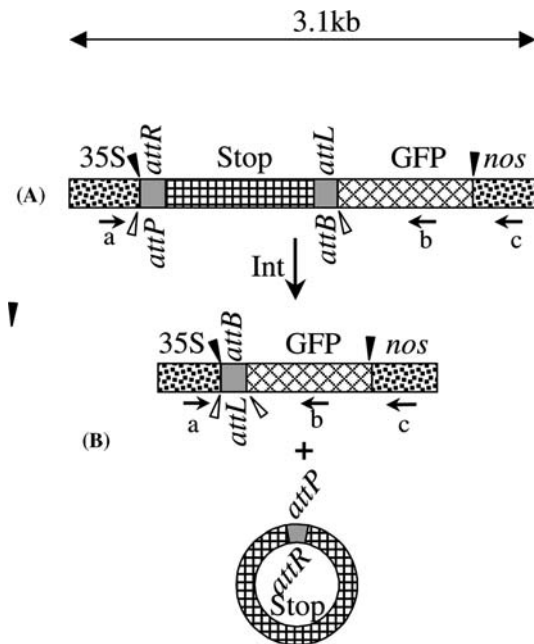


Figure 3. Microscopy of tobacco leaves injected with *Agrobacterium* transformed with GFP plasmids. (A) Light microscopy of leaves transformed with the GFP-Int fusion plasmid (pVB254). (B) Fluorescent microscopy of leaves transformed with the same plasmid. (C) Fluorescent microscopy of leaves transformed with the GFP-expressing plasmid (pBinGFP).



**Figure 4.** A schematic representation of the excision substrate (*attL* and *attR*) and of the integration substrates (*attP* and *attB*) cloned in the T-DNA of binary plasmids. (B) The excision products (*attB* and *attP*) and integration product (*attL* and *attR*) of the Int-promoted site-specific recombinations reaction. Stop is a transcription terminator derived from plasmid pBS302. The arrows show the primers used for the various PCR reactions. The closed triangles point to the *Eco*RI sites that are relevant for the Southern blot analysis of the excision reaction, and the open triangles show the *Eco*RI sites relevant for the Southern analysis of the integration reaction.

gene and the excision substrate yielded three PCR fragments as depicted for one of them in Figure 5A, lane f. The size of the largest fragment (S) is identical with that of the substrate (compare to lanes b and e). The smallest fragment (P) is identical with the product (compare to lane c) and the middle one (I) is identical to the *int* gene (compare to lane a). With these two primers ('a' and 'c' in Figure 4) the appearance of the *int* fragment is expected, because the *int* gene was also cloned between the 35S promoter and the *nos* terminator. In order to support this interpretation, a second PCR reaction was performed with DNA of the double-transgenic plant and the relevant control plasmids, using the same 35S oligomer ('a') as one primer and a second primer that represents a sequence of the GFP gene (arrow 'b' in Figure 4). This PCR is expected to eliminate the *int* product. The size of the two major products obtained from the double-transgenic plant (lane i

in Figure 5B) agrees with those of the substrate (lane g) and the expected product (lane h). The length of all PCR products indicated in parentheses in Figure 5A and B were all as expected. To verify the identity of the small PCR product (P) obtained from the plant DNA in lane i, it was extracted from the gel, purified, subcloned onto the vector pGEM-T Easy (Promega) and sequenced. The sequence agreed perfectly with that of 35S-*attB*-GFP (not shown) indicating that Int has catalyzed the expected excision reaction depicted in Figure 4. T2 progeny of the double-transgenic plants were plated on kanamycin to look for segregants that carried either the substrate or the product alone. Several progeny showed the substrate alone [the DNA of one of them was used as the control (lane e in Figure 5)], others carried the substrate and product, but among 250 offspring tested none was found to carry the product alone, indicating that the recombination was a rare event. DNA of segregants that carried *int*, the substrate and product was analyzed in a Southern blot to confirm that both the substrate and the expected excision product were chromosomally located (Figure 5C). The templates in the Southern analysis were control plasmids and genomic DNA all cut with *Eco*RI (closed triangles in Figure 4). The probe was a 150 bp-long DNA fragment that carried the sequence of *attB* flanked with residual linker restriction sites. As positive controls we used restricted DNA of the two plasmids that were used in the PCR reactions. One (pAR2), that carried the substrate (S in Figure 5C, lane a), and the other (pPG290) that carried the recombination product (P in lane b). As negative control we used genomic DNA of an untransformed plant (lane c). Lane d shows the presence of the substrate in the DNA of a plant with the substrate alone and lane e shows the presence of substrate and the product in the DNA of a plant that carried the substrate and the *int* gene. We have no convincing explanation for the higher bands in the blot of the double-transgenic plant (lane e) since it did not appear in the other plants (lanes c and d). It could either be partially cut DNA or due to the *int* gene that carried some of the restriction linker sequence as did also the probe. The signal of the product P in the blot of the double transgenic plant is rather weak, indicating a low rate of recombination, however the blot confirms that both the substrate

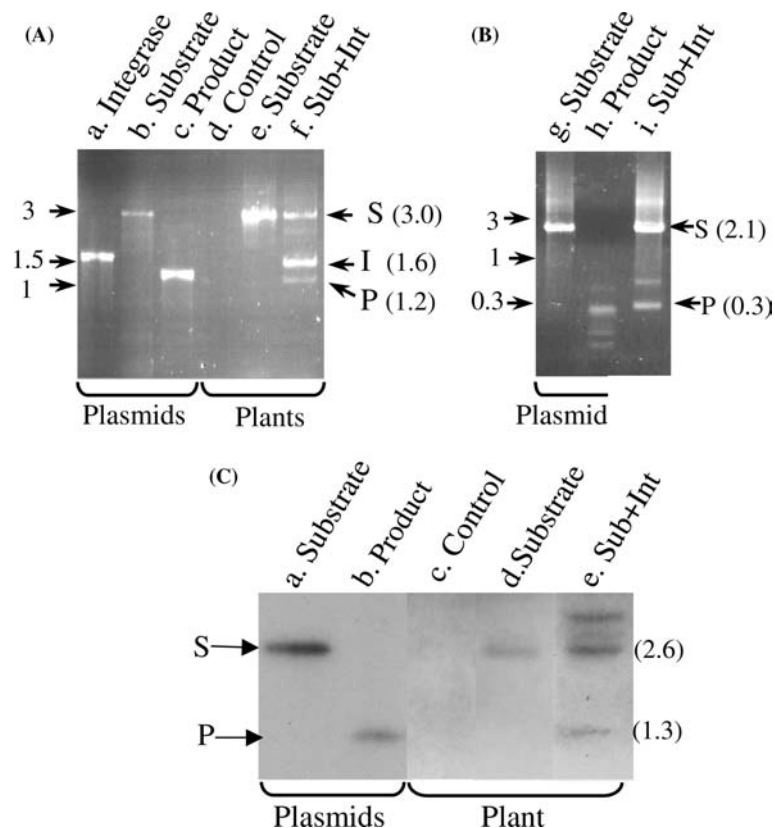


Figure 5. (A) PCR analyses of the excision reaction using oligomers a (oEY208) and c (oEY209) as primers. (B) PCR analyses of the excision reaction using oligomers a (oEY208) and b (oEY206) as primers. (C) Southern blot of the excision reaction (see text for further explanations).

and the recombination product are a part of the plant stable genome.

#### Integration activity in *Arabidopsis*

To test whether Int can also promote the integration reaction ( $attP \times attB$ ) in *Arabidopsis*, a similar T-DNA substrate was constructed on the kanamycin-resistant binary vector pBINPLUS (plasmid pPG279), except that the recombination sites were  $attP$  and  $attB$  (bottom sites in Figure 4A). Because these two sites are in the *cis* configuration (both on the same DNA) this recombination reaction actually excises a DNA fragment. Nevertheless it is considered as an integration reaction owing to the structure of the  $att$  sites that differs from  $attL$  and  $attR$ . The chromosomal product of the integration reaction is expected to carry an  $attL$  site between the 35S promoter and the open reading frame of the GFP (Figure 4B). Wild type *Arabidopsis* plants

were transformed with this integration substrate (pPG279) by selecting kanamycin-resistant progeny. Other plants were co-transformed with this integration plasmid along with the Int-expressing plasmid (pAR1) and progeny that were resistant to both hygromycin and kanamycin were selected. Figure 6A represents PCR products done with four transformants using oligomers 'a' and 'b' (Figure 4) as primers, and as before, the templates used were DNA of control plasmids and of genomic plant DNA. The control plasmids were the  $attP \times attB$  substrate (pPG279) (Figure 6A, lane a) and its product (pPG280) that was obtained from an *in vitro* recombination reaction (lane b). The genomic DNA templates were from an untransformed control plant (lane c), from a transgenic plant that carries the substrate only (lane d) and from a T2 double transgenic plant (lane e) showing the substrate and an expected product, each with its expected size. As before, the expected recombi-

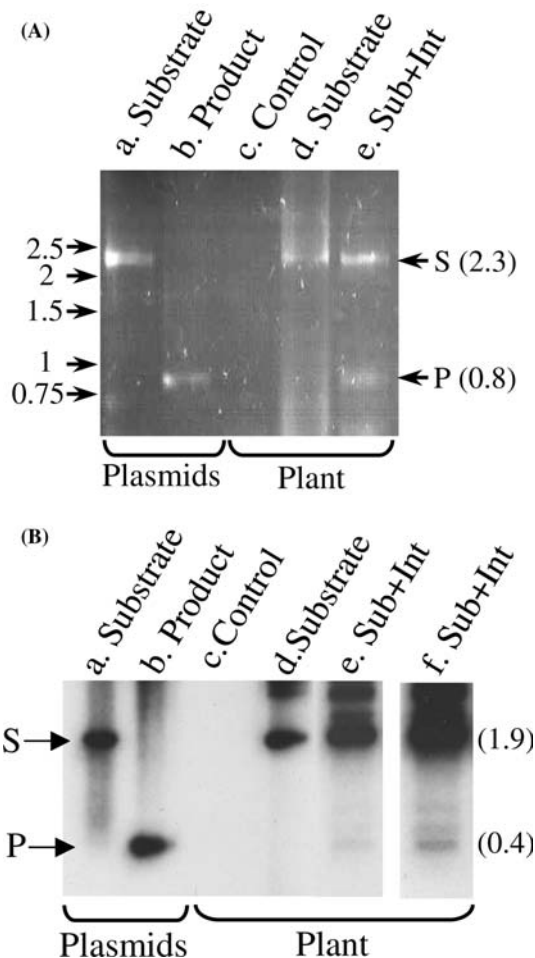


Figure 6. (A) PCR analyses of the integration reaction using oligomers a (oEY208) and c (oEY206) as primers. (B) Southern blot of the integration reaction (see text for further explanations).

nation product (P) of the double transgene was extracted from the gel, subcloned and sequenced. The sequence revealed the expected *attL*-GFP sequence (not shown), indicating that Int can also promote the *cis*-integrational recombination reaction in the plant milieu. A Southern blot analysis using *Eco*RI-restricted DNA (open triangles in Figure 4), was performed using DNA extracted from a T2 double transgenic offspring plant (Figure 6B), done as described above. The expected product (P) was evident in this plant that carried *int* and the *attP* × *attB* substrate (Figure 6B, lane e). Because the appearance of the product was rather weak, lane f shows a longer exposure of lane e. Thus, it can be

concluded that both the integration substrate and its product are stably located on the plant genome.

## Discussion

The expression and localization of Int in the nucleus is a prerequisite to its activity on chromosomally located *att* sites. We have shown that transiently expressed Int is localized in the nucleus of tobacco plants, as is the case in mammalian cells (Kolot and Yagil, 2003), and that transformed plants can stably express the protein. Since the recombination reactions were evident in *Arabidopsis*, it can be assumed that Int is likewise localized at least in part in the nucleus of these plants. The sequences of both recombination products, generated by PCR, have shown that Int can promote site-specific excisive and integrative recombination when compatible *att* sites were inserted into the plant genome in the *cis* configuration. The Southern blots done in the double-transgenic T2 plants have shown that both the substrate and the products are chromosomally located. Recent evidence has indicated that the single-stranded T-DNA that is transported from the *Agrobacterium* cells into the plant can become double-stranded prior to its chromosomal integration (Tzfira *et al.*, 2003). Therefore, it cannot be excluded that the Int-promoted recombination events could have occurred also prior to its insertion into the chromosome. However, due to the low frequency of the recombination such an event is very unlikely to occur. At present we do not know if the 35S-promoted Int is active in the germ line cells or if in each generation it is active only in somatic cells. Whichever the case may be, our results support the conclusion that Int can promote both types of site-specific recombination reactions at the chromosomal level.

The catalytic activity of Int in the plant cells takes place without the need to supply any of the accessory proteins (IHF and Xis) that are required in its natural milieu (the *E. coli* cell) or in the *in vitro* reactions (Abremski and Gottesman, 1982; Nash, 1983). Previous experiments have shown that both Int of HK022 and Int of  $\lambda$  are likewise active in mammalian cells without the need to supply the required accessory proteins. However, in the case of

Int- $\lambda$  only IHF-independent mutants were active (Christ *et al.*, 2002) whereas in the case of HK022 the wild type Int is active (Kolot *et al.*, 2003). *In vitro* experiments with the  $\lambda$  system have demonstrated that mammalian chromatin-associated high mobility group HMG proteins can replace IHF in excisive recombination (Segall *et al.*, 1994). These proteins also exist in plants (Grasser, 2003) and presumably have an identical function.

In the mammalian cells the expression of the GFP could be used as a quantitative reporter for the frequency of the recombination event. Each of the two integrases showed around 15–30% recombination activity when the *att* sites were located on extrachromosomal plasmids (Christ *et al.*, 2002; Kolot *et al.*, 2003), and only 0.01–0.1% when  $\lambda$  *att* sites are chromosomally located in *cis* (Christ and Dröge, 2002) and 2–4% when HK022 *att* sites are chromosomally located in *cis* (unpublished). For as yet unclear reasons the GFP did not express as a result of recombination in *Arabidopsis* and therefore the rate of recombination could not be estimated. The fact that among 250 of the T2 progeny plants none segregated with the product alone indicates that the efficiency of recombination is rather low. The quantitative results obtained in the mammalian cells (Kolot *et al.*, 2003) have indicated that the excision (*attL*  $\times$  *attR*) reaction is more efficient than integration (*attP*  $\times$  *attB*), the results of PCR and Southern blots obtained with *Arabidopsis* have shown a similar pattern.

So far we have only shown recombination reactions in the *cis* configuration, such reactions can be useful to eliminate undesired genes, such as genes for antibiotic resistance or genes that cause male sterility, as is already practiced by using the Cre-*lox* and FLP-*frt* systems (Luo *et al.*, 2000; Hoa *et al.*, 2002; Ow, 2002; Puchta, 2003). For the insertion or replacement of genes it still needs to be shown that the Int-catalyzed reactions take place also in the *trans* configuration (each *att* site on a different DNA molecule).

None of the transgenic *Arabidopsis* plants that expressed Int under the constitutive 35S promoter has shown any phenotypic aberrations indicating that the expression of Int may not be detrimental for plant growth. This is in contrast to Cre, whose expression did cause phenotypic aberrations when expressed constitutively, though in other plants (Que, 1998; Coppoolse *et al.*, 2003; Mlynárová and Nap, 2003).

In conclusion, the wild type Int of HK022 is a novel site-specific recombinase that is active when stably introduced into *Arabidopsis* plants, and therefore it has the potential to serve as an additional system for future site-specific gene manipulations in plants.

### Acknowledgements

B. Epel provided us with plasmid pBinGFP and Angela Cohen improved the manuscript. Support was provided by the Israel Ministry of Agriculture to EY and AR (grant No. 261-0364-00) and by the Israel Science Foundation to EY and MK (grant No. 637/02).

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