Plant Cell Rep (2008) 27:1821–1831
DOI 10.1007/s00299-008-0604-z

GENETIC TRANSFORMATION AND HYBRIDIZATION

Expression of active *Streptomyces* phage phiC31 integrase in transgenic wheat plants

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Received: 2 May 2008 / Revised: 5 July 2008 / Accepted: 24 August 2008 / Published online: 17 September 2008

Abstract Site-specific recombination systems are becoming an important tool for the genetic modification of crop plants. Here we report the functional expression of the *Streptomyces* phage-derived phiC31 recombinase (integrase) in wheat. T-DNA constructs containing a phiC31 integrase transgene were stably transformed into wheat plants via particle gun bombardment. A plant-virus-based assay system was used to monitor the site-specific recombination activity of the recombinant integrase protein in vivo. We established several independent doubled haploid (DH) inbred lines that constitutively express an active integrase enzyme without any apparent detrimental effects on plant growth and development. The potential of phiC31 integrase expression in crop plants related to transgene control technologies or hybrid breeding systems is discussed.

Keywords *Streptomyces* phage phiC31 integrase · Site-specific recombination · *Triticum aestivum* · Virus-based activity assay · DH inbred lines · Wheat dwarf virus (WDV)

Introduction

During recent years, site-specific recombination systems were increasingly applied to genome manipulations of transgenic plants. Site-specific recombination involves exchanges between specific DNA target sequences catalyzed by specialized proteins, the site-specific recombinases. According to the relative orientation of the target sites, site-specific recombination results in a variety of DNA alterations like insertions or excisions, inversions or translocations (Lyznik et al. 2003; Ow 2002). In plant biotechnology, this type of recombination is used in numerous research projects for the removal of selectable markers of transgenic plants (Ow 2007), specific integration of transgenes (Albert et al. 1995; Chawla et al. 2006; Vergunst et al. 1998) or the resolving of complex integration patterns (De Buck et al. 2007; Srivastava et al. 1999; Srivastava and Ow 2001). In other approaches, site-specific recombinase-systems are applied to convert genetic constructs from an inactive state into an active state, a method termed "gene switch". A site-specific recombinase-induced activation of genetic constructs can be performed by removing sequences that interrupt the reading frame of a gene (Hoa et al. 2002; Luo et al. 2000;

M. Rubtsova and K. Kempe contributed equally to the paper.

Communicated by H. Jones.

Electronic supplementary material The online version of this article (doi:10.1007/s00299-008-0604-z) contains supplementary material, which is available to authorized users.

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Tungsuchat et al. 2006) or by decrypting genetic constructs through flipping of DNA sequences (Gleba et al. 2004a).

In plant systems, the most commonly used site-specific recombination systems are Cre-lox of phage P1 and FLP-FRT of yeast. The application of Cre was reported for numerous studies performed in species including *Nicotiana tabacum* (Albert et al. 1995; Corneille et al. 2001; Dale and Ow 1991; Jia et al. 2006; Lutz et al. 2006; Mlynarova et al. 2006; Mlynarova and Nap 2003; Nanto and Ebinuma 2007; Tungsuchat et al. 2006), *Arabidopsis thaliana* (De Buck et al. 2007; Tremblay et al. 2007; Vergunst et al. 1998), rice (Chawla et al. 2006; Hoa et al. 2002; Sreekala et al. 2005), maize (Zhang et al. 2003), petunia (Que et al. 1998), and wheat (Srivastava et al. 1999). The FLP system was analyzed in maize (Lyznik et al. 1993, 1996), rice (Hu et al. 2008; Lyznik et al. 1993; Radhakrishnan and Srivastava 2005), tobacco (Gidoni et al. 2001), and *Arabidopsis* (Luo et al. 2000). A combined use of Cre and FLP recombination systems was reported by Luo et al. (2007) and Djukanovic et al. (2006).

However, Cre and FLP catalyze a reversible reaction between two recombination sites since the recombination site sequences are not altered during the process. Thus, the efficiency of processes for genetic engineering is often limited. As an example, during integration process, the DNA is readily re-excised in the continuous presence of the site-specific recombinase by the reverse reaction. In order to minimize reversibility of Cre-mediated site-specific recombination, mutant *lox* sites have been engineered (Albert et al. 1995; Araki et al. 1997). The challenging part of the approach was to minimize the reverse reaction by mutating the *lox* sites without inhibiting the forward reaction in order to obtain the desired site-specific recombination event at a reasonable frequency. The mutated *lox* recombination sites were successfully used to stabilize target integration by polyethylene-glycol-mediated transformation in tobacco (Albert et al. 1995) and by biolistic transformation in rice (Srivastava et al. 2004).

Another irreversible site-specific recombination system (IRS) is the integrase-*att* system from the *Streptomyces* phage phiC31 (Thorpe and Smith 1998). Naturally, phiC31 integrase mediates insertion of the phage genome into its bacterial host. It specifically unites two recombination sites (the bacterial *attB* and the phage attachment *attP* site) in a synapse and catalyses site-specific recombination reactions (Smith et al. 2004). Since *attB* and *attP* are not identical, the hybrid recombination products *attL* and *attR* are not substrates for the phiC31 integrase protein. Therefore, unlike the native Cre-lox and FLP-FRT systems, the phiC31 integrase alone cannot reverse the recombination reaction. Here, the recombination product remains stable unless an additional phage excisionase protein is provided. Hence, integrase-*att* is a useful choice of site-specific recombination technologies to create a "fixed state". In plants, phiC31 integrase was applied in tobacco for stable plastid transformation (Lutz et al. 2004) or for irreversibly excising plasmid marker genes (Kittiwongwattana et al. 2007). Recently, a new hybrid breeding system was demonstrated in *Arabidopsis* (Gils et al. 2008). Here, a cytotoxic barnase protein is produced in the tapetum tissue by in vivo complementation of two inactive barnase protein fragments, thus leading to pollen ablation and male sterility. The complementary gene fragments are located on the same locus on homologous chromosomes ("linked in repulsion"). This was achieved by the stable integration of a T-DNA construct in the *Arabidopsis* genome that carries both loci flanked by *att* recombination sites and the subsequent derivatization of the T-DNA through phiC31 integrase-mediated site-specific deletions.

Certainly, the long-term goal is to develop analogous concepts for commercially important crops like wheat. Therefore, crop varieties are needed that reliably express a suitable site-specific recombinase.

In this paper, we describe the transformation and expression of a *Streptomyces* phage phiC31 integrase transgene in lines of wheat (*Triticum aestivum* L.). For a rapid integrase activity monitoring, an encrypted vector expressing a Green Fluorescent Protein (GFP; developed by Icon Genetics GmbH, Halle/Saale, Germany) was delivered into the candidate plants by biolistic particle gun bombardment. Upon site-specific recombination catalyzed by the phiC31 integrase protein, a reconstitution of the vector function amplifying GFP expression is achieved, thus proving the presence of active integrase protein.

In order to analyze the expression of the stably integrated phiC31 integrase transgene in a constant genetic background and to avoid transgene segregation, we produced DH inbred lines of wheat.

Since wheat is the most-important food plant in the world, implementations of site-specific recombination systems would be of significant interest towards the optimization of this crop. Our results show that the production of an active phiC31 integrase can be stably established in wheat.

Materials and methods

Plant material and growth conditions

Spring wheat (*Triticum aestivum* L., cultivar "Bobwhite") was used throughout this study. Plants were grown under greenhouse conditions with 16 h of light at 20°C and 8 h of darkness at 16°C. For DNA isolation, plant tissues were harvested, frozen in liquid nitrogen, and stored at −80°C.

Standard molecular biology techniques

Standard molecular biology procedures were carried out according to Sambrook et al. (1989).

Description of the plant transformation plasmids

All vectors described in this paper (Fig. 1) have been made available by Icon Genetics GmbH. Plasmids are pBIN19-based binary vectors and were constructed using standard recombinant DNA methods.

pICH14313 and 13130

Vector pICH14313 (GenBank accession number AM887683; Gils et al. 2008) contains the *Streptomyces* phage phiC31 integrase coding sequence (Thorpe and Smith 1998) fused at the C-terminus to a SV40 T-antigen nuclear localization signal (amino acids PKKKRKV; Andreas et al. 2002). Vector pICH13130 is equal to pICH14313 except that the maize spm promoter was replaced by the ubiquitin I promoter from maize (Christensen and Quail 1996). Additionally, a 300 bp sequence derived from an intron of the *Petunia hybrida* Psk7 gene (GenBank accession number AJ224165) was introduced into the phiC31 integrase sequence at amino acid position 49. Since the vectors have originally been developed for analyzes in dicotyledonous plants, they contain a neomycinphosphotransferase (NPT) gene cloned between CaMV 35S promoter and terminator (Odell et al. 1985).

pICH12875

For selection in wheat, vector pICH12875 was co-bombarded with pICH14313 or pICH13130, respectively. pICH12875 contains an HPT (hygromycin-phosphotransferase) gene (Malpartida et al. 1983) that was inserted between a maize ubiquitin promoter and a nos terminator sequence. For expression enhancement, a 150 bp intron sequence derived from the *Petunia hybrida* gene Psk7 was inserted into the HPT coding sequence at amino acid position 76.

pICH16710

pICH16710 is a wheat dwarf virus- (WDV-) based amplification vector (Matzeit et al. 1991). WDV is a monopartite geminivirus (genus *Mastrevirus*) that infects monocotyledonous plants (Gutierrez 2000; Lazarowitz 1992). We replaced the sequence encoding the viral capsid protein (CP) of the wildtype virus by a GFP marker gene (GFP-S65T mutant, with the serine at position 65 exchanged by a

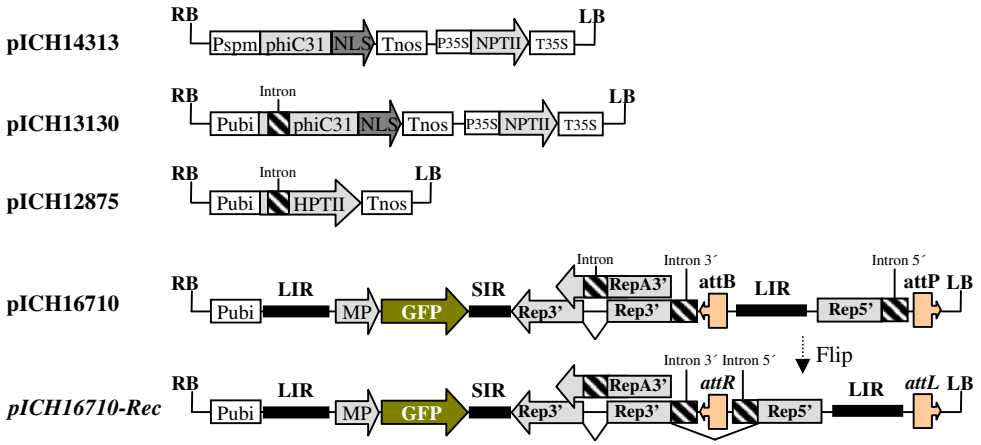

Fig. 1 Vector constructs. All elements are cloned between the right border (*RB*) and left border (*LB*) of pBIN19-based binary T-DNA vectors. Intron sequences are indicated as *dashed boxes*. phiC31 integrase vectors pICH14313, pICH13130 and transformation vector pICH12875. The phage phiC31 integrase (*phiC31*) is controlled either by an spm promoter from maize (*Pspm*; pICH14313) or a maize ubiquitin promoter (*Pubi*; pICH13130). Further abbreviations: *Tnos*, nopaline synthase terminator; *NLS*, nuclear localization signal; *Intron*, intron sequence derived from the *Petunia hybrida* Psk7 gene; *HPTII*, hygromycin phosphotransferase gene (Malpartida et al. 1983); *NPTII*, neomycinphosphotransferase gene (Matsumura, et al. 1984); *P35S* and *T35S*, CaMV 35S promoter and 35S terminator, respectively (Odell et al. 1985). pICH16710. Polypeptides Rep and RepA are essential for the replication of the viral DNA. The proteins are encoded by two distinct overlapping open reading frames. Rep is expressed after a splicing reaction of the transcript. The structure of the construct is depicted before (pICH16710) and after recombination (*pICH16710-Rec*). *GFP*, green fluorescent protein; *MP*, movement protein; *LIR*, large intergenic region; *SIR*, small intergenic region (LIR and SIR are the non coding regions in mastraeviruses that play an important role in the DNA replication cycle; Gutierrez 2002); *RepA3′*, 3′-terminal part of the RepA protein; *Rep3′*, 3′-terminal of the replication initiator protein; *Rep 5′*, common 5′-terminal part (110 inverted amino acids) of the RepA protein and the replication initiator protein; *Intron 5′*, *Intron 3′*, split *Petunia hybrida* Psk7 gene intron; *attP* and *attB*, *Streptomyces* phage phiC31 recombination sites; *attL* and *attR* are produced by recombination of *attP* and *attB*. Further details are explained in the text

threonine). The expression of GFP is depending on a site-specific recombination between *attP* and *attB* (Fig. 1). Once the inactive plasmid pICH16710 is delivered into a plant cell and exposed to active phiC31 protein, the flipping reaction reconstitutes the open reading frame of the genes encoding proteins essential for the replication of the virus (Rep, RepA), thus enabling the virus to initiate autonomous replication. GFP is expressed from the CP subgenomic promoter located at the 3′-terminus of the MP (movement protein) sequence. GFP-expression is restricted to single cells because the virus-replicates do not move intercellularly.

Genetic transformation of wheat plants via biolistic particle bombardment

Callus culture maintenance

Immature seeds of wheat were surface-sterilized by immersing them in 70% ethanol for 3 min. The procedure was followed by incubation in 2.5% sodium hypochlorite solution, including 0.01% SDS, with shaking at 125 rpm for 7 min and subsequently by three washing steps in sterile distilled water. Immature embryos (1.0–2.5 mm in length, semitransparent) were excised aseptically and placed, with scutellum-side up, on MS culture medium (Duchefa, M0222; Murashige and Skoog 1962), containing 30 g/l sucrose, 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.25% phytagel for solidification. Embryos that develop compact nodular calli were selected using a stereomicroscope and used for bombardment 14–21 days after isolation. The cultures were kept in the dark at 25°C.

Microprojectile bombardment of immature embryos

The gold coating procedure was done according to Sanford et al. (1993) and following the original protocol of Bio-Rad (Munich, Germany). In some experiments, the coating procedure was modified according to an advanced protocol of Eliby et al. (2000).

Standard procedure

For particle coating, 50 µl of gold suspension (0.6 Micron gold in 50% glycerol, 60 mg/ml) was mixed with 10 µl (1 µg/µl) plasmid-DNA, 50 µl 2.5 M $CaCl_2$ and 20 µl 0.1 M spermidine. In case of co-transformation, plasmids pICH13130 or pICH14313 together with pICH12875 were mixed in a 1:1 ratio (5 µg each). The mixture was shaken for 2 min, followed by incubation at room temperature for 30 min, brief centrifugation and washing with 70 and 99.5% ethanol. Finally, the gold particle pellet was resuspended in 60 µl of 99.5% ethanol. For one bombardment procedure, 6 µl of the suspension was used. All manipulations were done at room temperature.

Microprojectile bombardment was performed utilizing the Biolistic PDS-1000/He Particle Delivery System (Bio-Rad). Prior to the bombardment, the immature embryos were pre-treated for 4 h on MS medium supplemented with 100 g/l sucrose.

Approximately, 50 embryos were placed in the centre of a plate to form a circle with a diameter of about 10 mm. The shooting was carried out using a helium pressure of 900 psi, with 15 mm distance from a macrocarrier launch point to the stopping screen and 60 mm distance from the stopping screen to the target tissue. The distance between rupture disk and launch point of the macrocarrier was 12 mm. Finally, 16 h after treatment, the calli were transferred to MS medium containing 60 g/l sucrose and grown in dark conditions for 1 week at 25°C.

Selection and regeneration

Embryogenic calli were transferred to MS medium with 2 mg/l 2,4-D and 150 mg/l hygromycin B for selection 7 days after bombardment. The cultures were kept in the dark at 22°C. After 5–6 successive callus selection steps (total time 4–6 months) callus tissue was subcultured in MS regeneration medium supplemented with 1 mg/l kinetin, 7 mg/l zeatin. Regenerating plantlets were transferred to jars with half strength hormone-free MS medium containing 50 mg/l hygromycin B for plant selection. Fully developed plantlets were acclimated for 7–10 days at room temperature in liquid medium containing fourfold diluted MS salts. Plants with developed roots were transferred into soil and grown under greenhouse conditions to maturity.

Transient phiC31 integrase activity assay

Before bombardment, leaves of transgenic plants were kept for 6 h on MS medium supplemented with 100 g/l sucrose. Particle coating and bombardment of leaves were performed as described for callus. In total, 10 µg pICH16710 plasmid-DNA was delivered into the leaves by shooting each leaf sample twice. The helium pressure for the transient assay bombardment was 1,100 psi.

Before visual inspection, the samples were kept in the dark first for 24 h at 24°C and subsequently at 4°C.

GFP signal detection

Leaf tissue expressing GFP was viewed under UV illumination generated by a microscope Leica DM IL with filter sets for GFP plant fluorescence (excitation filter 470_40 nm; barrier filter, 525_50 nm).

Molecular analysis of transformants

Isolation of total plant DNA

For DNA isolation, 300 mg leaf material of young plants was shock-frozen. Homogenization was carried out using a TissueLyser© from Qiagen (Hilden, Germany). Total plant DNA was isolated following a modified protocol from Dellaporta et al. (1983).

PCR-amplifications

For detection of the integrase gene, PCR was performed with primers *phiC31integrase FW* (CCGACCACGAAGA TTGAGGG) and *phiC31integrase REV* (GTTGCTTCCGG AAGTGCTTCC). The resulting fragment of 580 bp was labeled radioactively and used as a probe (Probe-INT, Fig. 4c) for Southern blot hybridization. A second probe for Southern blot analyzes was produced by using the primers *NPTIIFW* (GGATTGAACAAGATGGATTGC AC) and *NPTIIRev* (CTCGTCAAGAAGGCGATAGAA GG), resulting in a fragment of 785 bp (Probe-NPT, Fig. 4c).

The molecular proof of site-specific recombination was carried out using the primers *GFP Fw* (GGGATCACTCT CGGCATGGAC) and *Rep 5′ Rev* (CATCCTAACATTCA AGCTGCCA).

PCR analyzes were done in a thermocycler (DNA Engine™ PTC-0200, Bio-Rad). Amplification was carried out for 35 cycles (94°C for 1 min; 55°C for 1 min; 72°C for 1–2 min).

Southern blot analyzes

Total DNA (10 µg) was digested with *Hin*dIII, which releases a 969 bp fragment containing the integrase sequence that is covered by the probe-INT (Fig. 4c). For estimation of the integrase copy number, the DNA was digested with *Nco*I and hybridized with the NPT probe. This strategy results in fragments containing the homologous vector sequence and a genomic DNA content of unpredictable size. DNA fragments were separated using 0.8% agarose gels and transferred onto a nylon membrane (Pall Biodyne B, USA) according to the standard procedure (Southern 1992). The membranes were hybridized with [^{32}P] labeled DNA fragments.

Inbred line production by doubled haploid (DH)-technology

We produced DH inbred lines using a modified protocol from Matzk and Mahn (1994). Transgenic Bobwhite lines were cultured in substrate (Fruhstofer Type P) at a 16 h light, 20°C/8 h dark, 16°C regime in the greenhouse. Two days before pollen shedding, the anthers were removed and the spikes were bagged. Approximately 2 days later, maize pollen was applied onto well developed stigmata. The spikes were bagged for 2 days before being dipped in a 100 mg/l Dicamba solution. After 3 weeks the caryopses were harvested and sterilized in sodium hypochlorite (Dan Chlorix). Eventually, the embryos were aseptically removed, plated on Gamborg B5 medium and cultured at 20°C in the dark. After germination, plantlets were transferred to the greenhouse. About 4 weeks later, the plants were treated by root dipping in a 0.125% colchicine solution, supplemented with 2% DMSO. After 5 h of incubation, the plantlets were thoroughly washed by tap water and re-potted in soil.

Results

Generation of transgenic wheat plants

Wheat plants were transformed by biolistic particle bombardment. Either vector pICH13130 or pICH14313 was bombarded together with plasmid pICH12875 in co-transformation experiments (Fig. 1). For pICH14313, 25 hygromycin resistant T_0 plants were identified. In case of pICH13130, 24 transgenic T_0 plants were produced (data summarized in supplementary material).

Monitoring the phiC31 integrase activity by in planta engineering of a functional WDV-based replicon

The experimental strategy for phiC31 integrase activity detection is described in Fig. 1. The complementary sense major transcript of vector pICH16710 (toward the left) encodes the RepA protein (Dekker et al. 1991). As a result of a splicing event, the altered transcript encodes the replication initiator protein Rep (Schalk et al. 1989). The Rep protein is essential for viral replication as it is responsible for initiating DNA replication during a rolling circle amplification stage. The virion sense transcript (toward the right) encodes the movement protein (MP) and GFP (that replaces the natural capsid protein CP).

A region spanning 110 amino acids of the 5′-Rep sequence is cloned in reverse ("flipped") orientation. Thus, the viral amplicon is not functional and the expression of GFP is inhibited. Consequently, delivery of pICH16710 into leaves of untransformed plants does not result in GFP expression (Fig. 2a). The flipped Rep coding sequence is placed between inversely arranged *attP* and *attB* recombination sites. These sequences serve as targets for the *Streptomyces* phage phiC31 integrase expressed either from pICH14313 or pICH13130 transgenes. Intramolecular

recombination between *attB* and *attP* should result in an inversion (flipping) of the intervening DNA sequence and a reconstitution of a translatable reading frame for the Rep gene (the recombination product is designated as pICH16710-Rec, Fig. 1). Finally, for obtaining a perfect fusion of the 3′- and 5′-parts of the Rep protein by splicing, sequences derived from the third intron of the petunia Psk7 gene were cloned next to *attB* and *attP* (Marillonnet et al. 2004).

phiC31 integrase expression in wheat plants

Expression from T-DNA pICH14313

As pICH14313 was successfully used to induce site-specific recombination in *A. thaliana* (Gils et al. 2008), the first plant transformation experiments were carried out using this plasmid. In total, 25 hygromycin resistant primary transformants were assayed by bombarding them with the vector pICH16710. Visual inspection carried out 4–7 days after bombardment revealed that 18 out of 25 transformed plants displayed GFP-signals in the bombarded leaf tissue (supplemented data). Therefore, it can be concluded that these lines express active phiC31 integrase protein.

However, the number of cells that exhibit GFP signals was low (typically 5–30/cm^2 leaf area). Furthermore, the GFP signals were usually weak and hardly distinguishable from background fluorescence in many cases. An example of a phiC31 integrase-expressing line transformed with pICH14313 is given in Fig. 2b.

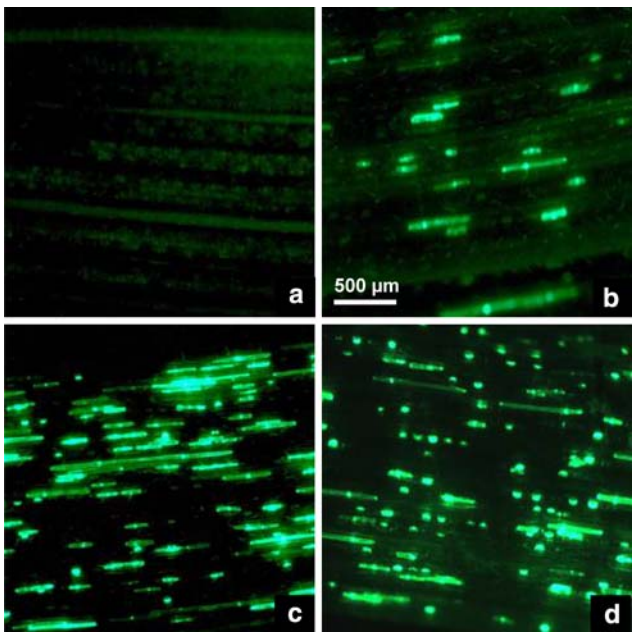

Fig. 2 Integrase activity assay. Vector pICH16710 was delivered into leaf tissue of wildtype control plants (**a**), plants that carry the vector pICH14313 (**b**), T_1 progeny of a primary pICH13130 transformant (**c**), and of line DH13130-5 (**d**). The pictures were taken 5 days (**a**, **b**) or, respectively, 48 h (**c**, **d**) after bombardment under UV light

The analysis of generations T_1 and T_2 led to similar results. Although the phenotype is stable and expression was monitored in some plants, the analysis was inconvincible in terms of signal strength and number of expressing cells.

Expression from T-DNA pICH13130

A different plasmid construct was also transformed into wheat plants. In case of vector pICH13130, the expression of phiC31 integrase is controlled by the maize ubiquitin 1 promoter (pICH14313, maize spm promoter). Additionally, the pICH13130 phiC31 integrase coding sequence contains an intron (Fig. 1; "Materials and methods"). We were able to obtain 24 hygromycin resistant T_0 plants from transformation experiments.

After bombardment with vector pICH16710, first GFP signals were detectable after 24–36 h. The fluorescence reached a maximum after approximately 5 days before it started to decline. Plants transformed with vector pICH13130 displayed intensive signals that could be identified unambiguously (Fig. 2c) and that were always stronger than those produced in plants transformed with pICH14313. The number of GFP-expressing cells reached 1,000–1,500/cm^2 leaf area. According to the assay, 20 of 24 primary transformants (T_0) produced detectable active integrase protein. In case of two lines analyzed further, a distinct integrase expression was obtained in plants of three generations (T_0–T_2, supplemented data).

We were able to detect integrase activity in 3 weeks–3 months old leaf tissue. In general, the number of GFP signals declined with the age of the bombarded plant material. In 1–2% of control experiments (bombardment of pICH16710 into leaves of wildtype plants and into leaves of a wheat line transformed with a control vector that carries only an herbicide resistance gene but no integrase transgene), a small number of GFP-expressing cells could be observed ($\leq$5 cells/cm^2). Plants that have been bombarded with empty control vectors or uncoated gold particles never exhibited GFP expression.

Molecular proof of site-specific recombination

In order to provide molecular evidence for the site-specific recombination process, PCR analyzes were performed. Two days after delivery of pICH16710 into leafs of wildtype and transgenic lines carrying pICH13130, genomic DNA was isolated from the bombarded tissue and analyzed by PCR. Primers were designed in such a way that an amplification product can be only obtained in case of an

integrase-induced recombination of pICH16710 (resulting in vector *pICH16710-Rec;* Fig. 3a). PCR fragments of the expected size (1419 bp) were only produced in case of tissue that carries both the stably integrated phiC31 integrase vector and the bombarded plasmid pICH16710 (Fig. 3b). Therefore, it can be concluded that the expression of GFP is dependent on phiC31-mediated inversion of the Rep 5′ fragment to the correct orientation.

The PCR-product was subcloned and sequenced. Its DNA sequence confirmed the predicted structure of the recombined control vector *pICH16710-Rec* (see Fig. 1). The amplification product contains 214 bp of the 3′ GFP coding sequence (including the binding site for the primer GFP Fw), the viral SIR sequence (168 bp), 792 bp of the 3′ Rep protein encoding sequence, and the 3′ part of the *Petunia hybrida* Psk7 intron. The sequence is followed by an *attR* sequence (hybrid site-specific recombination product that consists of a 5′ *attB* and a 3′ *attP* region) and the sequence region that was inverted through phiC31-mediated site-specific recombination, containing a 5′ part

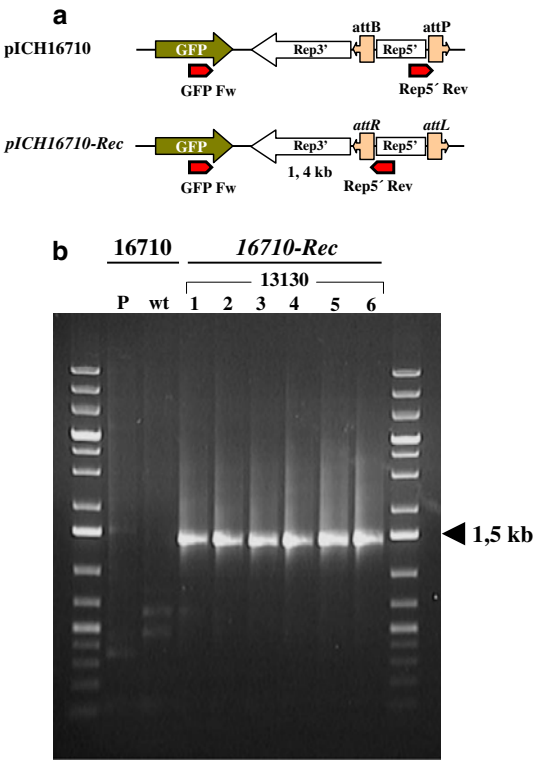

Fig. 3 PCR analyzes of the phiC31-induced recombination process. **a** Schematic illustration of the positions of primer binding sites (symbolized by *arrows*) before (pICH16710) and after recombination (*pICH16710-Rec*). PCRs were carried out on pICH16710 plasmid DNA (P) and on total DNA from untransformed wheat plants (wt) that has been bombarded with pICH16710 (**b**). Total DNA of five T_1 plants that displayed strong GFP-expression after delivery of pICH16710 was analyzed (pICH13130-2, *lanes 1 and 2*; pICH13130-21, *lane 3*; pICH13130-30, *lane 4*; pICH13130-65, *lane 5*; and pICH13130-63, *lane 6*

of the *Petunia hybrida* Psk7 intron and 36 bp of the 5′ part of WDV Rep protein (including the inverted binding site for primer Rep 5′ Rev).

phiC31 expression in doubled haploid (DH) inbred lines

Out of 24 primary transformed individuals carrying pICH13130, progeny of selected T_0 and T_1 plants with high integrase activity was analyzed. Two individual plants from two T_1 lines (13130-30-1; 13130-21-11, supplementary data) that showed the most pronounced GFP-expression in the assay experiments were chosen as donor plants for DH production.

Inbred line production from the donor plants 13130-30-1 and 13130-21-11 by doubled haploid (DH)-technology resulted in ten doubled haploid wheat lines (Table 1). Four of these lines produced seed and their T_1 generation was examined using pICH16710. Lines DH13130-**1,** DH13130-**5** and DH13130-**9** are derivatives of the T_1 donor plant 13130-30-1; DH13130-**10** resulted from DH production of the T_1 donor plant 13130-21-11. In case of lines DH13130-5, -9 and -10, GFP expression was detected in all individual plants that were analyzed in generations T_0 and T_1. For pICH13130-5, T_2 progeny plants were analyzed. Again, a pronounced GFP expression was visible. No obvious changes in expression could be observed between the primary DH plants (T_0) and the T_1 and T_2 individuals. An example of the phiC31 integrase expression in a T_1 plant of the line DH13130-5 is given in Fig. 2d.

None of the plants expressing integrase protein has shown any phenotypic differences compared to wildtype plants grown under the same conditions, indicating that the expression of the recombinase has no drawbacks for plant development.

Molecular analysis of DH lines

Southern blot analyzes of T_1 progeny plants from lines DH13130-**5**, 13130-**9** and DH13130-**10** confirmed the presence of a phiC31 integrase DNA fragment of the expected size whereas line DH13130-**1** lost the integrase transgene through segregation during the DH production process (Fig. 4a, b). Furthermore, it could be shown using NPTII probe, that the transgenic DH lines DH13130-**5** and 13130-**9** carry the T-DNA in a single copy configuration (Fig. 4b, c).

Discussion

The function of *Streptomyces* phage phiC31 integrase systems in plants was reported in earlier publications.

Table 1 Integrase activity of pICH13130 DH lines

Donor plant	T_0		T_1		T_2	
	DH line DH13130	GFP	Presence of integrase	GFP	Presence of integrase	GFP
pICH13130-30-1	**1**	−	−	−(3)	N.D.	N.D.
	2	+				
	3	−				
	4	+				
	5	+	+	+ (14)	N.D.	+ (10)
	9	+	+	+ (15)	N.D.	N.D.
pICH13130-21-11	*6*	+				
	7	−				
	8	+				
	10	+	+	+ (25)	N.D.	N.D.

All progeny plants are derived from self-fertilization of the parental individuals. Only T_0 lines indicated with bold figures gave rise to progeny that was further analyzed. In brackets, total number of plants analyzed. All progeny plants from a particular line produced either completely active integrase (+) or did not produce integrase (−). *N.D.* not determined

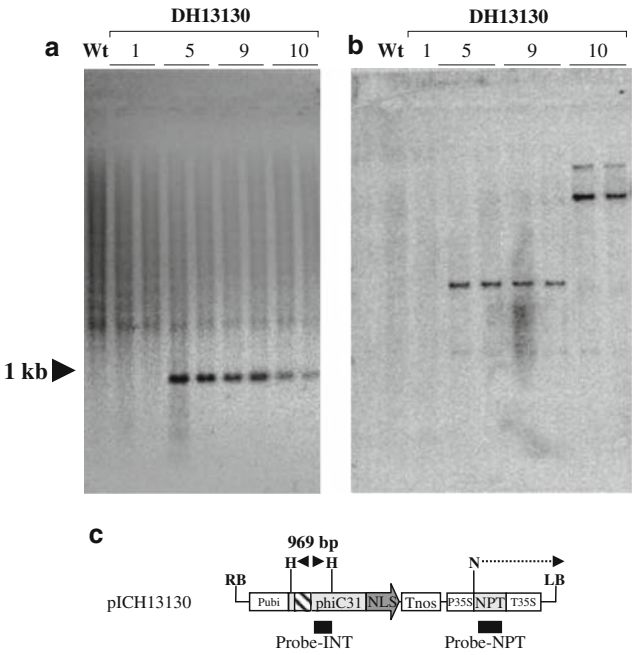

Fig. 4 Southern blot analyzes of phiC31 DH lines. Total DNA of pICH13130 DH lines (T_1) and wildtype control plants was digested with *Hin*dIII (**a**) or *Nco*I (**b**) and analyzed with a probe homologous to the phiC31 integrase sequence (**a**, Probe-INT) or to the NPT sequence (**b**, Probe-NPT). Regions of homology are shown by *rectangles* in the schematic illustration (**c**) where H and N indicate the sites for *Hin*dIII and *Nco*I restriction enzymes, respectively

phiC31 integrase is used for plastid transformation and marker gene excision in *N. tabacum* (Lutz et al. 2004; Kittiwongwattana et al. 2007), removal of transgene fragments in *A. thaliana* (Gils et al. 2008) and transient approaches to engineer viral RNA amplicons for protein production in planta (Marillonnet et al. 2004). Yet, to our knowledge, there is no phiC31 integrase expression system established in monocotyledonous plants. In this study, we succeeded in producing transgenic wheat lines that stably express a functional phiC31 integrase enzyme from a presumable single-copy transgene. In wheat cells, phiC31 expression was sufficient to catalyze site-specific recombination on viral DNA-based recombination sites.

We have chosen the phiC31 integrase/*att* system since it mediates a non-reversible recombination process. The recombination product is flanked by the hybrid sites *attL* and *attR*. These sites are not target for the phiC31 integrase. Thus, the system leads to site-specific recombination products that are predicted to be stable. This is an essential condition for the establishment of efficient biotechnological approaches.

In order to reach a high practicability in applied systems, it is necessary to monitor the recombinase activity by an easy-to-use assay prior to the selection of appropriate integrase lines. We were able to reliably prove the presence of an active integrase in wheat plants in less than 3 days by using a viral-based assay system. Amplicons of viral origin are particularly favorable to reach a high expression or to design a process with a short expression time and a high throughput (Gleba et al. 2004b). Theoretically, a single recombination event is sufficient to reconstitute the viral amplicon, thus making the system extremely sensitive. We assume that the insignificant rate of weak signals detected in some negative controls is a result of spontaneous rearrangements of pICH16710 during the delivery process.

The strong phiC31 integrase expression resulting from transformation with pICH13130 is presumably caused by the use of the maize ubiquitin 1 promoter which is known as a strong constitutive promoter in wheat (Clausen et al. 2000; Rooke et al. 2000). In case of transformants carrying pICH13130, the integrase expression declined with the age

of the material used in the transient assay. This observation is in agreement with publications that describe a maximum of the ubiquitin promoter activity in rapidly dividing cells (Cornejo et al. 1993; Clausen et al. 2000, Rooke et al. 2000). However, as an alternative explanation, it cannot be ruled out that the viral system itself replicates less in older leaves.

Site-specific recombination can be manifoldly applied in systems designed for the control of transgene activity or transgene flow (Gleba et al. 2004a; Luo et al. 2007; Mlynarova et al. 2006). Here, two states of a transgene are possible, the "on" and the "off" state. Both possibilities are implemented in systems that can be switched by the activity of a site-specific recombinase at a desired time or developmental stage of the plant. Switching is achieved either by sexual crossing to a plant that expresses a recombinase, a second round of gene transfer or, alternatively, through transcriptional activation of the recombinase by using inducible promoters. Currently, a specific approach of a phiC31 recombination for a combined transgene flow and transgene activity control was reported for *Arabidopsis* (Gils et al. 2008). In this feasibility study, a multi-component system was established for hybrid seed breeding. A key issue of the technology is the production of complementing genetic loci that are located on identical chromosomal positions but on homologous chromosomes ("linked in repulsion"). Here, the derivatization of a precursor-T-DNA ("pro-locus") construct is achieved by deleting alternative sequences that are flanked by *att* recombination sites. The deletions are induced by crossing a plant carrying the precursor construct to a plant that expresses an active phiC31 integrase.

Certainly, the feasibility of such processes in commercial valuable crop species will depend on crop lines that reliably express active site-specific recombinases. We hope that phiC31 lines like the ones described in this paper will contribute to the establishment of a new generation of crop optimization concepts like for example advanced hybrid breeding systems.

There remain certain limitations in connection with the significance of the transient viral assay system. In most biotechnological applications, the recombinase acts upon intrachromosomal DNA. It was postulated that a condensed chromatin structure can lead to a reduced accessibility of the target DNA to the recombinase enzyme (Mengiste et al. 1999; Puchta 2003). Such inhibiting effects are excluded from the transient test system that is based on an extrachromosomal site-specific recombination. Therefore, the results revealed for the different phiC31 integrase wheat lines might not be totally representative for all intrachromosomal recombination scenarios that will possibly occur in later applications. However, in earlier experiments, transgenic *Arabidopsis* lines containing vector pICH14313 were crossed with lines that carried a stably integrated T-DNA locus with *att* sites. In the progeny of such crosses, a high frequency of site-specific recombination could be obtained at the locus (Gils et al. 2008). This demonstrates that the genetic constructs used in this study are principally suitable for an efficient induction of site-specific recombination at intrachromosomal T-DNA loci in plants. Nevertheless, in future, it would be preferable to set up an assay for phiC31-mediated site-specific recombination of a stably integrated T-DNA that is exposed to an integrase transgene encoded on a second chromosomal locus. In addition, future systems should also enable to investigate developmental-, tissue- and environmental-specific integrase activity in order to assay the technological value of the phiC31 system in wheat.

The phiC31 integrase system is expected to be functional in other monocotyledonous species and in commercial wheat varieties. We succeeded in transforming phiC31 integrase vectors into rice (*Oryza sativa*), pearl millet (*Pennisetum glaucum*) and varieties of commercial winter wheat provided by the Nordsaat Saatzucht GmbH, Böhnshausen, Germany (unpublished data). The results obtained with those lines are in accordance with those obtained for the wheat cultivar Bobwhite described in this paper. Plants carrying pICH13130 synthesized active integrase protein as indicated by GFP expression in leaf tissue after bombardment of pICH16710. None of the transgenic plants that constitutively express phiC31 integrase displayed any phenotypical aberration, indicating that the expression of the phiC31 integrase has no apparent negative effects on plant growth and fertility. This observation is important since site-specific recombination systems may induce genomic rearrangements that result in detrimental effects on the plant phenotype. It was reported that expression of Cre led to aberrant phenotypes in some plant species belonging to the Solanacea family (tomato, tobacco and petunia; Coppoolse et al. 2003; Mlynarova and Nap 2003; Que et al. 1998). However, there are no reports about similar effects in Cre expressing wheat plants (Srivastava et al. 1999). In conformity with this study, Ream et al. (2005) revealed no evidence of ectopic recombination and no apparent phenotypical aberration in Cre expressing maize plants. Together with our results, the studies indicate that site-specific recombinases can be expressed in monocotyledonous plants without inducing severe phenotypical effects.

In conclusion, it was shown here that the expression of functional phiC31 integrase can be efficiently established and maintained in transgenic plants of an important crop species. We anticipate that wheat lines expressing an active site-specific recombinase can be broadly applied in the field of plant genetic engineering and crop improvement.

Acknowledgments The authors thank Icon Genetics GmbH (Halle/Saale, Germany) for kindly providing the plasmid-vectors used in this study. We particularly thank Dr. Serik Eliby for his contribution in vector construction and his distinguished advices for wheat transformation. Also, we thank Dr. Romy Kandzia for vector cloning and Dr. Sylvestre Marillonnet for many helpful conversations. We are mostly grateful to Corinna Moritz for excellent technical assistance and greenhouse management. The support provided by Dr. Heike Schmuths (Saaten-Union Resistenzlabor GmbH) is gratefully acknowledged. Additionally, we like to thank Wolf v. Rhade and Dr. Ralf Schachschneider (Nordsaat GmbH) for constant encouragement. Especially, the authors wish to thank Dr. Renate Schmidt for comments on the manuscript and for many inspiring discussions. The research was funded by the Bundesministerium für Bildung und Forschung (BMBF, GABI-FUTURE grant 0315043A) at the Leibniz Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK) Gatersleben.

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