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ORIGINAL ARTICLE

Molecular and FISH analyses of a 53-kbp intact DNA fragment inserted by biolistics in wheat (*Triticum aestivum* L.) genome

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Abstract

Key message **A large, 53-kbp, intact DNA fragment was inserted into the wheat (*Triticum aestivum* L.) genome. FISH analyses of individual transgenic events revealed multiple insertions of intact fragments.**

Abstract Transferring large intact DNA fragments containing clusters of resistance genes or complete metabolic pathways into the wheat genome remains a challenge. In a previous work, we showed that the use of dephosphorylated cassettes for wheat transformation enabled the production of simple integration patterns. Here, we used the same technology to produce a cassette containing a 44-kb *Arabidopsis thaliana* BAC, flanked by one selection gene and one reporter gene. This 53-kb linear cassette was integrated in the bread wheat (*Triticum aestivum* L.) genome by biolistic transformation. Our results showed that transgenic plants harboring the entire cassette were generated. The inheritability of the cassette was demonstrated in the T1 and T2 generation. Surprisingly, FISH analysis performed on T1 progeny of independent events identified double genomic insertions of intact fragments in non-homoeologous positions. Inheritability of these double insertions was demonstrated by FISH analysis of the T1

generation. Relative conclusions that can be drawn from molecular or FISH analysis are discussed along with future prospects of the engineering of large fragments for wheat transformation or genome editing.

Keywords Wheat · Biolistic transformation · FISH · Large fragment · Gene cassettes

Introduction

Wheat, rice and maize are the main crops that provide food for humans and feed for animals. Alone, wheat provides 20% of the calories consumed by humans (http://faostat.fao.org/site/339/default.aspx). Until recently, one of the breeders' goals was to increase productivity to get past the predicted population peak in 2050. Recently published results (Gerland et al. 2014) suggest that the world population is unlikely to stop growing this century and can be expected to increase to 10 billion in 2100. Therefore, yield improvement appears to be a major goal for agriculture for the next 20 years. Even if classical breeding is still the most widely used method for wheat improvement worldwide, biotechnologies can provide research teams with functional validation tools for rapid understanding of gene functions, and breeders with new tools to achieve their selection objectives more efficiently. Although wheat was the last of the major cereals to be transformed, first by biolistics (Vasil et al. 1992) and some years later by *Agrobacterium*-mediated transformation (Cheng et al. 1997), advances in tissue culture, combined with improvements in transformation technology, have resulted in increased transformation efficiency, and today, the routine introduction of a transgene in this crop is feasible (for reviews see Sparks and Jones 2014; Spark et al. 2014). The efficiency of wheat

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transformation has been improved over the past 20 years with the goal of transferring a combination of one or two genes to the selective marker gene, but certain research programs require the transfer of large fragments, for example gene clusters for disease resistance, genes with particularly long 5′ regulatory sequences (Saidi et al. 2007; Hu et al. 2008) or for the importing of entire metabolic pathways that can require up to nine genes (Naqvi et al. 2010). To conserve the entire functionality of the transferred DNA, care must be taken to transfer a linear, unbroken large fragment with no rearrangements.

The transformation of plants using large DNA fragments was achieved using binary bacterial artificial chromosome (BIBAC) technology, biolistic transformation or bioactive beads transformation technology. Hamilton et al. (1996) described the introduction of 30 and 150 kb of yeast and human genomic DNA, respectively, into the tobacco genome using a binary-BAC system strategy. Using this strategy, five tobacco independent events were obtained, which appeared to contain the entire intact 150-kb human DNA fragment. Additional results in tomato showed that transgenic events obtained using the same BIBAC strategy (Hamilton 1997) were stable through several generations without gene silencing (Frary and Hamilton 2001). In the same way, transformation-competent artificial chromosomes (TAC) that can accept and maintain large genomic DNA fragments in both *Escherichia coli* and *Agrobacterium tumefasciens* were developed in *Arabidopsis* (Liu et al. 1999) and wheat (Liu et al. 2000). However, BIBAC and TAC clones that contain fragments of repetitive or single-copy DNA larger than 100 kb were shown to be unstable in *Agrobacterium*, including *recA* deficient strains (Song et al. 2003). The same results were obtained for a 83,102 bp *Arabidopsis* BIBAC clone also shown to be unstable in *Agrobacterium* (Chang et al. 2011) and for a 92-kb BIBAC clone that was shown to be subject to long stretches of deletions in *Agrobacterium* (Nakano et al. 2005). In the same publication, these authors analyzed transgenic rice plants produced by *Agrobacterium*-mediated transformation with a large T-DNA insert containing a 92-kb region of the wheat genome by fluorescence in situ hybridization on extended DNA fibers (fiber FISH). Their results suggest that the large T-DNAs integrated by *Agrobacterium*-mediated transformation tend to be rearranged in transgenic rice plants (Nakano et al. 2005). On the other hand, Shi et al. (2011) reported that, after being cultured for 96 h in LB medium, maize B73 BIBAC clones were stable in *Agrobacterium*. Recently, Wang et al. (2015) succeeded in transforming rice with a 164-kbp maize BIBAC clone, but the inheritance and stability of the transgene in the T1, T2 and T3 generations remains unclear. One way to succeed in stable transfer of large fragments into plant genomes without the use of *Agrobacterium* could be to use biolistic transformation. Ercolano et al. (2004) used this technology to introduce a 106-kb BAC plasmid. The authors used PCR to check that the *R1* gene and most of the BAC sequences were present in the resulting transformed plants. However, the plasmid was transferred as a circular molecule, which had to be broken to be inserted, and so there is no evidence—and little likelihood—that the 106-kb fragment was intact. The same conclusions can be drawn for rice, which was transformed with circular BACs containing random genomic DNA (122–157 kbp) or centromere-specific sequences (74–102 kbp) (Phan et al. 2007), and for tobacco, which was transformed with a circular BIBAC containing 83 kb of *Arabidopsis* genomic DNA (Chang et al. 2011). An alternative method using bioactive beads was applied by Wada et al. (2009) to transfer a ∼80-kb circular BAC containing the *Hardness* (*Ha*) locus of *Aegilops tauschii* into the rice genome. Once again, the probability that the large DNA fragment was unbroken is very low because a circular BAC was used for transformation. Large linear DNA fragments of, respectively, 80 and 150 kb or 23 and 50 kb cloned into YAC (yeast artificial chromosome) vectors were successfully used to transform tobacco cells (Mullen et al. 1998), or tobacco plastid DNA (Adachi et al. 2007) using biolistic technology. Despite this success, chimaerism, instability and technical difficulties in the preparation and purification of YAC (Monaco and Larin 1994) have drastically limited the development of this kind of library. So, the transfer of large unbroken DNA fragments into the plant genomes without rearrangements remains a challenge. Recently, we developed a strategy that involves the transfer of dephosphorylated linear fragments (dephosphorylated cassettes) for increased production of transgenic wheat with simple insertions (Tassy et al. 2014). In the present study, we tested the efficiency of the same strategy for the transfer of large DNA fragments into the wheat genome. A 53-kb linear cassette was produced, purified and dephosphorylated to limit concatemerization and to produce more simple integration patterns. This cassette contained a 44-kbp BAC insert originating from *A. thaliana* to minimize the risk of cross hybridization with wheat genomic DNA, flanked by one selection gene and one reporter gene. Our results showed that transgenic plants harboring the entire linear cassette were generated. The inheritability of the components of the cassette is demonstrated in the T1 and T2 generations.

Materials and methods

Plasmid construction

A vector containing the cassette used for wheat transformation, named pLF*g*44*p*, was constructed based on the vector pBelobac11 (NEB-E4154S) in the three following steps:

Construction of the [*Asc*I-gus-*Not*I-pmi-*Asc*I] cassette

The [*Asc*I-gus-*Not*I-pmi-*Asc*I] cassette (Supplementary data 1) was constructed using Gateway MultiSite technology (Life technologies). A *Not*I restriction endonuclease cutting site was inserted between the *gus* and *pmi* cassettes to facilitate subsequent cloning of the *Not*I-*Not*I Arabidopsis fragment. Construction of the 3′ selection cassette containing the maize ubiquitin promoter, the first intron of the ubiquitin gene, the *pmi* (*manA*) coding sequence and the terminator sequence of the nopaline synthase gene of *A. tumefasciens* is described in Tassy et al. (2014). The central cassette containing the *Not*I restriction enzyme site was assembled by combining the two following complementary primers 5′ > 3′GGGGACAAGTTTGTACAAAAAAGCA GGCTGCGGCCGCACCCAGCTTTCTTGTACAAAGTG GT and 5′ > 3′ CCCCTGTTCAAACATGTTTTTTCGTC CGACGCCGGCGTGGGTCGAAAGAACATGTTTCAC CA. The 5′ cassette containing the *gus* reporter gene was constructed in the same way as the *pmi* gene. The Gateway MultiSite (Life Technology) combination of the three cassettes was performed following the manufacturer's instructions (Supplementary data 1). The resulting [*Asc*I-gus-*Not*I-pmi-*Asc*I] construction was cloned in the Gateway pDEST R4-R3 vector (Life Technology).

Subcloning of the [*Asc*I-gus-*Not*I-pmi-*Asc*I] cassette on a modified *pBeloBac11* vector

First, the pBeloBac11 vector (NEB-E4154S) was engineered to replace the unique *Not*I cloning site by an *Asc*I site. The original pBeloBac11 plasmid was linearized using *Not*I. The double strand DNA resulting from the hybridization of the following synthetic oligonucleotides [5′NotAsc] (5′GGCCATGA**GGCGCGCC**CT3′) and [3′*Not*I *Asc*I] (5′GGCCA**GGGCGCGCC**TCAT3′) was cloned into the *Not*I site. This fragment included an *Asc*I site (bold nucleotides) and the four protruding bases GGCC, that were cohesive with *Not*I GCGGCC ends but that did not reconstitute the *Not*I site. Ligation reaction was performed using T4 DNA ligase supplemented with *Not*I nuclease to avoid self-ligation of the *Not*I digested pBeloBac11 plasmid. The resulting vector was named [pBeloBac11_Asc].

The modified plasmid was introduced into *E. coli* cells (DH10b Electromax Invitrogen), by electroporation, using a BioRad MicroPulser according to the instruction manual. Transformed cells were selected on LB-agar plates containing 12.5 µg/ml of chloramphenicol. Plasmid DNA was extracted from recombinant colonies using the Qiagen Miniprep Kit. *Asc*I and *Not*I digests allowed us to check for the presence of the *Asc*I site (linearization of the plasmid) and the absence of *Not*I site (undigested plasmid).

Second, the [*Asc*I-gus-*Not*I-pmi-*Asc*I] cassette was cloned into the [pBeloBac11_Asc] plasmid. pDEST R4-R3 vector containing the [*Asc*I-gus-*Not*I-pmi-*Asc*I] cassette was submitted to *Asc*I digest (1 h, one unit of enzyme per µg of plasmid DNA). Restriction fragments of 2.5 kb (vector) and 8.9 kb (cassette) were separated by electrophoresis on 1% agarose gel (50 mA, 5 V/cm). An agarose plug containing the 8.9-kb fragment corresponding to the cassette was cut onto the gel. DNA was recovered using the QIAquick Gel Extraction Kit (Qiagen) according to the instruction manual. [pBeloBac11_Asc] vector was digested by *Asc*I and dephosphorylated as described in Sambrook el al. (1989). Finally, the purified cassette was cloned into the dephosphorylated vector. Ligation reaction was introduced into *E. coli* DH10b Electomax (Invitrogen). Transformed cells were selected on LB-agar medium containing chloramphenicol 12.5 µg/ml. Plasmid DNA was extracted using BAC or BiBAC miniprep DNA protocol (Sambrook et al. 1989) and screened for the presence of the cassette inserted into the [pBeloBac11_Asc] vector by *Xho*I digest and fingerprinting. The resulting vector named pLF*gp* (pLF for plasmid Large Fragment, *g* for *gus* and *p* for *pmi*) is presented in Fig. 1a.

Purification and subcloning of the 44-kb Arabidopsis genomic fragment

The BAC DNA (ref. F12K22) was provided by the *Arabidopsis* Biological Resource Center. *Arabidopsis* inserts were originally cloned into a pBeloBACKan vector as partial *Eco*RI fragments. We previously checked that there were no specific genes or sequences in the insert and no *Not*I enzymatic site. Using the NCBI-BlastN program (Altschul et al. 1990), we also checked that there is no homology between this *Arabidopsis* sequence and the wheat genome to avoid false positives by cross hybridization. BAC DNA was extracted using the QIAGEN Large-Construct Kit (ref. 12462) following the manufacturer's instructions. DNA was submitted to *Not*I enzymatic digestion for 3 h at 37 °C using one unit of enzyme per µg of DNA. BAC vector (pBeloBacKan, 8774 bp) and 44-kb *Arabidopsis* fragments were separated by pulse field gel electrophoresis on a Bio Rad CHEF Mapper in 0.5X TBE buffer, with the following setting: auto algorithm 5–50 kb—1% agarose gel—14 °C—20 h 30 min. The *Arabidopsis* DNA fragment was cut into the gel, recovered by electro-elution and dialysis as described in Peterson et al. (2000). The vector pLF*gp* was digested by *Not*I in the same conditions as for the BAC DNA and dephosphorylated. The 44-kb fragment was ligated on the pLF*gp* vector as described in Peterson et al. (2000). The ligation reaction product was cloned on DH10b Electromax *E. coli* (Invitrogen). The
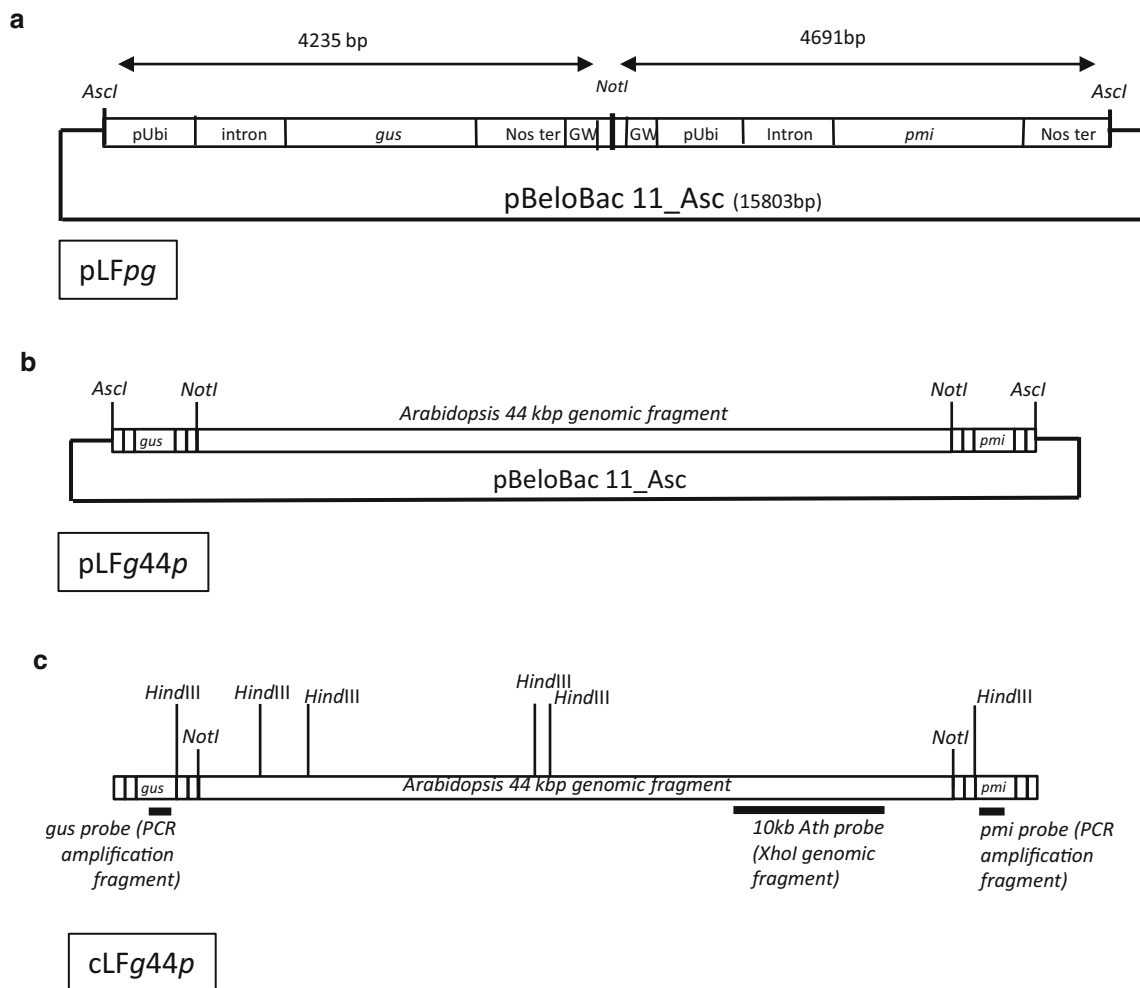
Fig. 1 Construction of the cLFg44p cassette. **a** pLFgp (plasmid Large Fragment pmi gus) was based on pBeloBac 11_Asc vector. It contained a cassette including a unique NotI site flanked by pmi and gus reporter genes and two AscI sites. The two reporter genes were under the control of maize ubiquitin promoter followed by the first intron of the maize ubiquitin gene. The unique NotI enzymatic site located between the two reporter genes will be used for subsequent subcloning of the Arabidopsis genomic fragment. The two AscI flanking sites will be used for cassette release. This cassette was constructed using the MultiSite Gateway technology. **b** pLFg44p plasmid. The 44-kbp Arabidopsis BAC insert was released by NotI digest, purified and cloned into the unique NotI site of the pLFgp plasmid. **c** The final cLFg44p cassette was released from the pLFg44p plasmid by AscI digest. Bold lines under the cassette represent the different probes used for Southern analysis. Gus and pmi probes were obtained by PCR amplification on pLFg44p plasmid using previously described primers. XhoI digest of the pLFg44p plasmid generated a fingerprint containing a unique XhoI-XhoI fragment named 10 kpb Ath probe. This fragment was recovered by gel-elution after agarose gel electrophoresis

presence of the 44-kb insert was checked by XhoI (fingerprint), NotI and AscI digests. One out of 96 colonies gave the right digest profile for the three endonucleases. The resulting plasmid was named pLFg44p (Fig. 1b).

Production of cLFg44p DNA cassette

The clone containing pLFg44p plasmid was cultured overnight in 500 ml bottles containing 250 ml of LB medium supplemented with 12.5 ug/ml chloramphenicol. Plasmid pLFg44p was recovered and purified using the QIAGEN Large-Construct Kit (ref. 12462), according to the instruction manual. AscI restriction enzyme digest was performed overnight using one unit of enzyme per μg of plasmid DNA. The resulting 53-kb cassette cLFg44p (Fig. 1c) was separated from the 6.9-kb plasmid by electrophoresis on pulse field gel and purified as previously described. Finally, the cassette was dephosphorylated as described in Tassy et al. (2014) and the concentration was adjusted to 30 ng/μl.

Plant material and transformation protocol

Triticum aestivum cv. Bobwhite S26 was used for all experiments. Transformation experiments and plantlets regeneration were performed as described in Tassy et al.

(2014), according to Pellegrineschi et al. (2002) and Wright et al. (2001).

Histochemical GUS assay

Expression of the *gus* gene was checked using the X-Gluc substrate with young leaves of transgenic plant as described in Jefferson (1987). Leaf tissues were cut into small pieces and incubated for 24 h at 37 °C in X-Gluc buffer. Chlorophyll was extracted from the tissues by incubation in 70 °C ethanol at 37 °C to be able to see the blue staining of the transformed tissues.

PCR analysis

Wheat genomic DNA was isolated from young leaves using a modified cetyltrimethylammonium bromide (CTAB) method (Tassy et al. 2006). The *gus* gene was detected by PCR amplification using 50 ng of total genomic DNA as template and *Taq* DNA polymerase (Q-BIO gene) following the manufacturer's instructions, using the primers [GUS A forward 5′ TGC CAG GCA GTT TTA ACG ATC 3′]–[GUS B reverse 5′ AAT CAG GAA CTG TTC GCC CTT 3′], for the *GUS* assay, and [PMI-1 forward 5′ GCA CAG CCA CTC TCC ATT CA 3′]–[PMI-2 reverse 5′ ATC GGA GTT TGC CAT CAC TTC 3′] for the *gus* and *pmi* assay, respectively.

The PCR reaction mixture (50 μl) contained 1X Taq polymerase buffer (Qiagen) containing 1.5 mM MgCl2, 150 μM of each dNTP, 20 pmol of each primer, and one unit of Taq polymerase (Qiagen). PCR was performed using the MJ research PCR thermocycler with one cycle at 94 °C for 3 min, 34 cycles at 94 °C for 45 s, 62 °C for 45 s, 72 °C for 90 s and a final extension step of 72 °C for 5 min for the *pmi* assay and with one cycle at 94 °C for 3 min, 30 cycles at 94 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min and a final extension step of 72 °C for 5 min for the *gus* assay. The 543-bp and 710-bp PCR products for *pmi* and *gus* genes, respectively, were detected by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

Southern blot analysis

The DNA probes used for Southern blot analysis were produced as follows: *gus* and *pmi* probes (Fig. 1c) were generated by PCR amplification using primers GUSA/GUSB; PMI1/PMI2 described above and Gateway pDEST R4-R3 vector containing the [*Asc*I-*gus*-*Not*I-*pmi*-*Asc*I] cassette as DNA template. The PCR products were purified after agarose gel electrophoresis, using the QIAquick Gel Extraction Kit (Qiagen ref. 28704). The *Arabidopsis* 10-kb probe (Fig. 1c) was produced by *Xho*I digest of the F12K22 Bac and the 10-kb fragment was recovered after electrophoresis on 1% agarose gel. Purification was performed as described above. The cLF*g*44p cassette (Fig. 1c) was used as probe for in situ hybridization.

Plant genomic DNA was isolated from young leaves using a modified CTAB protocol (Tassy et al. 2006). Aliquots (15 μg) were digested for 6 h using 10 units per μg of genomic DNA of the appropriate restriction enzyme. Restriction fragments were then separated by electrophoresis in 1% agarose gel in 1X TAE (Tris–Acetate EDTA) buffer, or by pulse field gel electrophoresis as previously described, and blotted by alkaline/saline transfer on a nucleic acid transfer membrane (Amersham hybond N+). Probes were labeled with ^{32}PdCTP, using random hexamer primer method (Megaprime DNA Labeling System GE healthcare ref RPN1606). Southern hybridization was carried out as previously described in Barret et al. (1998).

FISH analysis

Root preparation

Root tips for cytological studies were obtained from T1 plants. T1 seeds were first sterilized in a 5% sodium hypochlorite solution and then germinated on moist filter paper in a Petri dish for 24–48 h at room temperature. The seedlings were then left at 4 °C for 48 h, and at room temperature for 24 h. Young roots were harvested when they were about 2–3 cm long. They were pre-treated in ice water for 24 h to accumulate mitotic cells in metaphase before fixation in 3/1 ethanol/glacial acetic acid solution. The material was fixed at least 48 h before preparation of microscope slides.

Slide preparation for in situ hybridization

The best slides were selected by phase contrast microscopy. They were prepared as described in Mirzaghaderi (2010), Mirzaghaderi et al. (2010).

DNA probes

The pLF*g*44*p* cassette labeled with Biotin Nick Translation Kit (Roche 11,745,824,910) according the manufacturer's instructions was used as FISH probe. To identify A, B or D genomes, two specific probes labeled with fluorescein were added: clone TAS40 (NCBI accession number DX373040) containing a 1-kb fragment from the 3B chromosome from *Triticum aestivum* cv Courtot and specific to the B genome (Paux et al. 2006), and clone pAs1 probe containing a 1-kb fragment from *Aegilops squarrosa* (Rayburn and Gill 1986) specific to the D genome.

Fluorescence in situ hybridization

FISH was performed as described in Schwarzacher and Heslop-Harrison (2000).

Results and discussion

Functionality of *gus* and *pmi* genes on the [*Asc*I-*gus*-*Not*I-*pmi*-*Asc*I] cassette

Before starting the final construct with the 44-kbp *Arabidopsis* insert, we checked the functionality of both *gus* and *pmi* genes of the [*Asc*I-*gus*-*Not*I-*pmi*-*Asc*I] cassette. To this end, 200 immature wheat embryos were bombarded with the [*Asc*I-*gus*-*Not*I-*pmi*-*Asc*I] cassette and 200 more were bombarded without DNA as control. The embryos were first analyzed for transient *gus* expression. All the embryos bombarded with the cassette presented GUS phenotype 3 days later, we could thus conclude that the *gus* reporter gene cassette was functional. Ten plantlets were regenerated by in vitro culture on mannose selective medium, and nothing on control experiment, indicating the functionality of the *pmi* expression cassette. Among the 10 plantlets, four presented stable GUS expression in young leaves and roots. Recovery of plants expressing both reporter genes indicated that the *gus* and *pmi* genes in the [*Asc*I-*gus*-*Not*I-*pmi*-*Asc*I] cassette were functional. The plasmid containing the [*Asc*I-*gus*-*Not*I-*pmi*-*Asc*I] cassette was used to clone the BAC insert of Arabidopsis (*Not*I-*Not*I fragment) in the *Not*I restriction site to obtain the pLF*g*44*p* plasmid (Fig. 1).

Cassette cLF*g*44*p* production process: quality checking

*Asc*I digest of the pLF*g*44*p* plasmid liberated the *Asc*I-*Asc*I cLF*g*44*p* cassette (Fig. 1c). The quality of this cassette was checked by agarose gel electrophoresis (Fig. 2). We obtained a single band of the right size (53 kbp), without low-molecular weight degraded DNA, thereby demonstrating that it is possible to obtain large pure intact DNA fragments using PFEG technology combined with electro-elution dialysis.

Wheat transformation with cLF*g*44*p* cassette and functional characterization of T0 transgenic plants

Wheat transformation experiments were performed with the *cLF*g*44p* cassette. The total size of this linear, dephosphorylated cassette was 53kbp. We chose to transfer the linear cassette instead of circular plasmid to avoid random breaking of the insert structure.

We first bombarded 800 immature embryos in two independent transformation experiments. Twenty-four plantlets were regenerated on mannose selective medium. The transformation efficiency (the number of plantlets regenerated to the number of embryos shot) was 3%, which is similar to our usual results. In our hands, 98% of the transgenic wheats obtained after mannose selection were transgenic for the *pmi* gene (data not shown), so we postulate that all the 24 transgenic events were PMI positive (right border of the cassette). Analysis by GUS assay shown that nine out of the 24 transgenic events (38%) presented GUS expression (left border of the cassette). After the first round of analysis, nine plants were identified that presented evidence for the presence of the two extremities *gus* and *pmi* of the transferred cassette, which was a good indicator of the presence of the 44-kbp *Arabidopsis* genomic fragment. Two of these plants died at the end of in vitro culture process. The seven remaining plants were tagged T0_I to T0_VII and subsequently underwent molecular analysis. A third batch of transformation experiment was performed 6 months later to validate our first transformation results (Supplementary data 2).

Molecular analysis of T0 transgenic events

Genomic DNA of the seven selected T0 plants was extracted from young leaf tissue, digested by three different restriction enzymes and analyzed by Southern blot to characterize the integration profiles of the DNA cassette in the transgenic plants.
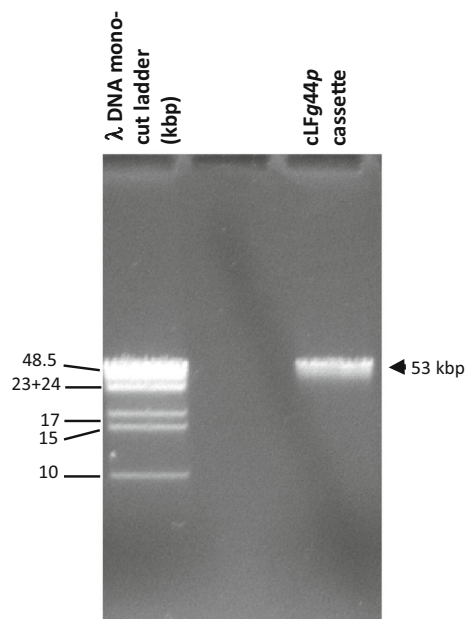

Fig. 2 Gel electrophoresis of the purified 53-kbp cassette cLF*g*44*p*. Lambda DNA mono-cut ladder is a high molecular weight ladder (New England Biolabs)

Genomic T0 DNA was first digested by *Xho*I and hybridized with the cLF*g44p* cassette as probe. Fingerprints obtained for T0 plants were compared to the fingerprint generated by the cLF*g44p* cassette itself. The theoretical profile of a complete copy insertion was attempted to present the 9 *Xho*I internal restriction fragments plus two supplementary bands corresponding to the extra-fragments of unknown size hybridized by the borders of the probe (Fig. 3; Table 1). Five out of the seven T0 plants showed a correct fingerprint. In the second step, the number of copies of the transgene was estimated. Genomic DNA from the T0 plants was digested with *Hind*III and hybridized by *gus* probe (Fig. 1 for *gus* probe and *Hind*III restriction map, and Fig. 3 for Southern analysis). In this configuration, each band resulted from an insertion event of the cassette, depending on the position of the first *Hind*III site on the flanking genomic DNA. Therefore, the number of bands was directly correlated with the minimum number of copies of the cassette inserted into the wheat genome. T0 plants I, II, VI and VII presented a single band on Southern autoradiography (Fig. 3; Table 1), suggesting that a single copy of the *cLFg44p* cassette was inserted into the genome of these plants. These results are consistent with the results obtained by Fu et al. (2000), Yao et al. (2006) and Tassy et al. (2014) showing that the use of linear DNA led to a small number of copy insertions. In a second experiment, the same Southern blot was hybridized by the *pmi* probe (Fig. 1) and only T0 plants VI and VII presented a single band pattern (data not shown). Taken together, these experiments suggest that T0 plants number VI and VII had single-copy insertions of the cLF*g44p* cassette.

The intactness of the 53-kbp cassettes inserted into T0 plants was assessed by probing genomic DNA digested by *Asc*I with a 10-kb *Arabidopsis* probe (Fig. 1). As *Asc*I did not cut into the cassette (Fig. 1), we attempted to obtain a fragment larger than the entire cLF*g44p* DNA. *Asc*I-digested T0 genomic DNAs were separated using pulse field gel electrophoresis to allow efficient separation of large DNA fragments. T0 plants VI and VII have a single band (Fig. 4). These two bands were larger than the cLF*g44p* control. There was no detectable hybridization signal for T0 plants I–V, which was not consistent with the fingerprints observed for these T0 plants on *Xho*I Southern blot (Fig. 3). We hypothesize that the Southern PFG electrophoresis was underexposed and that the bands of the T0 events I–V remained under the detection limit, which could be the consequence of the large size of the wheat genome (16 Gbp) that limits the number of copies on the blot. The same results were observed for the third transformation batch (Supplementary data 2). Moreover, PFG electrophoresis was used to separate large fragments that were difficult to handle and to transfer on the blot, which could limit their level of detection. Results

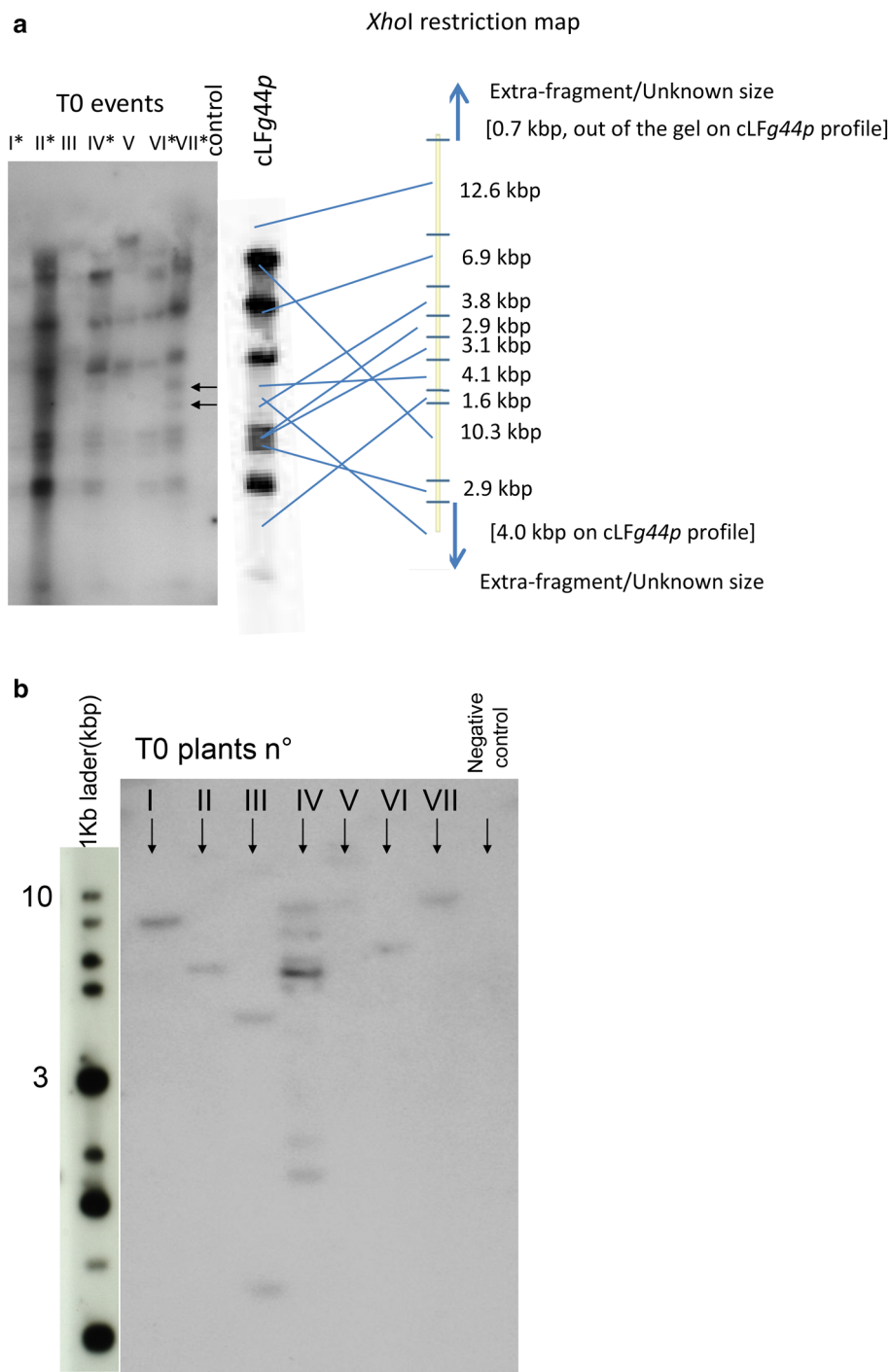obtained using molecular analyses are summarized in Table 1.

Two out of the seven T0 plants (events VI and VII) gave correct profiles in all the Southern analyses. Two additional fragments ($\sim$5 and $\sim$6 kbp) were clearly visible on event VII. The size of these fragments, which were larger than the borders of the cassette, and their specificity to the particular event VII, was consistent with the hypothesis of extra-border fragments. According to these data, we postulated that a single intact copy of the cLF*g44p* cassette was inserted in these two plants. Subsequent analysis was performed to check the heritability and the stability of the insertion in the T1 generation for events VI and VII.

Stability and segregation of the 53-kb insert

Primary transformed plants (T0) were grown in the glasshouse and self-pollinated to produce T1 seeds for genetic and functional analysis of the transferred cassette. Six T1 seeds of each VI and VII T0 event were sown. Inheritance and structural stability of the transgene on T1 progeny were checked by Southern analysis (Fig. 5). The Southern hybridization profiles of T1 plants were the same as those of the corresponding T0 plants, indicating that the large fragment was stably inherited in the T1 generation. Moreover, the two additional $\sim$5 and $\sim$6-kbp bands identified in plant T0VII that could correspond to extra-border fragments were clearly detected in T1VII progeny (Figs. 3, 5, fragments A and B, and Table 2), showing that the integration site was stable.

Fragment C (Fig. 5; Table 2) was not detected in the T0 VII plant but was clearly detected in plants T1VII-1 and T1VII-4, with no detectable modification of the original T0 pattern. Even if the initial *Xho*I profile remained intact, we cannot exclude the possibility that fragment C corresponded to duplication or rearrangement of the cassette fragment. Fragments D and E (Fig. 5; Table 2) were faintly detected in T0 VII plants and in the T1 progeny, and could correspond to artefactual, low specific hybridizations, or to additional borders fragments indicating two independent insertion events. For event VI, a faint fragment F was detected on the T0 VI fingerprint, clearly detected on T1 VI-1, VI-3, VI-5 and VI-6 plants, and not detected on plant VI-4 (Fig. 5; Table 2). This fragment appeared to be in segregation, and could correspond to an extra-border fragment. Indeed, the hypothesis of a single-segregating fragment was not compatible with the hypothesis of a single integration site (in which case, the entire fingerprint would have disappeared from the T1 VI-4 plant), indicating that the entire cassette could have been integrated in two or more loci. Functional analysis by GUS assay showed that *gus* expression was inherited in T1 progeny (data not shown). We checked also that the intact cassette fragment

Fig. 3 **a** Southern blot analysis of T0 plants digested by *Xho*I using the whole cLF*g44p* cassette as probe. Plant genomic DNA was digested by *Xho*I and restriction fragments were separated by agarose gel electrophoresis. *Xho*I digest of the cLF*g44p* cassette was used as control. Southern experiments were performed using the whole cLF*g44p* cassette as probe. Size of the *Xho*I restriction fragments of cLF*g44p* are indicated on the restriction map. T0 plants with correct fingerprint are tagged with an asterisk. *Arrows* indicate the two extra-fragments of event VII. **b** Southern blot analysis of T0 plants for insertion copy number. Genomic DNA from T0 plants was digested by *Hind*III endonuclease. Restriction fragments were separated by agarose gel electrophoresis, transferred onto a membrane and hybridized by a *gus* probe. The number of bands is directly correlated with the number of copies of the cassette inserted into the wheat genome

a T0 events

I* II* III IV* V VI* VII* control

cLF*g44p*

*Xho*I restriction map

Extra-fragment/Unknown size
[0.7 kbp, out of the gel on cLF*g44p* profile]

12.6 kbp
6.9 kbp
3.8 kbp
2.9 kbp
3.1 kbp
4.1 kbp
1.6 kbp
10.3 kbp
2.9 kbp

[4.0 kbp on cLF*g44p* profile]

Extra-fragment/Unknown size

b 1Kb lader(kbp)

T0 plants n°

I II III IV V VI VII

Negative control

10

3

was present in T1 VI-3, -5 and -6 plants (supplementary data 3).

In conclusion, even if some faint additional bands were detected by Southern analysis, the profile generated by *Xho*I in T0 plants was definitely transmitted to the T1 progeny, confirming the stability of the integrated fragment. In addition, we confirmed these results by *gus* and *pmi* PCR analysis, and by histochemical GUS assay on T2 progeny of VI and VII T1 lines (Supplementary data 4).

Cytogenetic structural analysis of T1 progeny by fluorescence in situ hybridization (FISH)

T1 plants of the event VI were analyzed by FISH using the cLF*g44p* cassette as probe. Two loci were detected by FISH, one on chromosome 4B and one on chromosome 6B. On chromosome 4B insertion occurred on the telomere, even though on chromosome 6B, insertion occurred in the middle of one arm of the chromosome (see Fig. 6 for plant

Table 1 Molecular analysis of T0 plants

Enzyme>	XhoI	HindIII	HindIII	NotI
Probe>	cLFg44p	gus	pmi	10 kb Ath
Plants				
T0_I	Fingerprint ok	1	–	0
T0_II	Fingerprint ok	2	5	0
T0_III	Fingerprint missing	3	–	0
T0_IV	Fingerprint ok	4	–	0
T0_V	Missing bands	3	2	0
T0_VI*	Fingerprint ok	1	1	1
T0_VII*	Fingerprint ok	1	1	1

The numbers refer to the number of bands detected on Southern blots. HindIII and NotI restriction maps are presented in Fig. 1c. The XhoI restriction map is presented in Fig. 3. Probes are presented in Fig. 1c. Genomic DNA from T0 plants was digested and restriction fragments were separated by agarose gel electrophoresis (XhoI and HindIII) or PFGE (NotI and AscI) before blotting

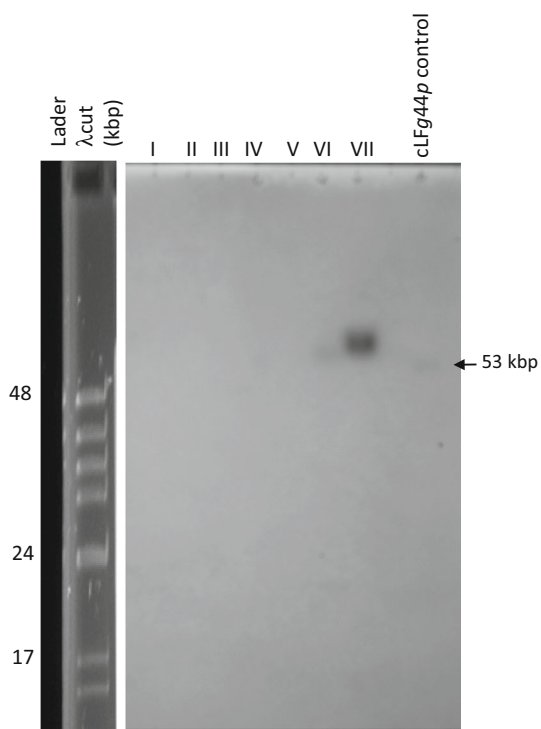Plants T0_VI and T0_VII (*) presented a correct profile in all the molecular analyses

Fig. 4 Southern analysis of DNA from T0 plants digested by AscI and probed with 10-kb Arabidopsis insert. Large DNA fragments generated by the 8-bp site AscI endonuclease were separated by PFGE

T1 VI-6 and supplementary data 5). FISH performed on individual plants of the T1 VI population showed that the two loci segregated independently (Supplementary data 5 and Table 2). Interestingly, the T1 plant VI-4 was detected as potentially null segregant for the 6B loci because the

hybridization signal was only detectable on the two 4B chromosomes on a complete mitotic plate. This plant consequently showed a single insertion of the cassette in the genome, and showed a complete fingerprint of the cassette on XhoI Southern blots (Fig. 5), indicating that that the insertion contained the complete cassette with no major rearrangements. Taken together, these data suggest that the T1 plants VI-4 contained intact insertion of the entire cLFg44p cassette on chromosomes 4B. Fragment F, which segregated in XhoI Southern blot (Fig. 5), was not detected in plant VI-4, suggesting that this fragment could correspond to an extra fragment of the transformation event located on chromosome 6B (Table 2).

FISH analysis of the progeny of event VII also showed two insertion loci on chromosomes 2B and 2D (Supplementary data 6). Plant T1 VII-6 was a null segregant for the 2B locus, and consequently presented a single insertion on chromosome 2D. XhoI Southern showed a complete fingerprint of the cassette with no major rearrangements. These data suggest that plant T1 VII-6 contained a complete and intact cassette inserted on chromosome 2D. Fragments D and E were missing on XhoI Southern only in plant T1 VII-6, suggesting that these two fragments were extra-fragments of the insertion event located on chromosome 2B. In the same way, fragments A and B in Fig. 5 could correspond to extra-border fragments of the insertion located on chromosome 2B. The reason for the presence of fragment C in T1 plants VII-1 and VII-4, and its absence in T0 VII plant remain unclear. One possible explanation is that a rearrangement or a partial duplication of the integrated cassette occurred, but the probability that it occurred twice in independent gametes is small. Another possible explanation involves incomplete digest by XhoI of the DNA of the two T1 plants VII-4 and VII-6 that could generate one supplementary fragment. Another technical explanation could be that this fragment was difficult to detect at the heterozygous stage.

FISH analysis of the T1progeny of two other transgenic events I and III detected insertions at one and two loci, respectively (data not shown). These insertions were located on three different chromosomes, indicating that the cassette was randomly inserted in the genome. Concerning the number of insertion loci, FISH analyses detected two independent insertion loci for events III, VI and VII, and one for event I. In a previous work (Tassy et al. 2014) made with gene cassettes of about 3 kbp, we detected only 13% of the transgenic events that segregated in a non-Mendelian fashion, which could indicate multi-locus insertions. Using Agrobacterium, Cheng et al. (1997) found 32% (16/50) of aberrant segregation and Stoger et al. (1998) found 20% (14/70) using bombardment. Even if we checked only four independent events, it was surprising to find 75% of the plants with two independent insertion sites of the cassette.

Fig. 5 Southern blot analysis of T0 and T1 plants, using 15 µg of genomic DNA digested by *Xho*I and probed with cLF*g*44*p* cassette. cLF*g*44*p* probe is presented in Fig. 1c and cLF*g*44*p*/*Xho*I restriction profile is presented in Fig. 3. *a–e* Fragments of particular interest
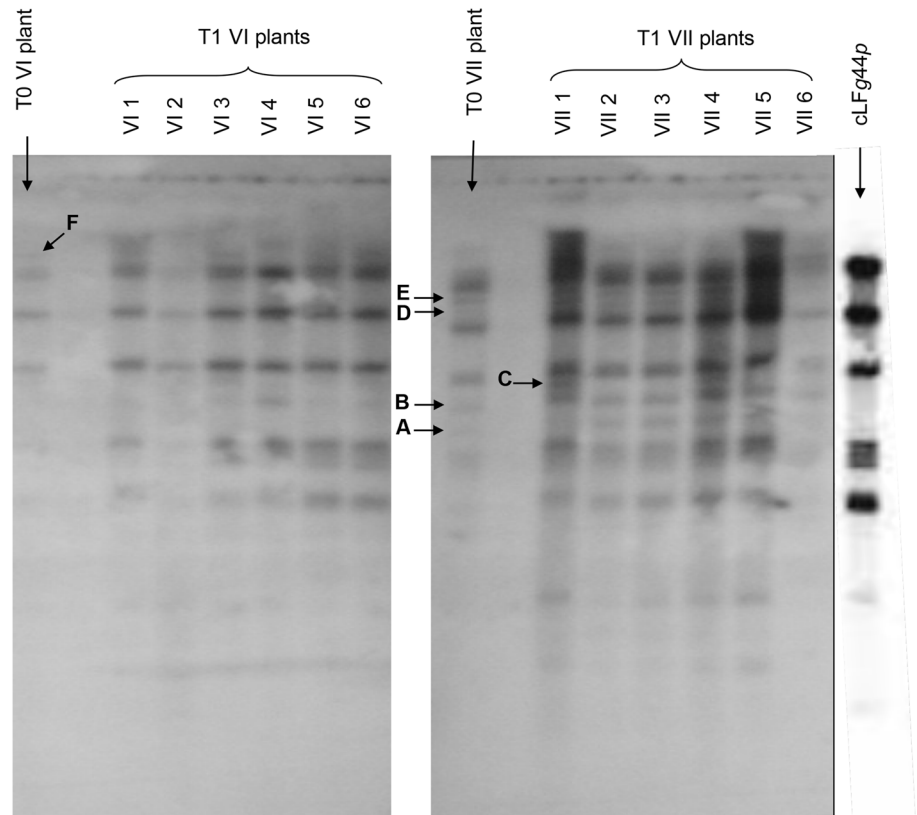

Table 2 a Summary of RFLP fragments identified on *Xho* I Southern blot (Fig. 5) and b FISH analysis of T1 progeny of T0 events VI and VII using cLF*g*44*p* cassette as probe

<Fragments	VI-T0	Event VI T1 progeny						VII-T0	Event VII T1 progeny					
		VI-1	VI-2	VI-3	VI-4	VI-5	VI-6		VII-1	VII-2	VII-3	VII-4	VII-5	VII-6
(a)														
F	+	+	−	+	−	+	+							
E									+	+	+	+	+	+	−
D									+	+	+	+	+	+	−
C									−	+	−	−	+	−	−
B									+	+	+	+	+	+	+
A									+	+	+	+	+	+	+

In situ hybridization of T1 progeny

VI-1	VI-2	VI-3	VI-4	VI-5	VI-6	VII-1	VII-2	VII-3	VII-4	VII-5	VII-6
(b)											
4B/–	4B/M	M/M	4B/4B	4B/–	4B/–	M/M	2B/–	M/M	2B/–	2B/2B	–/–
6B/–	6B/6B	6B/6B	M/M	6B/–	6B/–	M/M	2D/–	M/M	2D/–	2D/M	2D/M

Letters C–G tagged polymorphic fragments on Fig. 5

M Missing data (undetected or unidentified chromosome)

Two hypotheses offer a possible explanation. First, we postulate that FISH is a more powerful method to detect multi-insertions than Southern or genetic analyses on segregating populations. Southern detection by fingerprinting (Figs. 3, 5) of multi-locus insertions is based on the detection of extra-border fragments, two for one locus,
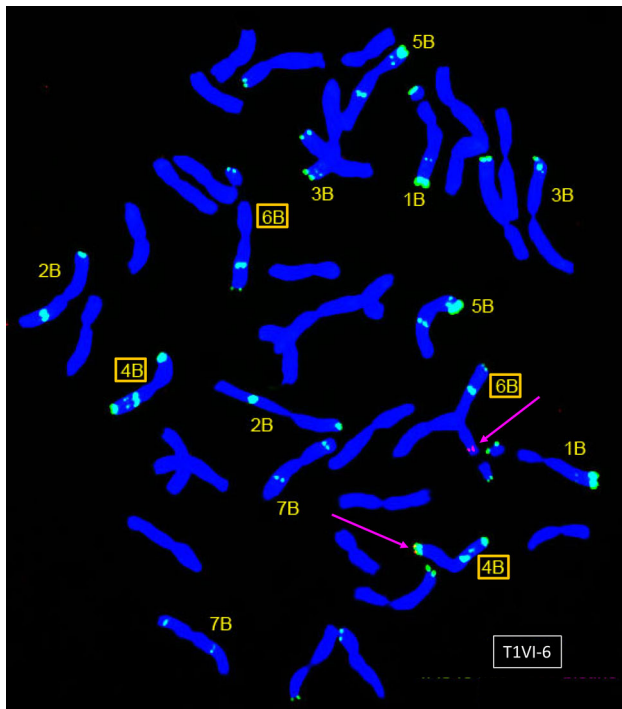
Fig. 6 FISH analysis of T1 plant VI-6. *Red spots* represent the cLF*g*44*p* biotin labeled probe. *Green spots* represent probes specific to genome B (TAS43) or D (PAS1), labeled with fluorescein (color figure online)

four for two loci, but these extra-fragments hybridize with the radioactive probe only on part of their sequence and are consequently very often poorly detected on the autoradiography. It is also very difficult to use Southern analysis hybridized with a cassette-end probe (in our case the *gus* or *pmi* probe in Fig. 1) to distinguish between a duplicated insertion at the same locus and at an independent locus, and also to distinguish between one or more fragments (Fig. 3) when only very faint bands are present on the autoradiography. In the case of genetic analysis, the goal is to detect the statistical difference between segregation of one locus (12/16–4/16) and two loci (15/16–1/16), which for a χ^2 test to have 90% power to detect a significant difference (with alpha = 5%), requires analyzing more than 500 plants. Using a large BAC fragment as probe and FISH, we succeeded in identifying plants harboring two insertion sites, each at the homozygous or heterozygous stage. FISH analysis has also been performed on wheat using shorter probes like cDNA probes (Danilova et al. 2014). Under the first hypothesis, we postulate that in many cases the number of independent insertions is underestimated because the technologies used for the analysis of transgenic plants fail to detect simple insertions at different loci. In our work, we detected four independent events using molecular analysis, but after FISH analysis, seven independent transgenic loci were found to be present in T1 progeny. The second hypothesis is that transformation with large DNA fragments improves multi-locus insertions. The size of the DNA molecule that could potentially form a secondary structure may trigger alternative pathways for DNA integration, which, in turn, could integrate single copies of the DNA at different loci instead of one or more copies at a single location. Validation (or invalidation) of this hypothesis requires new experiments.

After segregation in T1, the three heterozygous mono-locus events tested by Southern fingerprinting (T1 plants VI-1, VI-4 and VII-6) had the correct profile, suggesting the cassette was very often integrated at the two loci with no rearrangement. The two T0 events VI and VII that could have been tested by PFG Southern showed no HMW hybridization fragment to suggest a multi-copy integration locus. Taken together, these results show that, at least for the three T1 plants VI-1, VI-4 and VII-6, a single copy of the cassette was integrated at a single locus, with no detectable rearrangement.

Conclusion

We have demonstrated that biolistic technology can be used to transfer large intact DNA fragments into the wheat genome. Three T1 lines showing a single locus/simple copy insertion of a complete cLF*g*44*p* cassette were generated in our experiments, demonstrating the advantages of biolistic techniques for the transfer of large undisturbed fragments of DNA into the wheat genome. Moreover, only DNA of interest was transferred, with no other undesirable sequence, and with no detectable DNA rearrangements due to the use of Agrobacterium. The transferred fragment was correctly transmitted to the progeny, and expression of the *gus* reporter gene was detectable in T1 and T2 plants. We have also shown that it is very difficult to accurately estimate the number and the integrity of copies of a transgene using only molecular biology techniques, especially in plants with a very large genome like wheat. In this case, the use of FISH as a complement to molecular analysis can provide more precise information.

Our results show that biolistics is one of the best technologies for the introduction of undisturbed DNA fragments into the wheat genome, for example wheat fragments to generate cisgenic (Holme et al. 2013) wheats with no foreign DNA.

Our work also provides evidence that using biolistic technology makes it possible to transfer large undisturbed DNA fragments into the nucleus of the wheat cell. This paves the way for interesting CRISPR/Cas9-mediated targeted integration (Wang et al. 2014) of large DNA fragments containing, for example, clusters of disease resistance genes or new metabolic pathways for synthetic

biology. In the same way, CRISPR-Cas9-assisted homologous recombination (Li et al. 2013) would require long stretches of DNA as a donor for the replacement of a complete allele, for example a gene and its complete regulations. Moreover, the DNA fragments, coated on gold beads, are probably supplied to the nucleus at much higher concentrations than by Agrobacterium-mediated transformation, thereby providing a large quantity of matrix for further integration or recombination events.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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