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TECHNICAL NOTE

Introduction of large DNA inserts into the barley pathogenic fungus, *Ustilago hordei*, via recombined binary BAC vectors and *Agrobacterium*-mediated transformation

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Abstract Genetic transformation of organisms with large genome fragments containing complete genes, with regulatory elements or clusters of genes, can contribute to the functional analysis of such genes. However, large inserts, such as those found on bacterial artificial chromosome (BAC) clones, are often not easy to transfer. We exploited an existing technique to convert BAC clones, containing genomic DNA fragments from the barley-covered smut fungus *Ustilago hordei* to binary BACs (BIBACs) to make them transferable by the *Agrobacterium tumefaciens* T-DNA transfer machinery. Genetic transformation of *U. hordei* with BAC clones using polyethylene glycol or electroporation is difficult. As a proof of concept, two BAC clones were successfully converted into BIBAC vectors and transferred by *A. tumefaciens* into *U. hordei* and *U. maydis*, the related corn smut fungi. Molecular analysis of the transformants showed that the T-DNA containing the BAC clones with their inserts was stably integrated into the *U. hordei* genome. A transformation frequency of approximately 10^{-4} was achieved both for *U. hordei* sporidia and protoplasts; the efficiencies were 25–30 times higher for *U. maydis*. The combination of in vivo recombineering technology for BAC clones and *A. tumefaciens*-mediated transformation of *Ustilago* species should pave the way for functional genomics studies.

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Introduction

Smut fungi of the order Ustilaginales are basidiomycete plant pathogens that occur throughout the world and are responsible for significant crop losses worldwide. Many *Ustilago* species infect members of the gramineae family, including cereal crops and grasses that are used as food and feed. Most smut fungi infect the ovaries of grains and grasses, destroying the seeds and thus considerably reducing yield (Thomas and Metcalfe 1988). The corn smut *Ustilago maydis* as an exception can produce large tumors on floral and vegetative tissue in which black-colored teliospores develop. *U. maydis* is the best-studied smut and has become a model for basidiomycete plant–pathogenic fungi, due to the availability of many molecular tools, good genetics and a complete genome sequence (Kamper et al. 2006; Brefort et al. 2009). *U. hordei*, a close relative of *U. maydis*, causes covered smut on barley and oats. The fungus sporulates during flowering and replaces the seeds with thick-walled teliospores. Teliospores overwinter under the seed hull of healthy seeds or in infested soil and germinate under favorable condition with the seed. Upon germination, teliospores undergo meiosis giving rise to haploid basidiospores that segregate 1 to 1 for mating types *MAT-1* and *MAT-2* (Bakkeren and Kronstad 1994). Cells of opposite mating types, *MAT-1* and *MAT-2*, can recognize each other through a pheromone/receptor system (Bakkeren and Kronstad 1996) and form dikaryotic hyphae by fusion. These dikaryotic hyphae penetrate coleoptiles directly only at the seedling stage and establish themselves in the meristematic region of the growing point. After

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weeks, when the inflorescences develop, the fungal biomass increases and teliospores are formed in the developing seeds, thereby completing the life cycle (Hu et al. 2002). The barley-*U. hordei* pathosystem is an excellent model system for small grain-infecting smut fungi, complementing the *U. maydis*-corn model, because, in contrast to the latter pathosystem, it displays race cultivar-based resistance (Tapke 1945; Linning et al. 2004). The genome of *U. hordei* has been sequenced (Laurie, Bakkeren, Kahmann, Schirawski et al. unpublished).

An understanding of cell development and pathogenicity in *U. hordei* will require an efficient genetic transformation procedure for gene complementation, gene replacement and other genome manipulations. It is also desirable to transfer large DNA fragments containing complete genes with regulatory sequences necessary for gene function, or complete clusters of genes, to assess the location of specific functions/genes on genome-size fragments as represented by bacterial artificial chromosome (BAC) inserts (Shizuya et al. 1992). For example, *U. hordei* harbors gene clusters coding for related predicted secreted proteins that could be effectors during the interaction with its host, similar to the clusters described in *U. maydis* (Kamper et al. 2006; Ali, Laurie, Bakkeren, unpublished).

There is no efficient and reproducible transformation system for *U. hordei*. Current methods use partial protoplasts and the addition of 1% polyethylene glycol (PEG) followed by electroporation (Bakkeren, unpublished). The generation of partial protoplasts involves the use of lytic enzymes, which have to be calibrated for each enzyme batch rendering this method not very reproducible. *Agrobacterium tumefaciens* is a well-known plant pathogen causing crown gall disease on plants by transferring a part of its tumor-inducing (Ti) plasmid DNA to plant cells and integrating this stably into the host genome. Any DNA between specific 25 base-pair imperfect repeats, termed left and right border sequences, can be transferred. Such constructs can be located on smaller replicating plasmids, so-called binary vectors (Hoekema et al. 1983; Lee and Gelvin 2008). Commonplace in plant biotechnology as the agent of choice for genetic transformation, the *Agrobacterium*-mediated transformation (AMT) system has been exploited extensively for fungal transformation as well (Bundock et al. 1995; de Groot et al. 1998; Michielse et al. 2005a; Michielse et al. 2005b; Amey et al. 2002; Sugui et al. 2005; Tucker and Orbach 2007). Compared to conventional transformation methods, AMT is very efficient for many filamentous fungi and has worked well for the development of transformation systems for fungi, which are refractory to transformation using conventional methods, such as *Agaricus bisporus*, *Aspergillus giganteus* and *Helminthosporium turcicum* (Chen et al. 2000; Mikosch et al. 2001; Meyer et al. 2003; Degefu and Hanif 2003; Michielse et al. 2005b). Among basidiomycetes, AMT is very efficient in *Cryptococcus neoformans*, *Cryptococcus gattii* (McClelland et al. 2005) and *U. maydis* (Ji et al. 2010) and was recently also used successfully for the genetic transformation of the flax rust, *Melampsora lini* (Lawrence et al. 2010). AMT is not only more efficient compared to conventional methods, but often results in single copy integration events at random sites in the fungal genome, which is desirable for creating insertion mutations (Mullins et al. 2001; Combier et al. 2003; Takahara et al. 2004). An additional advantage of AMT, compared to classical mutagenesis is the ease with which fungal sequences flanking the T-DNA insertion site can be recovered and identified (Bundock and Hooykaas 1996; de Groot et al. 1998; Bundock et al. 2002; Leclerque et al. 2004). For example, sporulation-deficient mutants, pathogenicity-deficient mutants, antibiotic-deficient mutant and mutants altered in pigmentation have been obtained in several fungi (Rogers et al. 2004; Li et al. 2005; Blaise et al. 2007).

Conventional cloning methods for the generation of transformation constructs using restriction enzymes are often inefficient and time consuming, especially for BAC clones because of their big sizes and few convenient restriction sites (Nagano et al. 2007). Recombineering as an alternative method uses the DNA double-strand repair machinery and bacteriophage lambda RED recombination proteins (Lee et al. 2001) and does not require restriction sites for conventional cloning or specific recombination sites such as the ones required for 'Gateway™' cloning. Recombineering is therefore useful for cloning large DNA fragments and facilitates the cloning of whole or specific regions of BAC or PAC clones (Lee et al. 2001; Raymond et al. 2002).

Methods to transfer large genomic fragments in BAC clones to plants using AMT have been established many years ago when *Agrobacterium*-specific binary BAC vectors, so-called BIBAC vectors, were developed (Hamilton et al. 1996). Combining the above-mentioned techniques, Takken et al. (2004) recently developed a strategy to convert BAC vectors into fungal-specific BIBAC vectors suitable for *Agrobacterium*-mediated transfer into fungal strains. This was efficiently achieved by a one-step procedure making use of in vivo recombineering (Lee et al. 2001) of a linear 'fungal- and binary-specific' fragment into BAC clones.

In the present study, we investigated the feasibility of using this system in *U. hordei* and testing whether large DNA fragments could be delivered stably into its genome via AMT. We converted two BAC clones containing genomic inserts into BIBACs and showed that *A. tumefaciens* can deliver these genomic fragments stably into the genome of *U. hordei*.

Materials and methods

Strains and plasmid

E. coli strain SW102, a recombineering strain derived from strain DY380 (Warming et al. 2005), was obtained from Dr. N. Copeland (National Cancer Institute, Frederick, MD). Supervirulent *Agrobacterium* strain COR309 is a recA-deficient C58 nopaline strain UIA143 harboring disarmed pTiB6 derivative plasmid pMOG101 (Hamilton et al. 1996) and a special vir helper plasmid pCH32, which provides extra copies of the *virA* and *virG* two-component signaling genes. *Agrobacterium* strain COR308 is similar to COR309 except that it has disarmed pTi derivative plasmid pMP90 instead of pMOG101; they were obtained from Cornell University (http://www.biotech.cornell.edu/BIBAC/BIBACHomePage.html). *Ustilago hordei* haploid strain Uh4857-4 (alias Uh364, *MAT-1*) has been described (Linning et al. 2004) and *U. maydis* haploid strain 324 (a2b2) is identical to Um521 (Kronstad and Leong 1989). The REC plasmid pFT41 was obtained from Dr. F. Takken (Swammerdam Institute for Life Sciences, University of Amsterdam, The Netherlands; Takken et al. 2004). pUSBAC5 is a BAC vector derivative of pEcBAC1 (Frijters et al. 1997) converted for use in *Ustilago* species by introducing a specific hygromycin B-resistance cassette (Linning et al. 2004).

Recombineering

Target constructs, pUSBAC5, pUSBAC5_2-1 and pUsBAC5_1-6, were transformed into recombineering *E. coli* strain SW102, selected on Luria–Bertani (LB) plates supplemented with chloramphenicol (Cm) 20 μg/ml at 30°C to prevent premature induction of the phage recombineering genes (RED gene). Details of the protocol can be found in Lee et al. (2001). The recombining REC part was amplified from pFT41 by polymerase chain reaction (PCR) with primers cat-f2 (CCGTTGATATATCCCAATGGC) and catR (ACAAACGGCATGATGAACCT) using TaKaRa LA Taq^{TM} polymerase (TAKARA Bio INC) and the following program on a MyCycler (BioRad): an initial denaturation step of 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 40 s at 58°C and 10 min at 68°C, with a final extension at 68°C for 15 min. The PCR product was digested with *Dpn*I to remove the template and purified on an agarose gel using the QIAquick Gel extraction kit (QIAGEN) according to the manufacturer's instruction. The SW102 cells harboring the target BAC vectors were grown in LB medium supplemented with 20 μg Cm/ml at 30°C to an optical density at 600 nm (OD600) of 0.6–0.9, and incubated for 15 min at 42°C to induce the phage recombination RED genes, the expression of which was under control of a temperature-sensitive λ-repressor. Cells were cooled immediately by chilling on ice for 20 min, then centrifuged at 4,000 rpm for 10 min and washed three times with sterile ice-cold water. Cells were resuspended in an appropriate volume of ice-cold sterile water. Cells were either used fresh for electroporation or mixed with 30% sterile glycerol and stored at −80°C for future use. The electroporation of 40 μl cells in a 0.2 cm gap electroporation cuvette was carried out in a Gene Pulser (BioRad) with 120 ng of REC DNA, using a pulse of 2.5 kV (at 25 μF and 200 Ω). Immediately after electroporation, 0.5 ml of SOC medium (2% Bacto-tryptone, 0.5%, Bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose, pH 7.0) was added and cells were incubated at 32°C for 1 h with gentle shaking (120 rpm) to initiate recombineering, and recover and express antibiotic resistance. Cells were subsequently plated on LB medium containing 50 μg/ml kanamycin (Km) and incubated overnight at 30°C. Transformants were tested for sensitivity to chloramphenicol; correct recombination of the REC vector into the BAC vector disrupts the chloramphenicol acetyl transferase (CAT) gene. Further verification of generated BIBAC vectors was carried out by restriction enzyme digests and PCR.

Fungal transformation

BIBAC constructs were introduced by standard electroporation into *A. tumefaciens* strain COR309 or COR308, and a fresh colony was grown overnight at 28°C in LC medium (0.8% NaCl w/v, 1% Bacto-tryptone w/v, 0.5% Bacto-yeast extract w/v) supplemented with 5 μg tetracycline/ml to select for the helper plasmid pCH32 and 50 μg kanamycin/ml to maintain the BIBAC construct. As much as 5 ml of BIBAC-containing *A. tumefaciens* culture was spun down for 10 min at 4,000 rpm and the pellet resuspended in 5 ml induction medium (IM, minimal medium as in Takken et al. (2004), supplemented with 40 mM MES, 0.5% glycerol, 0.2% glucose). After centrifugation as above, the pellet was resuspended to an OD600 of 0.4 in 5 ml IM containing the appropriate antibiotics and supplemented with 200 μM acetosyringone (AS, PhytoTechnology Laboratories, Shawnee Mission, KS). Cells were incubated at 28°C for 6–8 h to reach OD600 of 0.5. Control cells were treated identically in the same medium, but without AS. *U. hordei* strain Uh364 and *U. maydis* strain 324, grown in 5 ml complete medium (CM; Holliday 1974) for 2 days, were used to re-inoculate 20 ml fresh CM to an OD600 of 0.15 and subsequently grown to an OD600 of 0.5. *U. hordei* was always incubated at 22°C and *U. maydis* at 28°C unless mentioned otherwise. Both *U. hordei* and *U. maydis* cell cultures were diluted tenfold in IM and mixed with an equal volume of AS-induced *A. tumefaciens* culture

and 200 µl of the mixture was plated onto ME-25 filters (Schleicher and Schuell, 0.45 µm pore size, 47 mm diameter), which were placed on co-cultivation medium plates (IM, but with 0.1% glucose and 200 µM AS added); negative controls contained no AS. The membranes were air dried briefly for 10–60 min and incubated at 20–24°C for 2–5 days on co-cultivation media. To select for transformants, membranes were transferred to CM plates containing Cefotaxime 200 µg/ml to kill off *A. tumefaciens* and 300 µg/ml hygromycin B (hyg B, Calbiochem EMD Biosciences, Inc. La Jolla, CA, USA) to select for fungal transformants. After 4 days on selection media, individual transformants were transferred to CM medium supplemented with 300 µg/ml hyg B.

Analysis of transformants

Genomic DNA isolation and PCR amplification

Individual putative *U. hordei* transformants were grown in 5 ml CM medium supplemented with 300 µg hyg B/ml for 2 days. Subsequently, 1 ml of this culture was inoculated in 100 ml fresh CM medium with the same antibiotic. Cultures were spun down and pellets frozen rapidly in liquid nitrogen for direct use or storage at −80°C. Frozen pellets were ground to a fine powder in liquid nitrogen and used for DNA extraction using the DNeasy Plant Maxi kit (QIAGEN) following the manufacturer's instructions. PCR was used to confirm the presence of T-DNA by amplifying an internal 1,023-bp fragment of the hygromycin B phosphotransferase open reading frame using primers hyg B-F (GTACCATGGAAAAGCCTGAACTCACCGCGACG) and hyg B-R (GCATCTAGACTCTATTCCTTTGCCCTC GGAC). The cycling conditions were as follows: an initial denaturation step of 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C and 1 min at 72°C, and a final extension at 72°C for 10 min. To verify the presence of an intact T-DNA insertion, another PCR reaction was performed using primers near the left border: cat-f2 (see above) and LB-r2 (CACAGCGACTTATTCACACGA). An intact left T-DNA border would result in the amplification of a 302-bp fragment. The cycling conditions were the same as above except for an annealing temperature of 64°C and extension time of 30 s.

DNA gel blot hybridization

For DNA blot analysis, 8 µg of genomic DNA was digested with *Ava*I or *Bgl*II run out slowly on a 0.8% (w/v) agarose gel in 1× Tris–borate–EDTA (TBE) buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA pH 8.0), and transferred overnight to a Hybond-N+ membrane as recommended by the supplier (Amersham Biosciences/GE healthcare). Two hybridization probes were used: for the left border of the T-DNA, a fragment of 302 bp was amplified from BIBAC_2-1 by PCR using *Taq* polymerase and primers cat-f2 and LB-r2, and for right border of the T-DNA, a fragment of 564 bp was generated by PCR using primers cat-r2 (ACAAACGGCATGATGAACCT) and Rb-r2 (CACAGCGACTTATTCACACGA). These fragments were gel purified using a QIAquick Gel extraction kit (QIAGEN) following the manufacturer's instruction. Probe labeling was carried out with αP^{32}-dCTP, using the Rediprime II Random Prime Labeling system (Amersham Biosciences/GE healthcare) and hybridized to the membrane using ULTRA-hyb buffer (Ambion) at 42°C according to the manufacturer's instructions. The blots were washed twice for 5 min with 2× SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0) and 0.1% w/v sodium dodecyl sulfate (SDS) followed by two washes each of 15 min in 0.1× SSC and 0.1% SDS. All washes were carried out at 42°C and the blots were exposed to Kodak Biobax film (Kodak Canada, Toronto, ON, Canada).

Results

Recombineering

The recombineering technology is based on the RED homologous recombination system and uses functions that are provided by a defective λ prophage that is present on chromosomal DNA of *E. coli* strain SW102. These λ prophage gene products supply the functions that protect and integrate the linear introduced REC DNA into BAC vectors (Warming et al. 2005). To convert BAC vectors into BIBACs, *Agrobacterium*-specific functions such as a bacterial selectable marker (kanamycin resistance), T-DNA specific border sequences BL and BR and a broad-host range origin of replication need to be introduced on the linear, recombining part of the REC vector. Flanking this transforming fragment are 40-bp ends that provide the homologous termini for integration into the CAT resistance gene. Such an REC vector was developed by Takken et al. (2004) for use in ascomycete fungi by introducing a fungal-specific selectable resistance cassette to allow AMT of *Fusarium* and *Aspergillus* species. We used the pFT41 backbone to convert a previously developed *Ustilago*-specific BAC vector, pUsBAC5, already containing an *Ustilago*-specific hygromycin B cassette under control of the HSP70 promoter and terminator signals (Wang et al. 1988). Integration of the REC fragment from pFT41 would create a binary construct that has the complete pUsBAC5 construct including the hygromycin B cassette and any genomic insert residing on pUsBAC5, between the T-DNA borders BR and BL (Fig. 1).

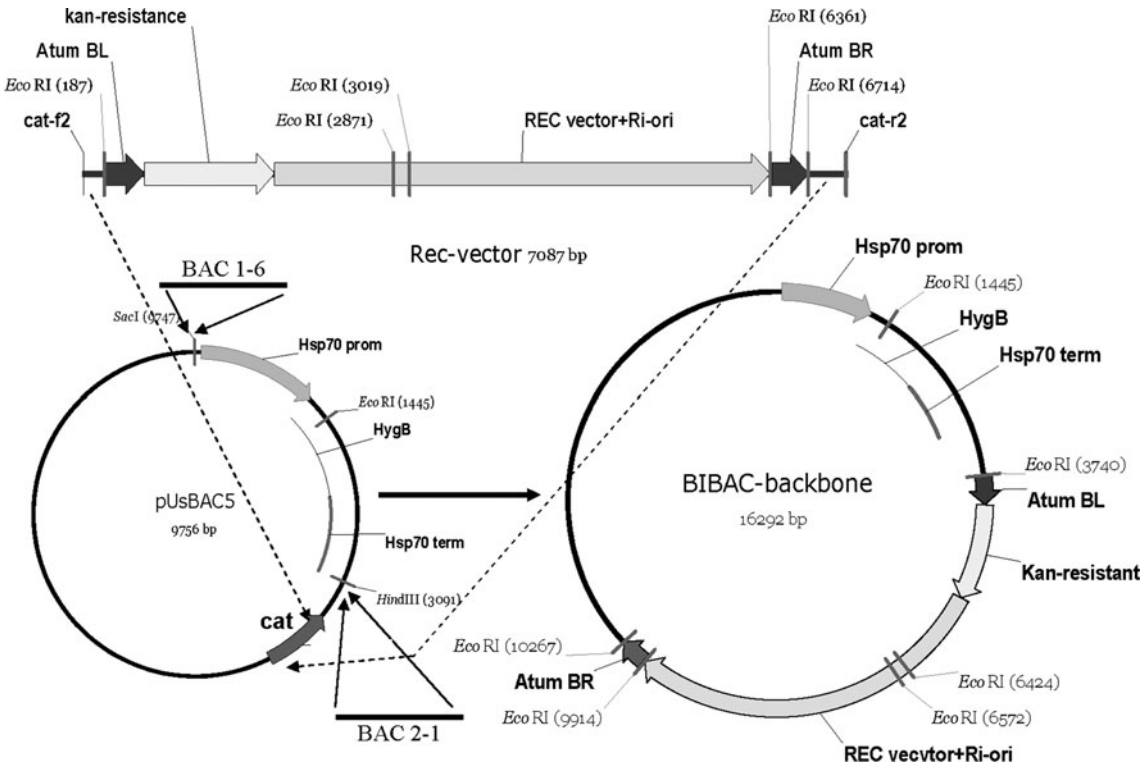

Fig. 1 Schematic representation of the BAC to BIBAC conversion method. Conversion of bacterial artificial chromosome vector, pUSBAC5, harboring genomic inserts, into a binary BAC (BIBAC) vector using a linear 7,087 bp, PCR-amplified DNA fragment (*REC vector*) from pFT41 (*top line*; Takken et al. 2004). The REC vector recombines into the chloramphenicol resistance gene (*CAT*) present on pUSBAC5, using homologous regions present on both left (cat-f2) and right end (cat-r2) of the REC vector (*dashed arrows*). Recombinants are selected for kanamycin resistance (*Kan*) present on the REC vector and screened for loss of chloramphenicol resistance indicating proper integration. The location of the *U. hordei* genomic fragments in the respective BAC clones, 1-6 in the *Sac*I site or 2-1 in the *Hin*dIII site, are indicated (*solid arrows*). The resulting BIBAC constructs are then transformed into a suitable *Agrobacterium* strain for subsequent transformation into *U. hordei*. Any DNA present between the right border (*BR*) and left border (*BL*) sequence elements is considered T-DNA and is transferred by *Agrobacterium* to the host. *U. hordei* transformants were selected on hygromycin B; a Ustilago-specific hyg B phosphotransferase cassette present on the T-DNA resulted from recombination of the REC vector with pUSBAC5

Two different target constructs were made in *Ustilago*-specific BAC vector, pUsBAC5, by inserting a 11-kb *Sac*1 and a 9.3-kb *Xba*1 fragment from the *U. hordei* avirulence gene 1 (*UhAvr1*)-containing genomic region (Linning et al. 2004). The 11-kb *Sac*I-fragment was cloned in the *Sac*I site of pUsBAC5, creating pUsBAC5_1-6, and the 9.3-kb *Xba*1 fragment was inserted in the *Hin*dIII site by partially filling in two base pairs in each of the five overhanging tails generated by *Hin*dIII and *Xba*I with the Klenow fragment of DNA polymerase 1 to create only 2-bp sticky overhangs (Korch 1987); ligation generated construct pUSBAC5_2-1. pUsBAC5 was used as an "empty vector" control. The introduction via electroporation of 120 ng of linear, PCR-amplified REC DNA into *E. coli* strain SW102, previously transformed with the BAC target constructs and heat induced to activate the λ RED genes, usually resulted in 50–100 kanamycin-resistant colonies. No colonies were obtained from cells not heat induced. Kanamycin-resistance colonies were tested for chloramphenicol sensitivity to select for proper recombination in the CAT gene of the BAC vector. In general, 25–30% of kanamycin-resistant colonies became chloramphenicol sensitive in RED-induced cells (Table 1). Figure 2 shows an *Eco*RI restriction enzyme pattern of BIBAC plasmids purified from colonies that were chloramphenicol sensitive and kanamycin resistant. The restriction analysis of these recombinants showed that the REC vector had integrated at the proper position without causing any rearrangements of the BAC clones. It appears that recombineering works well and allows for the conversion of *U. hordei* BAC library clones into BIBAC vectors.

Fungal transformation

Constructs BIBAC_2-1, BIBAC_1-6 and BIBAC_5 ("empty vector" control) were introduced into *A. tumefaciens* strain COR309 and COR308 via electroporation. These *Agrobacterium* strains are *recA*$^-$ and contain extra copies of *virA* and *virG* on a helper plasmid. Frary and Hamilton (2001) have shown that extra copies of these

Table 1 Recombineering and transformation efficiencies

BAC	RE (%)	Inoculation medium	Cocultivation medium	*U. hordei* TF (transformants/ 1.2×10^4 sporidia	*U. hordei* TF (transformants/ 1.2×10^4 protoplasts	*U. maydis* TF (transformants/ 1.2×10^4 sporidia
2–1	30	+AS	−AS	0	0	0.4
		+AS	+AS	1	1	32
1–6	25	+AS	−AS	0	0	0.5
		+AS	+AS	0.8	0.9	27
pUSBAC5	34	+AS	+AS	3	NT	40

Recombination efficiencies of conversion of BAC clones into binary BAC constructs in *E. coli* (RE), and transformation efficiencies (TF) of *U. hordei* sporidia, protoplasts and of *U. maydis* sporidia using AMT

AS medium with (+) and without (−) acetosyringone added, *NT* not tested

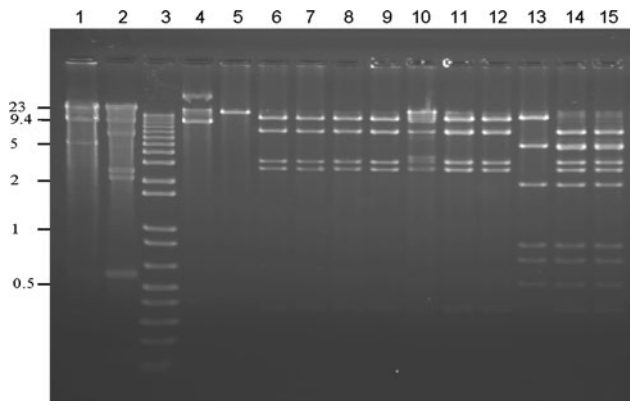

Fig. 2 Verification of conversion of BAC clones to BIBAC vectors. Ethidium bromide-stained 1.2% agarose gel showing *Eco*RI-digested pUSBAC5 (*lane 4*), pUSBAC5_2-1 (*lane 5*), seven independent BIBAC_2-1 clones (*lanes 6–12*), pUSBAC5_1-6 (*lane 13*) and two BIBAC_1-6 clones (*lanes 14* and *15*). The molecular markers are: 5 kb ladder (*lane 1*), *λHind*III fragments (*lane 2*) and 1 kb ladder (*lane 3*) with sizes indicated in kb on the *left*

acetosyringone (AS) inducer-sensing and signaling components are essential for successful transformation of plants with large pieces of DNA, such as those contained on BIBAC vectors. Co-cultivation of *Agrobacterium* cells harboring BIBAC_5, BIBAC_2-1 and BIBAC_1-6 with *U. hordei* strain Uh364 in the presence of AS led to the formation of hygromycin B-resistant colonies, while no colonies were obtained on co-cultivation medium without AS. We also included related corn smut pathogen *U. maydis* strain 324 (a2b2) for comparison. The AMT transfer efficiency of these BIBAC constructs into *U. hordei* is lower than that for *U. maydis* (Table 1). Interestingly, for *U. maydis*, some hygromycin B-resistant colonies were obtained in the absence of AS in co-cultivation medium, although AS was used in the induction medium. For *U. hordei*, hygromycin B-resistant colonies were obtained only when AS was used in both induction and co-cultivation media. We tested whether *U. hordei* cells without cell walls (protoplasts) would be more sensitive to the *Agrobacterium* T-DNA transfer machinery. AMT transformation efficiencies were found to be essentially the same as for sporidia (Table 1). The transformation frequency of BIBAC_5, the "empty vector" control that still harbored a T-DNA insert of approximately 10 kb, was on average three times higher in *U. hordei* than that of BIBAC_2-1 and BIBAC_1-6 (Table 1). Various parameters have been reported to affect AMT efficiencies in other fungi, such as drying of co-cultivation medium plates with *Agrobacterium* and fungal mixtures for various times (Almeida et al. 2007), the length of the cocultivation period (Rho et al. 2001), the use of different ratios of *Agrobacterium* to fungal cells (Michielse et al. 2005b) and various cocultivation temperatures (Michielse et al. 2005b). However, due to low overall transformation efficiencies, no significant differences could be measured.

Molecular analysis of fungal transformants

To test the mitotic stability of the transgenes, eight randomly selected hygromycin B-resistant transformants (selected on CM medium supplemented with 300 µg hygromycin B/ml) were transferred to selection-free PDA plates for five successive cycles (4 days of growth at 22°C per cycle). Cells from non-selective PDA plates were then transferred to 100 ml CM liquid medium supplemented with 300 µg hygromycin B/ml for total genomic DNA isolation. To confirm the presence of intact T-DNA, two PCR analyses were performed: one to test for the presence of the internal hygromycin B phosphotransferase gene and one for the left T-DNA border. Using the primers hyg B-F and hyg B-R, a PCR product of expected size (1,020 bp) was amplified from DNA of all eight putative transformants (Fig. 3a), which correlated with the observed growth in selective hygromycin B medium. *Agrobacterium* generates T-DNA directionally from an initial nick at the right border, which is then linked to the virD2 protein, ending at the left border. Integrated T-DNA therefore frequently has variable left border truncations in contrast to the right

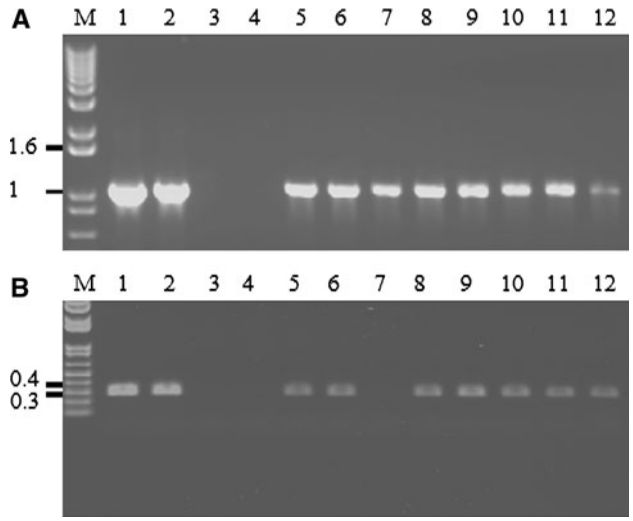

Fig. 3 PCR analysis of genomic DNA of six independent BIBAC_2-1 and two independent BIBAC_1-6 *U. hordei* transformants. **a** Ethidium bromide-stained 1% agarose gel showing the amplification of a 1,020 bp fragment constituting the hygromycin B phosphotransferase open reading frame, using primers hyg B-F and hyg B-R. **b** Ethidium bromide-stained 1% agarose gel showing PCR amplification products of 302 bp using primers LB-r2 and cat-f2, representing the T-DNA left border end. *Lane 1* BIBAC_1-6 vector (positive control), *lane 2* BIBAC_2-1 vector (positive control), *lane 3* Uh364 untransformed (negative control), *lane 4* Uh365 untransformed (negative control). *Lanes 5–10* independent Uh364 BIBAC_2-1 transformants (named A, B, C, D, E and F, respectively), *lanes 11 and 12* independent Uh364 BIBAC_1-6 transformants (named Y and Z, respectively). *M* 1 kb plus molecular weight DNA ladder. Size bars at the left side of the gels are in kbp

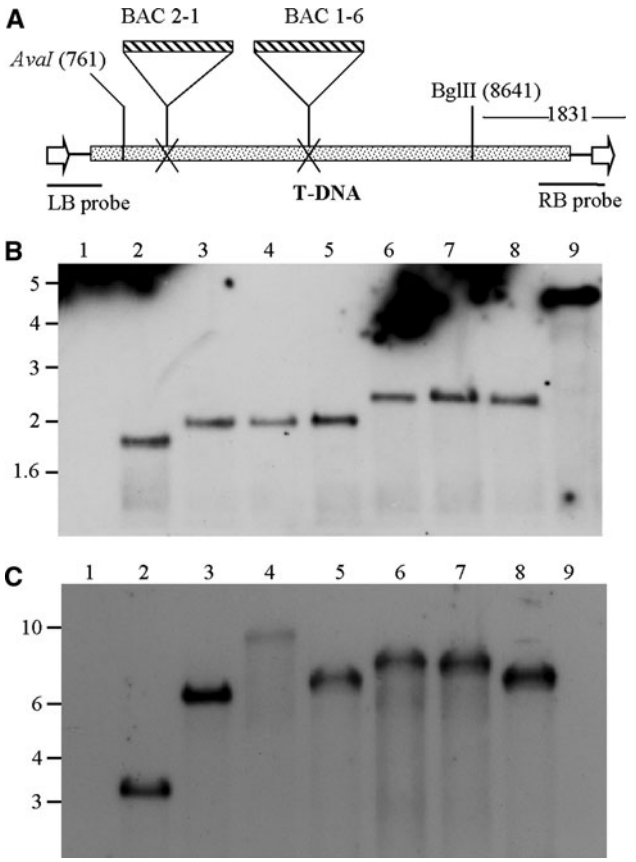

Fig. 4 DNA blot analysis of the genomic DNA of independent *U. hordei* BIBAC transformants. **a** Genomic DNA was digested with *Ava*I, which cuts 761 bp proximal to the *left border* of the T-DNA. The membrane was probed with a 302-bp fragment located within the *left border* of the T-DNA and the most proximal *Ava*I site (*black line* in the cartoon above the blot; the location of the genomic inserts, BAC_2-1 or BAC_1-6, is indicated). **b** Genomic DNA was digested with *Bgl*II, which cuts 1,831 bp proximal to the *right border* of the T-DNA. The membrane was probed with a 564-bp fragment located within the *right border* of the T-DNA and the most proximal *Bgl*II site (*black line* in the cartoon above the blot). *Lane 1* Uh364 untransformed (negative control), *lanes 2–7* Uh364 BIBAC_2-1 transformants A, B, C, D, E and F, respectively, *lanes 8* and *9* Uh364 BIBAC_1-6 transformants Y and Z. *Size bars* at the *left side* of the blots are in kbp

border junction, which is often more precise (Tinland 1996; Bundock and Hooykaas 1996; Zhong et al. 2007). The second PCR was performed to verify the presence of intact left border sequences by using primers LB-r2 and cat-f2, which is expected to amplify 302 bp immediately adjacent to the left border of the T-DNA. Seven out of the eight transformants amplified a PCR product of the expected size (Fig. 3b).

The genomic DNA of the eight selected, PCR-positive, stable *U. hordei* transformants was analyzed on DNA blots to determine the randomness of T-DNA integration and to assess copy number of the insertions. We analyzed both ends of the T-DNA insertion by hybridizing two separate blots with a left T-DNA border- or a right border-specific probe. Transformant C, which was negative for the left border in the PCR analysis, was positive on hybridization (Fig. 4). Single fragments were revealed for all transformants for both right border and left border junctions which, since the selected probes did not span the chosen restriction enzyme sites, indicated that the T-DNA had inserted as a single copy in each strain. At least seven of the transformants revealed junction fragments of different sizes indicating random insertion events at different locations in the genome. One transformant, Uh364 BIBAC_1-6 transformant Z, did not show a positive hybridization signal with the right border-specific probe (Fig. 4b); in this case, it is likely that the right border end of the T-DNA became truncated upon the integration event, thereby deleting the probe binding site. Truncation of T-DNA upon integration is not uncommon, but is normally more prominent at the left border junctions.

Discussion

The main objective of this study was to establish an AMT protocol for transferring large fragments of genomic DNA,

contained on BAC library clones, into the barley smut fungus, *Ustilago hordei*. To this end, we evaluated the use of an in vivo recombineering method for converting BAC library clones into binary BAC (BIBAC) vectors and to subsequently develop an AMT protocol for this fungus. The recombineering method is based on a modification of cloned DNA in *E. coli* via a λ RED-mediated homologous recombination and avoids the cumbersome restriction and ligation reactions usually carried out to modify DNA (Warming et al. 2005). The recombination of the REC vector into a BAC clone is based on the expression of RED genes from a stably integrated defective λ prophage under the control of a temperature-sensitive repressor, cI857. The REC vector provides all the required functions for the construction of a binary vector (Takken et al. 2004). Recombineering uses stretches of homologous DNA, which in this method are provided by the bacterial chloramphenicol resistance gene. Proper insertion of the REC vector into BAC clones results in the loss of chloramphenicol resistance, while resistance to kanamycin, present on the REC vector, is gained. This provides an easy tool to select for likely proper recombinants, which can then be verified by restriction enzyme analysis (Fig. 2). We obtained an efficiency of proper recombination of 25–34% based on the gain of kanamycin and loss of chloramphenicol resistance. This is comparable to the 40% reported for the conversion of *Fusarium oxysporum* and *Aspergillus awamori* BAC clones into BIBACs by recombineering (Takken et al. 2004). The remaining colonies, found to be both kanamycin and chloramphenicol resistant, may be the result of integration of the transforming REC fragment into BAC vector locations other than the CAT gene, including homologous stretches in the BAC genomic insert, or may be due to dimerization of the generated BIBAC with the original BAC clone and thus contain both selectable markers (Takken et al. 2004). Yu et al. (2000) reported that such dimerization can be suppressed by transformation of both REC and BAC plasmids at the same time.

Compared to plant transformation, AMT of fungi is relatively new (Bundock et al. 1995; de Groot et al. 1998) but has been very successful for a number of species, and the number of fungi that can be transferred by *Agrobacterium* has been increasing. However, optimization of the transformation protocol is required for each species (Michielse et al. 2005b; Mata et al. 2007). To our knowledge, this is the first report on AMT of the barley pathogen *U. hordei*.

U. hordei strains Uh364 and *U. maydis* strain 324 were transformed with either BIBAC_2-1, BIBAC_1-6 or "empty vector" control BIBAC_5, using two *Agrobacterium* strains, COR308 and COR309. Overall, the transformation efficiency of *U. hordei* was low compared to *U. maydis* using the same protocol. In a recent study, Ji et al. (2010) reported on the development of an efficient AMT method for *U. maydis*, employing a series of optimization steps. In our study, no difference was observed in the transformation efficiency when using the two *Agrobacterium* strains COR309 or COR308, indicating that the origin of the virulence functions on the Ti plasmid does not influence efficiency. The transformation efficiency of BIBAC_5 was at least three times higher than that of both BIBAC_2-1 and BIBAC_1-6; it was slightly higher for BIBAC_2-1 compared to BIBAC_1-6. This suggests that the size of the insert (T-DNA) in the vector could influence the transformation efficiency. Alternatively, the inserts in both BIBAC_2-1 and BIBAC_1-6 clones originate from a region in the *U. hordei* genome where many repeats and transposable element have been found and this could also be the reason for the low transformation efficiencies found. Such elements might inhibit integration or it may be (in addition) that such repeats present on these T-DNAs and homologies in the genomic DNA in *U. hordei* make integration in the genome less efficient as to avoid duplication (Yu et al. 2000). Takken et al. (2004) also observed a low transformation efficiency and truncation of integrated T-DNA when using BAC clones containing genomic DNA inserts with large stretches of homologous DNA, compared with the empty vector without any homologous region.

We evaluated the effect of acetosyringone (AS), a plant phenolic compound produced in wound sites of plants. It is an inducer of the vir genes in *A. tumefaciens* (Gelvin 2003) and serves as inducing agent for in vitro transformation. We obtained *U. hordei* hygromycin B-resistant colonies only when AS was used both in the induction and co-cultivation media, which suggest that AS is essential for AMT of *U. hordei*, a requirement found for the majority of fungal species (Michielse et al. 2005b; Zhang et al. 2008; Marchand et al. 2007; Duarte et al. 2007). The presence of AS in induction medium before cocultivation is not necessary for *Agrobacterium* growth, but its presence has been reported to improve transformation efficiency in *Aspergillus carbonarius*, *Fusarium oxysporum* and *Magnaporthe grisea* (Mullins et al. 2001; Morioka et al. 2006). Indeed, in parallel experiments of AMT transformation of *U. maydis* 324, we obtained a few hygromycin B-resistant colonies in the absence of AS in the co-cultivation media, although AS was included in the induction medium.

The mitotic stability of the transformants was verified by growth on non-selective media plates for five successive transfers and subsequent comparative growth on selective versus non-selective medium plates. Subsequent PCR amplification of the hyg B phosphotransferase gene and the left T-DNA border (Fig. 3) and analysis of the genomic DNA by DNA blot hybridization (Fig. 4) were consistent with stable T-DNA integration into chromosomal DNA and similar to other reports on T-DNA transfer to filamentous

fungi (Ji et al. 2010; Gelvin 2003; Covert et al. 2001). The copy number of integrated T-DNA was assessed by DNA blot analysis (Fig. 4) and a single copy was found for all eight positive transformants tested. Previous reports showed that several parameters affect the T-DNA copy number in fungal genome (Michielse et al. 2005b). For example, the addition of AS in IM and the length of cocultivation time seem to affect the number of T-DNA integrations per transformant (Combier et al. 2003; Rho et al. 2001). The addition of AS in IM as carried out in our experiments, reduced the occurrence of multiple integrations in the ectomycorrhizal fungus, *Hebeloma cylindrosporum* (Combier et al. 2003), while in *Magnaporthe grisea* the addition of AS in IM increased multiple integrations (Rho et al. 2001).

DNA blot analysis revealed at least seven fragments of different sizes among the eight transformants analyzed, suggesting a random mode of integration. It has been suggested that the mode of T-DNA integration either by homologous or non-homologous recombination depends on the organism (Bundock et al. 1995; Covert et al. 2001; van Attikum et al. 2001). Cloning and sequencing of the junctions between the integrated T-DNA and the genomic insertion sites would be necessary to verify true random integration and to assess the precise mode of integration.

The T-DNA transfer process in *Agrobacterium* starts at the right border after nicking and the attachment of a VirD2 protein on the 5′-end of the nascent single strand. The production of the T-DNA molecule proceeds until the left border sequence is reached and another nick is introduced, generating an unprotected end (Tzfira and Citovsky 2006). After transfer and during integration, deletion of T-DNA nucleotides occurs at the junctions of the T-DNA repeats, most frequently at the unprotected left border end in plants, yeast and other filamentous fungi (Tinland 1996; de Groot et al. 1998; Bundock and Hooykaas 1996; Zhong et al. 2007). Recipient insertion sites in the genomic DNA also often suffer deletions. PCR analysis revealed that the left border end was missing from only one of the transformants, but the DNA blot analysis showed that this must be a minor truncation of T-DNA at this left border (including a primer binding site) because the left border end probe still hybridized (Fig. 4).

In conclusion, the strategy of using recombineering to convert BAC library clones into BIBAC constructs and to use *A. tumefaciens* for the transfer of these BIBAC constructs to *U. hordei* is feasible. We have several BAC libraries containing *U. hordei* genomic inserts of various sizes (Bakkeren et al. 2006). One is derived from a strain, which has several avirulence genes and the complete genome of which has been sequenced (Laurie, Bakkeren, Kahmann, Schirawski, unpublished). The method described in this paper will facilitate the functional analyses of individual genes and whole gene clusters by complementation studies.

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