Curr Genet (1996) 30: 166–173 © Springer-Verlag 1996

ORIGINAL PAPER

Miranda D. van de Rhee · Odette Mendes
Marc W. T. Werten · Henk J. Huizing
Hans Mooibroek

Highly efficient homologous integration via tandem exo-β-1,3-glucanase genes in the common mushroom, *Agaricus bisporus*

Received: 6 February 1996 / 27 March 1996

Abstract Homologous integration was studied in the common mushroom, *Agaricus bisporus*, using a plasmid (pHAG3-1) carrying the hygromycin-resistance gene and a 3.2-kb genomic fragment from *A. bisporus*. Homologous integration was found in 30–60% of the transformants obtained with pHAG3-1 linearized at three different positions within the homologous sequence, generating either blunt, 5′- or 3′-protruding ends. The genomic fragment was found to contain two homologous open reading frames in tandem, which showed 60% similarity to exo-β-1,3-glucanases from *Saccharomyces cerevisiae* and *Candida albicans*. The level of the corresponding mRNA is low in the vegetative mycelium and relatively high in fruiting bodies. In the vegetative mycelium of a transformant with tandemly integrated pHAG3-1 plasmids at the homologous position, exoglucanase mRNA was strongly increased without any apparent effect on growth rate or morphology.

Key words *Agaricus bisporus* · Homologous recombination · Exo-β-1,3-glucanase · Transformation

Introduction

The homobasidiomycete *Agaricus bisporus* (the common mushroom) is the most important cultivated edible mushroom. Over recent years, more information has become available from this species on genes involved in basal metabolism (Harmsen et al. 1992), colonization of the compost substrate (Raguz et al. 1992; Perry et al. 1993; Chow et al. 1994), spore traits (Kerrigan et al. 1994) and quality parameters, such as browning of the fruiting body (Wichers et al. 1995). The application of recombinant DNA technology in this fungus has been hampered by its recalcitrance to DNA-mediated transformation (Li and Horgen 1993; Challen and Elliott 1994). Only recently, however, both a homo- and a hetero-karyotic strain of *A. bisporus* were stably transformed to hygromycin B resistance in our laboratory and transgenic fruiting bodies were obtained (Van de Rhee et al. 1996).

Initially, transformations were carried out with pAN7-1 (Punt et al. 1987), containing the *Escherichia coli* hygromycin B phosphotransferase (*hpt*) gene fused to a promoter and terminator from the ascomycete *Aspergillus nidulans*. Since only few transformants were obtained with this vector (1–5 per 10 µg DNA), several strategies were adopted to increase transformation efficiency. First, the *hpt* gene was fused to the *GPD2* (glyceraldehyde 3-phosphate dehydrogenase) promoter from *A. bisporus*. This, however, did not lead to any significant increase in transformation efficiency (Van de Rhee et al. 1996). Recent work has demonstrated that additional replacement of the *A. nidulans* terminator by the *A. bisporus GPD2* terminator, only yielded approximately twice as many transformants (unpublished results). In a number of fungi, transformation frequencies and/or integration were enhanced when fragments homologous to the recipient genome were incorporated in the vector (Orr-Weaver et al. 1981; Tsuge et al. 1990; Farman and Oliver 1992; Steiner et al. 1995). Double-strand breaks within the homologous region can increase the frequency of integration at the resident locus. The present paper describes the incorporation of a randomly selected 3.2-kb genomic fragment of *A. bisporus* (called AbGH3) into the pAN7-1 transformation vector. The resulting plasmid pHAG3-1 was linearized within the homologous region before transformation, in an attempt to enhance plasmid integration into the *A. bisporus* genome. However, transformation efficiency was again not enhanced, although it was found that plasmid pHAG3-1 was targeted to the homologous position in up to 60% of the transformants. The AbGH3 fragment was subsequently characterized by sequencing and Northern-blot analyses.

M. D. van de Rhee · O. Mendes · M. W. T. Werten · H. J. Huizing
H. Mooibroek (✉)
DLO-Institute for Agrotechnological Research (ATO-DLO),
P.O. Box 17, 6700 AA Wageningen, The Netherlands

Communicated by P. de Wit

Materials and methods

Strains and transformation. The homokaryotic *ade* mutant strain of *A. bisporus* (ATCC 24663) was cultivated and transformed as described by Van de Rhee et al. (1996). ATCC 24663 transformants and the heterokaryotic strain Horst U1 (ATCC 62462) were maintained on MMP agar medium (1% malt extract, 0.5% mycological peptone, 1.5% agar). Horst U1 fruiting bodies were obtained from the Mushroom Experimental Station, Horst, The Netherlands.

DNA manipulations. DNA manipulations and cloning were carried out using standard procedures (Sambrook et al. 1989) and *E. coli* strain JM109. The AbGH3 fragment was isolated from U1 as an *Eco*RI genomic fragment of approximately 3.2 kb and cloned in pUC8. It was isolated from this plasmid as a 3.2-kb *Hin*dIII fragment (*Hin*dIII sites were present in the pUC8 polylinker and inside the genomic fragment just upstream of one of the *Eco*RI sites) and cloned in the *Hin*dIII site of pAN7-1 (Punt et al. 1987), downstream from the *A. nidulans trpC* terminator, to create pHAG3-1. Subclones of the *Hin*dIII fragment were sequenced using the A.L.F. system (Pharmacia). Before transformation of *A. bisporus*, pHAG3-1 DNA was digested with *Kpn*I, *Spe*I or *Eco*RV, extracted with phenol-chloroform (1:1) to remove restriction enzymes, precipitated and dissolved in 10 mM Tris, 1 mM EDTA (pH 8.0). Ten micrograms of DNA were used per transformation.

Southern- and Northern-blot analyses. For DNA and RNA isolation, the vegetative mycelium was grown on MMP agar medium covered with cellophane. DNA was isolated from freeze-dried mycelium according to Raeder and Broda (1985). Southern-blot analyses were performed with 1 µg of DNA per sample using standard procedures (Sambrook et al. 1989). The AbGH3 sequence was labelled using digoxigenin-dUTP and detection was performed using Lumigen PPD chemiluminescence following the manufacturer's instructions (Boehringer Mannheim). RNA was isolated from the vegetative mycelium and from fruiting bodies (Van Tunen et al. 1988). Seven fruiting body stages were distinguished according to Hammond and Nichols (1976) and four mushrooms per stage were harvested and pooled after separation into cap and stipe. Northern blotting was performed with approximately 10 µg of total RNA per sample using standard procedures (Sambrook et al. 1989) with the ^{32}P-labelled AbGH3 sequence, exon 9 from *GPD*^{Ag2} (Harmsen et al. 1992), and a 5.3-kb *Eco*RI genomic fragment with rRNA genes of *Schizophyllum commune* monokaryon 4–39 (Ruiters and Wessels 1989) as probes. RNA size markers were from Gibco BRL.

Poly(A)$^+$ RNA isolation and RT-PCR. Poly(A)$^+$ RNA was isolated from total RNA derived from a mixture of stage 2 and 3 fruiting bodies of strain U1, using Hybond mAP paper (Amersham). Reverse transcription was performed for 1 h at 37°C in 20 µl containing 100 ng of poly(A)$^+$ RNA, 0.5 µg of oligo d(T)$_{12–18}$, 20 nmole of dNTPs, 35.7 U of RNA-guard (all from Pharmacia) and 200 U of Moloney MuLV reverse transcriptase (Gibco-BRL) in 1× Expand buffer 1 (Boehringer Mannheim). PCR was performed using 4 µl of the RT-reaction in the Expand system (Boehringer Mannheim) with primers 5′-GCGGGAGAGCCTTACATTCAG-3′ (nt 466 to 486 of the AbGH3 sequence) and 5′-CCCAGAACTACAAGCAGACTG-3′ (complementary to nt 1399–1419). The RT-PCR product was cloned into pGEM-T (Promega).

Results

Homologous integration of pHAG3-1

Plasmid pHAG3-1 (Fig. 1 A) was obtained by inserting a randomly selected 3.2-kb *Hin*dIII fragment of genomic *A. bisporus* DNA (called AbGH3) into transformation vector pAN7-1 (Punt et al. 1987). A restriction map of the

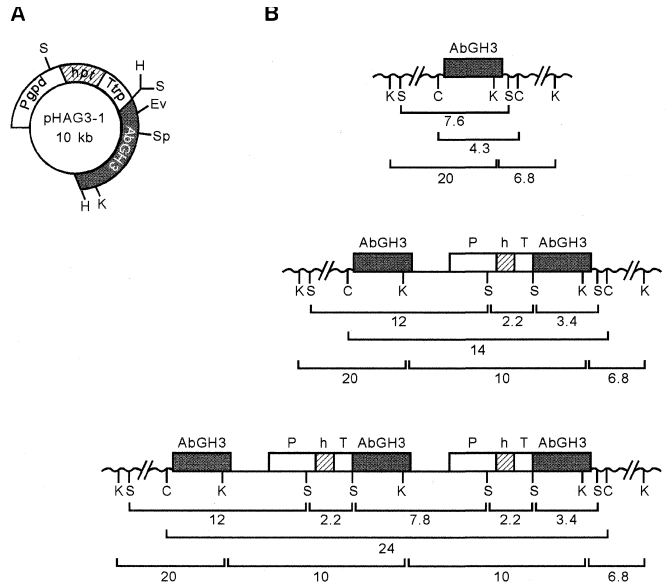

Fig. 1 A, B Map of plasmid pHAG3-1 **A** schematic representation of (from top to bottom): the AbGH3 locus in *A. bisporus* ATCC 24663, single-copy homologous integration and tandem homologous integration of pHAG3-1 into the AbGH3 locus **B** a*bbreviations: Pgpd or P* the *A. nidulans gpdA* promoter; *hpt or h* the *E. coli* hygromycin B phosphotransferase gene; *Ttrp or T* the *A. nidulans trpC* terminator; *AbGH3* the 3.2-kb *A. bisporus* genomic fragment. *Single line* pUC18 vector (**A, B**). *Wavy line* bordering *A. bisporus* genomic DNA (**B**). *Restriction enzyme sites: C Cla*I; *EV Eco*RV; *H Hin*dIII; *K Kpn*I; *S Sal*I; *Sp Spe*I. In **B** approximate restriction fragment sizes are given after digestion with *Sal*I, *Cla*I and *Kpn*I

AbGH3 region in *A. bisporus* was obtained by Southern-blot analysis (Fig. 1 B, upper panel). A homokaryotic adenine mutant strain (ATCC 24663) of *A. bisporus* was used for transformations with a *Kpn*I-linearized pHAG3-1 plasmid. *Kpn*I cuts at 369 bp from the 3′ end of the AbGH3 sequence in pHAG3-1 (Fig. 1 A). The numbers of hygromycin B-resistant transformants produced were comparable to those obtained with *Hin*dIII-linearized pAN7-1 lacking the AbGH3 sequence. Southern-blot analysis of the pHAG3-1 transformants with an *hpt* probe confirmed integration of the plasmid into the *A. bisporus* genome (data not shown). Subsequently, AbGH3 was used as a probe to determine whether the plasmid had integrated at the homologous position or ectopically. Homologous integration would lead to a duplication of the AbGH3 locus separated by vector sequences (Fig. 1 B, middle panel). Genomic DNA from non-transformed ATCC 24663 was digested with *Cla*I, which cuts neither the vector nor the resident AbGH3 locus, yielding a 4.3-kb hybridizing band (Figs. 1 B and 2 A, lane 3). Upon integration of pHAG3-1 into the AbGH3 region, this band was predicted to shift to a higher position (14 kb for a single-copy insertion, 24 kb for a tandem two-copy insertion, etc.). This was indeed found for 8 out of 13 transformants; representative examples are shown in Fig. 2 A. *Cla*I-digested DNA from transformants with homologous integration of multiple copies of pHAG3-1 are shown in lanes 5 and 7 (Ck10-3 and

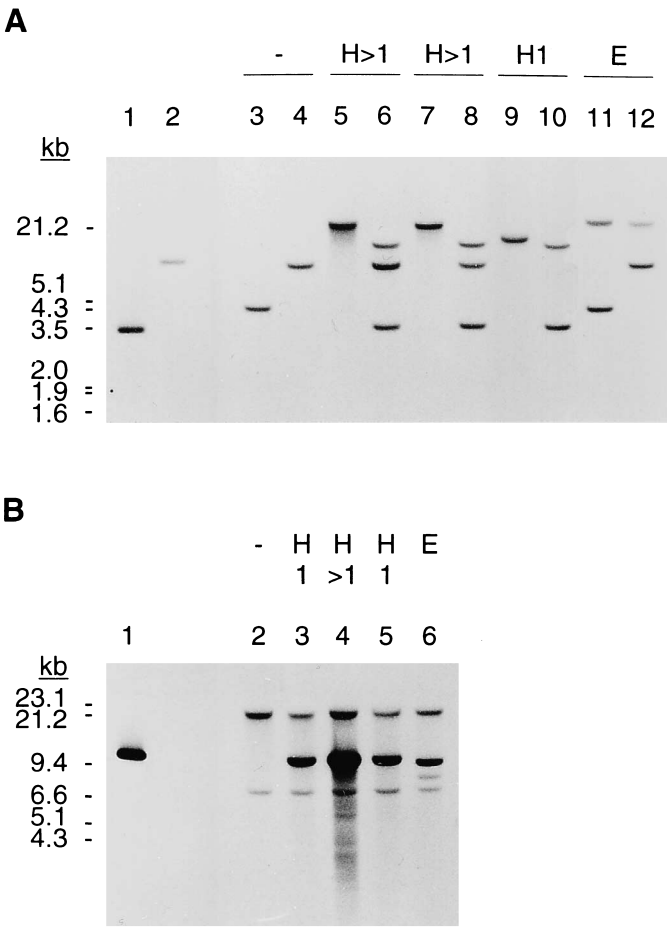

Fig. 2 A, B Southern-blot analysis of several pHAG3-1 transformants after digestion of genomic DNA by *Cla*I, *Sal*I (**A**) and *Kpn*I (**B**). **A** *lane 1*, *Hin*dIII-digested pHAG3-1; *lane 2*, *Sal*I-digested pHAG3-1; *lanes 3–4*, non-transformed ATCC 24663; *lanes 5–6*, transformant Ck10-3; *lanes 7–8*, Ck25-2; *lanes 9–10*, Ck10-6; *lanes 11–12*, Ck10-1. *Uneven lanes*, DNA digested by *Cla*I. *Even lanes*, digestion by *Sal*I. **B** *lane 1*, *Kpn*I-digested pHAG3-1; *lane 2*, non-transformed ATCC 24663; *lanes 3–6*, transformants Ck10-6, Ck25-2, Ck10-4, Ck10-1. *Abbreviations:* – non-transformed; *H>1* tandem homologous integration; *H1* single-copy homologous integration; *E* ectopic integration. Size markers are indicated on the left. Blots were hybridized to an AbGH3 probe

Ck25-2). In lane 9 (Ck10-6) a lower hybridizing signal was found, corresponding to a single-copy homologous integration. Lane 11 demonstrates an example of an ectopic integration (Ck10-1). In addition to the non-disrupted endogenous 4.3-kb *Cla*I fragment, a band of more than 20 kb was found, caused by ectopic integration of pHAG3-1.

These results were confirmed by *Sal*I digestion, which produced a 7.6-kb band in non-transformed control DNA (Fig. 2 A, lane 4). A single-copy homologous integration was predicted to result in bands of 12 and 3.4 kb hybridizing to the AbGH3 probe, due to the presence of two *Sal*I sites in the plasmid (Fig. 1 B). The *Sal*I site at the 5′ end of the AbGH3 sequence in pHAG3-1 is part of a pUC8 polylinker region which was introduced during the cloning procedure. Transformant Ck10-6 contained the predicted hybridization pattern (lane 10). Transformants Ck10-3 and Ck25-2 yielded an additional 7.8-kb band, due to the presence of the homologously integrated tandem copies (lanes 6 and 8). Transformant Ck10-1, on the other hand, gave a band of approximately 20 kb, corresponding to the ectopic integration of pHAG3-1, in addition to bands of 7.6 kb and 7.8 kb which co-migrated. The 7.6-kb band represents the non-disrupted AbGH3 locus, whereas the 7.8-kb band results from tandemly integrated pHAG3-1 copies elsewhere in the genome. The presence of tandem copies was confirmed by *Kpn*I digestion (see below). Hybridization with an *hpt* probe resulted in a 2.2-kb *Sal*I band in all transformants, as predicted (Fig. 1B, data not shown).

Transformant DNA was digested with *Kpn*I to determine whether this site was restored in *A. bisporus* upon integration of the linearized plasmid. Two bands of approximately 20 and 6.8 kb were found in all transformants analysed (Fig. 2 B, lanes 3 to 6), as well as in the non-transformed control (Fig. 2B, lane 2); these corresponded to the resident locus plus additional flanking sequences (Fig. 1B). In addition, a 10-kb band of plasmid-length was found in all transformants, indicating that the *Kpn*I sites were restored. Ck10-1 yielded an additional 7.7-kb band caused by ectopic integration.

Sequence of the AbGH3 fragment

Preliminary Northern-blot data indicated that a weak mRNA signal hybridizing to the AbGH3 probe was present in the vegetative mycelium of ATCC 24663 and that additional bands were visible in pHAG3-1 transformants. Since the AbGH3 fragment directed high-frequency homologous integration in *A. bisporus*, its sequence was elucidated to determine whether disruption of an interesting gene might have occurred in pHAG3-1 transformants. The AbGH3 fragment was found to harbour two open reading frames in tandem (Fig. 3) and which are opposite in orientation to the *hpt* gene in pHAG3-1. The ORFs showed homology to each other and to exo-β-1,3-glucanases from *Saccharomyces cerevisiae* and *Candida albicans* (Vazquez de Aldana et al. 1991; Chambers et al. 1993). The upstream gene, which was named *AbEXG1*, is interrupted by nine short introns (46–104 bp). The positions of introns 1–4 and 9 could be determined by comparison of the deduced amino-acid sequence to the yeast exoglucanases and by the presence of consensus sequences for intron splice sites. The positions of introns 5–8, however, were initially uncertain due to insufficient homology with the yeast exoglucanases. Therefore, fruiting-body RNA was reverse-transcribed into cDNA and the region from nt 446 to 1419 of *AbEXG1* was amplified by PCR, then cloned and sequenced, revealing the intron positions shown in Fig. 3.

The *AbEXG1* gene encodes a polypeptide of 419 amino acids (Mr 46, 680 Da) with a predicted signal peptide of 22 amino acids (Von Heijne 1986), suggesting an extracellular location for the encoded mature protein (Mr 44, 408 Da). Of the downstream gene, *AbEXG2*, only the

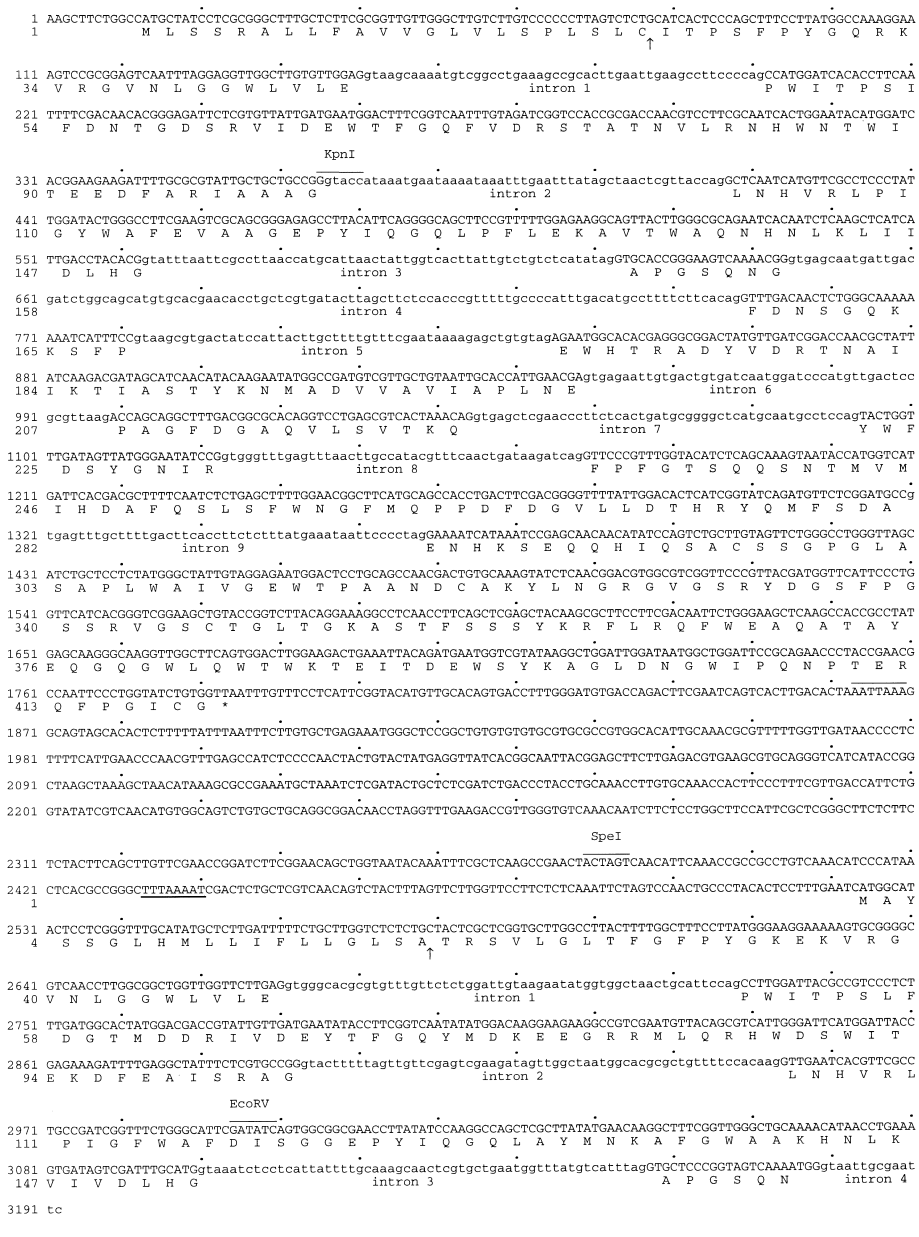

Fig. 3 Nucleotide sequence of the AbGH3 fragment and amino-acid sequence of the putative exo-β-1,3-glucanases [AbEXG1 and AbEXG2 (partial)]. Introns are shown in *lower case*. Predicted signal sequence cleavage sites in the proteins are indicated with *arrows*. A putative polyadenylation signal is *overlined* and a sequence resembling a TATA-box is *underlined*. Unique restriction sites (*Kpn*I, *Spe*I, *Eco*RV) are indicated. The nucleotide sequence data appeared in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under accession number X92961

N-terminal 159 amino-acid coding region is present on the *AbGH3* fragment, containing a predicted 19 amino-acid signal sequence. Intron positions are identical in the two genes. The amino-acid sequences of *AbEXG1* and *AbEXG2* show 61% identity and 81% similarity if conserved amino-acid changes are taken into account. Figure 4 shows that there are several blocks of identity to the yeast exoglucanases; these are dispersed over the entire reading frames, strongly suggesting that the predicted *A. bisporus* polypeptides are also exo-β-1,3-glucanases. The overall identity of AbEXG1 with the yeast exoglucanases is on average 42% (similarity 62%). The codon usage of the *AbEXG1* gene is not strongly biased against third position G (23%) or A (19%), contrary to the highly expressed GPD^{Ag2}, *cel1* and *lcc* genes of *A. bisporus* (Harmsen et al. 1992; Raguz et al. 1992; Perry et al. 1993). The G+C content is 49% in the coding regions of the two genes and 45% in non-coding regions. The intergenic region of 737 bp contains a putative polyadenylation signal and sequences resembling CAAT- and TATA-boxes.

Effect of linearization of pHAG3-1 on homologous integration

The *Kpn*I site used for linearization of pHAG3-1 in the experiments described above, was found to be located at the border of intron 2 of *AbEXG1* (nt 369). In order to determine whether the site of linearization influenced the frequency of homologous integration, pHAG3-1 was also linearized with *Spe*I (nt 2380) or *Eco*RV (nt 2996), cutting in the intergenic region and in exon 3 of *AbEXG2*, respec-

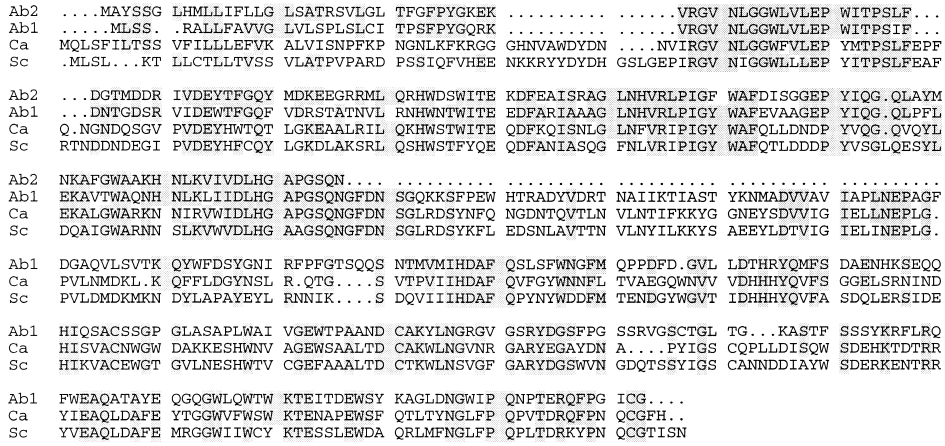

Fig. 4 Structural similarity of *AbEXG1* (*Ab1*) and *AbEXG2* (*Ab2*) gene products to yeast exo-β-1,3-glucanases. Multiple alignment compares the predicted *A. bisporus* polypeptides to each other and to exo-β-1,3-glucanases from *C. albicans* (*Ca*, encoded by the *XOG1* gene) and *S. cerevisiae* (*Sc*, *EXG1* gene). Identical residues are *shaded*; conserved changes are not indicated. Gaps are introduced to allow maximal alignment

Table 1 Frequency of homologous integration of pHAG3-1 in *A. bisporus* ATCC 24663

Transforming DNA[a]	Numbers of transformants[b]				
	Total	Ectopic integration	Homologous integration		
			Total	Tandem insertion	Single-copy insertion
* *Kpn*I	13	5	8	5	3
* *Eco*RV	15	7	8	2	6
* *Spe*I	9	6	3	3	0
Undigested	5	4	1	0	1

[a] Plasmid pHAG3-1 was digested before transformation with restriction enzymes cutting at positions indicated in Figs. 1A and 3
[b] Numbers of transformants obtained in 4, 4, 2 and 4 parallel transformation experiments with *Kpn*I-, *Eco*RV-, *Spe*I-digested and undigested pHAG3-1, respectively

tively (Fig. 3). *Kpn*I, *Spe*I and *Eco*RV generate 3′-protruding, 5′-protruding and blunt ends, respectively. Table 1 summarizes the results obtained by transformation of strain ATCC 24663. The distinction between ectopic and homologous integration and single-copy or tandem insertions at the homologous locus was made on the basis of separate *Cla*I- and *Sal*I-digestions for each transformant, as described above. The results show that linearized pHAG3-1 is targeted to the homologous locus in approximately 30–60% of the transformants. Homologous integration can occur independent of the site of linearization (inside or outside *AbEXG* coding regions; central in, or near the end of, the homologous sequence) and independent of the type of DNA ends formed. None of the transformants showed hybridization patterns indicative of concurrent targeted and ectopic integrations. Tandem plasmid copies were found in a large number of transformants with targeted insertions, as well as in transformants with ectopic insertions, but these were not studied systematically. Southern-blot analysis of three and one selected transformant(s) from *Eco*RV- and *Spe*I-digested pHAG3-1, respectively, showed that these restriction sites had been restored in *A. bisporus* as was found before with *Kpn*I sites (Fig. 2 B).

Undigested DNA yielded fewer transformants than linearized DNA, as we normally find in *A. bisporus*. One out of five transformants had integrated undigested pHAG3-1 at the homologous locus. The Southern-blot pattern of this transformant and one of the transformants obtained with *Kpn*I-digested pHAG3-1 indicated that they probably contained a mixture of non-transformed nuclei and nuclei with homologously integrated pHAG3-1 (data not shown).

Northern-blot analysis of the AbEXG genes

In order to study expression of the exoglucanase genes, Northern-blot analyses were performed on RNA isolated from the vegetative mycelium and seven fruiting body stages of strain U1. As shown in Fig. 5, the AbGH3 probe hybridized to a single mRNA band of approximately 1.45 kb. Exoglucanase mRNA was barely detectable in vegetative mycelium grown on complete medium (lane 1). Exoglucanase expression was considerably higher in fruiting bodies and increased after pinhead formation (lane 2) when the cap and stipe of the fruiting body were formed (stage 2, lanes 3 and 9). Expression did not vary much between cap and stipe and was rather constant in time with a marked decrease at the last stage (lanes 8 and 14).

The vegetative mycelium of strain ATCC 24663 contained very low levels of 1.45-kb exoglucanase mRNA, comparable to U1 (Fig. 6, lane 1). Two pHAG3-1 transformants, containing multiple integrations either ectopically (C10-1, lane 2) or at the homologous locus (C25-1, lane 3), showed additional smaller transcripts of approximately 0.7 and 0.9 kb, which may correspond to truncated exoglucanase mRNAs, originating from the partial *AbEXG2* gene present on pHAG3-1. In transformant C25-1, the level of full-length exoglucanase mRNA was highly increased compared to non-transformed mycelium. In some experiments, irreproducible hybridizing bands of more than 9.5 and less than 0.16 kb were found. Although the *AbEXG1* coding region is entirely present on pHAG3-1, it

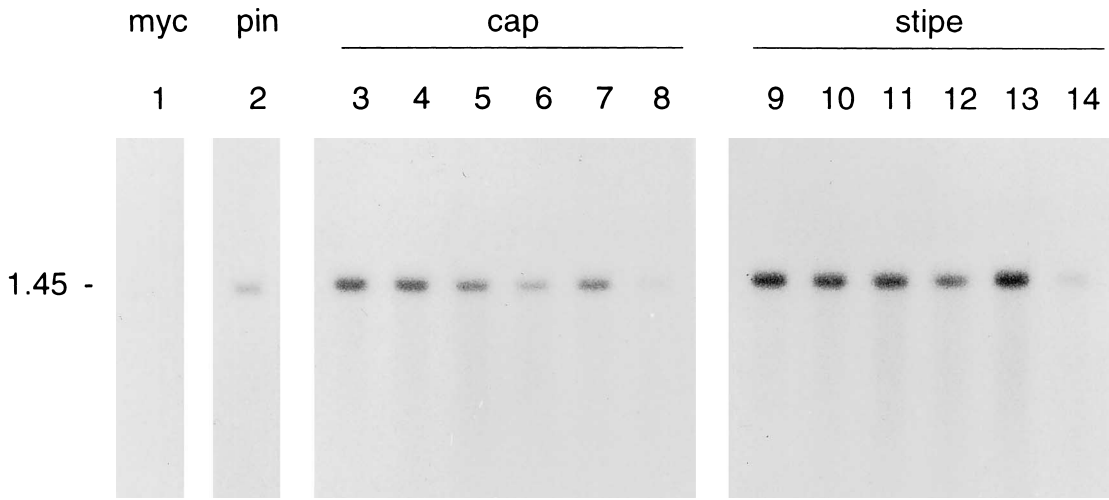

Fig. 5 Exoglucanase gene expression in vegetative mycelium and fruiting bodies of U1. RNA was isolated from vegetative mycelium (*lane 1*), pinheads (stage 1, *lane 2*) and from caps (*lanes 3–8*) and stipes (*lanes 9–14*) of stages 2 to 7 of fruiting body development, distinguished according to Hammond and Nichols (1976). Stages 2 and 3 correspond to commercially attractive mushrooms; at stage 4 the velum starts to tear and gills become visible. At stage 7, the cap surface curves upwards. The Northern blot was hybridized to the ^{32}P-labelled AbGH3 fragment. Equal RNA loading of lanes was confirmed by hybridization to an rDNA probe (data not shown).

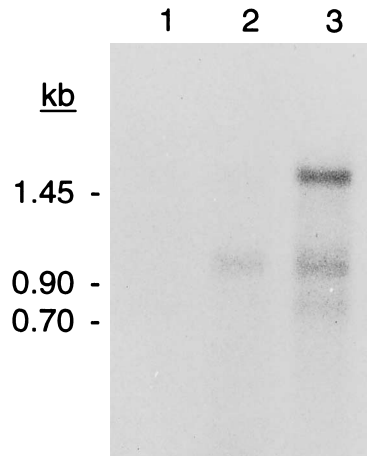

Fig. 6 Northern-blot analysis of pHAG3-1 transformants. RNA was isolated from the vegetative mycelium of non-transformed ATCC 24663 (*lane 1*) and of transformants carrying tandem copies of pHAG3-1 inserted ectopically (C10-1, *lane 2*) or at the homologous locus (C25-1, *lane 3*). The blot was hybridized to the ^{32}P-labelled AbGH3 fragment. Approximate sizes of bands are indicated on the left. Equal RNA loading of lanes was confirmed by hybridization to a *GPD* probe (data not shown)

lacks its promoter. Therefore, it is not clear whether the increased level of 1.45-kb mRNA in C25-1 is due to the activity of a cryptic promoter in the vector sequences or to altered regulation of expression of the resident *AbEXG* genes because of disturbance of the normal chromosomal context by insertion of pHAG3-1. C25-1 and C10-1 did not show an altered growth rate or an altered colony morphology under laboratory conditions.

Discussion

A 3.2-kb genomic *A. bisporus* fragment, directing high-frequency homologous integration, was shown to carry two tandem exo-β-1,3-glucanase genes (one partial). From *S. cerevisiae* three exo-β-1,3-glucanase genes with mutual homology have been isolated. *EXG1* encodes two glycosylated, extracellular isoforms (Vazquez de Aldana et al. 1991), whereas the *EXG2* gene product is a membrane-bound glycoprotein (Nebrada et al. 1986; Larriba et al. 1995). *SSG1* encodes a sporulation-specific extracellular enzyme (San Segundo et al. 1993). Disruption of the *SSG1* genes in diploids led to a delay in the formation of mature asci; additional disruption of the *EXG1* and *EXG2* genes did not result in an altered phenotype. The *S. cerevisiae BGL2* gene encodes a β-1,3-glucanase similar to plant endo-β-1,3-glucanases, but unrelated to the three genes described above (Klebl and Tanner 1989). The *C. albicans XOG1* gene, encoding an extracellular non-glycosylated exo-β-1,3-glucanase, is highly similar to the yeast *EXG* genes (Chambers et al. 1993). The *XOG1* gene is moderately expressed at the mRNA and protein levels; maximal mRNA expression occurs at the early phase of exponential growth. During secretion both the *EXG1* and *XOG1* primary gene products are processed in two steps involving the signal peptidase and a Kex2-like proteinase, resulting in mature polypeptides of 408 and 400 amino acids, respectively, similar in size to the predicted mature AbEXG1 protein of *A. bisporus*. The yeast genes do not contain introns. Recently, an endo-β-1,6-glucanase gene from the mycoparasitic fungus *Trichoderma harzianum* has been isolated (Lora et al. 1995). The encoded enzyme is processed at a Kex2 proteinase recognition site and shows 20–30% identity to yeast EXG1 and XOG1. Certain conserved domains were found, which are also present in the *A. bisporus EXG* genes. Chambers et al. (1993) and Lora et al. (1995) have noted the conserved sequences LEP and

NEP as well as the importance of glutamate residues for the catalytic activity of β-1,3- and β-1,4-glucanases and chitinase of plant, fungal and bacterial origin. However, according to Larriba et al. (1995), the conserved motif HHHY (particularly the second histidine and the tyrosine residue) is part of the active site of yeast EXG2. The LEP, NEP and HXY motifs are also present in AbEXG1 (residues 45–47, 205–207, 273–275) and the partial AbEXG2 sequence (only the LEP motif, residues 48–50).

The function of exo-β-1,3-glucanases remains unclear. They most likely play some role in the metabolism of β-glucan, an important structural cell-wall component, during processes such as apical growth, branching and spore formation. Exo-β-1,3-glucanase was proposed to be involved in the pathogenicity of *Cochliobolus carbonum* on maize plants by degrading callose, deposited in plant cell walls as a defensive barrier (Schaeffer et al. 1994). However, disruption of the *C. carbonum EXG1* gene by homologous integration did not reduce pathogenicity. Gene disruption studies in *C. carbonum* and in *S. cerevisiae* are confounded by the fact that residual exo-β-1,3-glucanase activity is detected in the mutants. Probably, other genes are still active and compensate for the mutated genes. The *A. bisporus EXG* mRNA level is very low in vegetative mycelium grown in vitro on nutrient-rich medium. Expression was not investigated in vegetative mycelium grown on compost, the substrate used for the production of fruiting bodies. However, the relatively high exoglucanase expression in different stages of the fruiting body suggests that exoglucanase may play a role in sporophore growth and development. This could be studied further by isolating and characterizing the corresponding enzymes and by gene disruption of the *AbEXG* genes. Alternatively, the C25-1 transformant, showing enhanced levels of exo-β-1,3-glucanase mRNA, can be mated with suitable strains to form fertile heterokaryons. Thus, the effect of exo-β-1,3-glucanase over-expression can be studied in the fruiting body.

In contrast to the yeast genes, the *A. bisporus EXG* genes carry many short introns, as is found in other *A. bisporus* genes (Harmsen et al. 1992; Raguz et al. 1992; Perry et al. 1993). The intron boundaries are generally in accordance with the consensus sequences GTNN(G/T) and (C/T)AG (Gurr et al. 1987; Perry et al. 1993). Like the *AbEXG* genes, the *GPD* genes of *A. bisporus* are also tandemly linked (Harmsen et al. 1992). The intergenic region between the *GPD* genes is only 265-bp long (including the untranslated 5′ leader sequence of the GPD^{Ag2} gene), yet this region was sufficient to drive expression of the *hpt* gene in *A. bisporus* transformants (Van de Rhee et al. 1996). The stop and start codons of the *AbEXG* genes are separated by 738 bp, which may well be sufficient to contain all promoter sequences for the *AbEXG2* gene. However, we have not yet proven that this gene is transcribed. In other *A. bisporus* genes, there is a preference for codons ending with C or T (68%; Van der Vlugt et al. 1993). We did not find such a strong bias in the *AbEXG1* gene (C+T = 58%), which may be an indication of low expression (Gurr et al. 1987).

We studied whether transformation frequency could be enhanced by linearizing plasmid pHAG3-1 within the AbGH3 fragment, thus providing recombinogenic ends with homology to the recipient DNA. However, the transformation efficiency was not enhanced by this strategy, although 30–60% targeting was observed. Apparently, other factors are limiting for transformation, e.g. the competence of the protoplasts or the state of the recipient genome. The frequency mentioned is possibly an underestimate of the recombination events taking place during *A. bisporus* transformation, since double cross-overs leading to gene replacement have gone unnoticed in our system. Homologous integration occurred with different sites of linearization, resulting in different DNA ends. Restriction sites used for linearization were reconstituted in all cases examined, indicating the accuracy of the recombination process in *A. bisporus*. In other fungi, e.g. *S. cerevisiae* (Orr-Weaver et al. 1981) and *Ustilago maydis* (Banks et al. 1992), restoration of restriction sites – and repair of larger gaps – in the incoming plasmid by recombination with the chromosome have been observed as well. Restriction enzyme-mediated integration has also been found to restore sites (Schiestl and Petes 1991), but this mode of integration does not apply here, since the restriction enzymes used for linearization of pHAG3-1 were removed before transformation.

Homologous integration and gene replacement occur with varying efficiencies in different fungal species. Little is known about the frequency of targeting in other homobasidiomycetes. Experiments with *Phanerochaete chrysosporium* and *Schizophyllum commune* selected only for gene replacements (Alic et al. 1993; Wösten et al. 1994). These occurred in 5–15% and 1% of the transformants, respectively. In *Agrocybe aegerita*, homologous integration via a promoter-like sequence was found in nearly 40% of the transformants tested, but was accompanied by rearrangements of the transformation vector (Noël et al. 1995). Homologous free DNA ends were not necessary for either gene replacement or homologous integration in the above systems. In *Coprinus cinereus* gene replacement and homologous integration were found in 5% of the transformants (Binninger et al. 1991). This low frequency was not enhanced by double- or single-strand breaks within the homologous region, single-stranded DNA, or a larger homologous fragment. It was proposed that in *C. cinereus* targeted interactions might be initiated on the chromosome, rather than on the incoming plasmid. The number of transformants obtained with circular pHAG3-1 in *A. bisporus* is too low to allow a comparison of targeting frequencies between circular and linearized DNA. Ligation between linearized plasmids was often observed in *C. cinereus*, leading to “head-to-tail” and “head-to-head” arrays at ectopic sites. Although we readily observed head-to-tail integrations at the homologous locus, no bands indicative of head-to-head or tail-to-tail integrations occurred. This suggests that homologous integration in *A. bisporus* only involves recombination events and not exclusive ligation between plasmid molecules prior to integration. Alternatively, these types of arrays may be unstable. In 7 out of 22 transformants with ectopic integration, bands which might indicate head-to-head or tail-to-tail arrays were found, but these were not investigated further.

The highly efficient homologous integration described here, opens perspectives for the use of gene targeting and gene disruption in common mushroom. To study promoter activity or over-expression of genes, it is very useful to target constructs to specific genomic sites to circumvent position effects. Future studies will have to show whether targeting is just as efficient when using other homologous genes and/or strains of *A. bisporus*.

Acknowledgements Plasmid pAN7-1 was kindly provided by C.A.M.J.J. van den Hondel. This work was supported in part by grant BIO91018 from the Dutch Ministery of Economic Affairs.

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