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Agrobacterium tumefaciens mediated transformation of *Agaricus bisporus*

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ABSTRACT: *Agrobacterium tumefaciens* is known to transfer parts of its tumor-inducing plasmid to filamentous fungi including *Agaricus bisporus.* We have used this transformation system to transform germinating basidiospores of *A. bisporus.* Analysis of hygromycin B-resistant clones showed that the T-DNA integrated at random sites and in multiple copies into the host genome. Furthermore we have been able to show that the integrated T-DNA is mitotically and meiotically stable. The data indicate that T-DNA transfer is an efficient system to transform the basidiomycete *A. bisporus.* We have tested whether it is possible to use the *A. tumefaciens* system to transform vegetative mycelium of a commercial *A. bisporus* strain. Our preliminary data suggest that T-DNA transfer could be used to directly transform commercial lines of *A. bisporus* or their constituent homokaryons.

1 INTRODUCTION

Application of molecular biology and biotechnology to the cultivated white button mushroom, *Agaricus bisporus,* has been limited thus far by the lack of an efficient transformation system. Although there have been considerable efforts in the past to develop such a transformation system the results have been disappointing. In previous attempts to transform *A. bisporus* a variety of methods (polyethylene glycol, electroporation and particle bombardment) have been used to introduce DNA into protoplasts, mycelium or basidiospores either without success or proofed to be hardly reproducible (Challen *et ai,* 1991; Royer & Horgen, 1991; Li & Horgen, 1993; Challen & Elliot, 1994; van de Rhee *et al.* 1996a, 1996b). In these reports several selectable markers have been used flanked by different regulatory sequences of host and non-host origin. The failure to develop a transformation system has been assumed to lie in factors as generation of false positives, low level of integration of transforming DNA, poor expression of foreign sequences, DNA modifications after integration or low competence of certain strains.

Here we report the application of a fundamentally different and promising strategy to generate stable transformants of *A. bisporus* using the plant pathogen, soil bacterium *Agrobacterium tumefaciens.* During crown gall tumor induction, *A. tumefaciens* transfers a part, the T-DNA, of its tumor inducing (Ti) plasmid to plant cells. The T-DNA then integrates into the plant nuclear genome. The induction of T-DNA transfer depends on a set of virulence *(vir)* genes, which are also located on the Ti plasmid. The *vir* genes are induced by compounds secreted from wounded plant cells, such as acetosyringone (AS) (Kado, 1991). This natural transformation system has been used in plant research for more than 25 years and has recently been used to transform filamentous fungi (de Groot *et al.,* 1998; Dunn-Coleman & Wang, 1998). In their report de Groot *et al.* (1998) showed that besides a variety of other fungi, *A. tumefaciens* can be used to transfer T-DNA to germinating basidiospores of *A. bisporus,* and to isolate transformants using hygromycin B resistance as a selectable marker.

We have adopted this method to confer *A. bisporus* to hygromycin B resistance using germinating basidiospores as well as vegetative mycelium. Transformants were analysed to obtain

more information on the fate of transforming DNA, the expression of heterologous genes and the mitotic and meiotic stability of foreign DNA in *A. bisporus.*

1 MATERIAL AND METHODS

2.1 *Strains and cultivation*

Spores from *A. bisporus* Horst® Ul fruit bodies where collected on glass petri dishes and stored at 4 °C. The standard growth medium for *A. bisporus* Horst® Ul was MMP-Agar (Sonnenberg *et al,* 1988) and incubation was performed at 24 °C. Mycelium for DNA extractions was grown on MMP-Agar with cellophane sheets. Mycelium for protoplasting was grown under the conditions described in Sonnenberg *et al.* (1988). Lab scale fruiting experiments were performed according to van Gils (1988). The *Agrobacterium* strain containing the binary vector pUR5750 used for transformation was kindly provided by the Unilever Research Laboratory, Vlaardingen, The Netherlands and has been described in de Groot *et al.* (1998). Bacterial cultivation was performed as described in de Groot *et al.* (1998) on *A. tumefaciens* minimal medium (MM) (Hooykaas *et al.,* 1979). For induction of virulence and T-DNA transfer *A. tumefaciens* was grown on induction medium (IM) with 200 mM AS and for negative controls without AS (Bundock & Hooykaas, 1996).

Selection for transformed mycelium colonies was performed after several rounds on MMP-Agar containing cefotaxime (200 mM) and hygromycin B (30 µg/ml).

2.2 *Transformation of A. bisporus Horst® Ul*

Media, growth of vegetative mycelium, fruiting and isolation of single spore isolates were done as described previously (Sonnenberg *et al.,* 1996).

For the transformation of vegetative mycelium, colonies where harvested from cellophane sheets. The mycelium from 5-10 plates was blended in 10 ml MMP in a Waring blender for 30 s and incubated for 2 d. Mycelium was harvested by filtration and resuspended in 3 ml IM, 2 ml of induced *A. tumefaciens* culture, prepared as in de Groot *et al.* (1998), was added and 1 ml of this mixture was transferred to cellophane sheets on IM plates and further incubated as described (de Groot *et al.*. 1998).

2.3 *Isolation of DNA, southern hybridisation and PCR*

A. bisporus mycelium was harvested from MMP plates, freeze dried and 100 mg was used for DNA isolations as described previously (Sonnenberg *et al.,* 1999). Following digestion with restriction enzymes DNA was size separated on agarose gels and transferred to nylon membranes as described in Mikosch (1999). Hybridisation was performed under conditions recommended for the DIG hybridisation system by Roche (Mannheim, Germany) using a 1800 bp DIG-labelled DNA probe covering the hyg^R gene and the trp^c terminator region of the plasmid pUR5750.

For PCR 50 ng of template DNA and the Platinum Taq polymerase from Life Technologies Ltd (Paisley, Great Britain) was used. PCR primers for the amplification of the hyg^R gene have been, forward: CATGCCTGAACTCACCGCGA, reverse: TCGAGTGGAGATGTGGAGTG. PCR primers for the amplification of the plN150 locus (Sonnenberg *et al.,* 1996), which is linked to the mating type, have been, forward: GCCATACTCTCGTCGAGACA, reverse: CTGCCGTGATAGGTTCACTG. PCR conditions were chosen as recommended by Life Technologies Ltd.

2.4 *Protoplasting for the isolation of homokaryons and CHEF analysis*

Protoplasting was performed according to a modified protocol of Sonnenberg *et al.* (1988) published elsewhere (Sonnenberg *et al.,* 1991). For regeneration and isolation of homokaryons protoplasts have been regenerated on MMP-Agar containing 0.6 M sucrose and the slow growing colonies have been used for further characterisation. For CHEF analysis protoplasts where embedded in agarose as described (Sonnenberg *et al.,* 1991). Electrophoresis parameters were

100 V, 400 to 800 s pulse for 96 h; 50 V, 2400 to 3300 s pulse for 72 h; 100 V, 300 to 400 s pulse for 24 h and 100 V, 500 to 600 *s* pulse for 24 h. Following electrophoresis the gel was stained with ethidium bromide for documentation and blotted to nylon membrane for hybridisations as in 2.3.

3 RESULTS

3.1 *Transformation ofgerminating basidiospores ofA. bisporus Horst Ul*

Cocultivation of germinating basidiospores of *A. bisporus* Horst® Ul and A *tumefaciens,* under T-DNA transfer inducing conditions, led to the formation of hygromycin-resistant colonies. Repeated transfer of putative transformants to fresh selective and non-selective medium confirmed the stability of the resistant-phenotype. Genomic DNA of selected transformants was isolated for Southern blot analysis and karyotype characterisation. From heterokaryotic transformants both parental karyotypes were isolated by protoplasting for separate Southern and CHEF analysis. To verify the meiotic stability of integrated copies of the selectable marker single-spore isolates have been prepared from fruit bodies of recombinant heterokaryotic clones and their genomic DNA was isolate for further analysis.

3.1.1 *Integration ofT-DNA into the genome of A. bisporus*

For Southern blot analysis genomic DNA from selected transformants was digested with *Bgl II,* which does not cut within the T-DNA. To confirm genomic integration, uncut DNA was hybridised with a hyg^R probe.

Hybridisation patterns (Fig. 1) show that the T-DNA integrates into the genome of recombinant clones with copy numbers between one and eight and that the majority of clones have integrated two to four copies. This indicates that the T-DNA integrates at random locations throughout the genome *ofA. bisporus.* Furthermore, the size distribution of fragments suggests integration at different loci in different transformants.

The homo- or heterokaryotic nature of the transformed offspring was analysed using a PCR scoreable marker linked to the mating type. Single spore isolates, homoallelic for such markers are usually homokaryotic (Kerrigan *et al.,* 1992). As expected for an organism with a secondary homothallic life cycle (Raper *et al.,* 1972), most of the transformants were heterokaryotic.

3.1.2 *Mitotic and meiotic stability ofintegrated T-DNA*

Transformed clones were repeatedly transferred to non-selective medium in order to investigate the mitotic stability of the integrated T-DNA. Following several rounds of growth on medium without hygromycin B the clones were transferred to selective medium. All tested transformants still showed the ability to grow on selective medium indicating that hyg^R is mitotically stable. Clones grown on selective or non-selective medium never showed a formation of sectors with different growth rates or colony morphology as it was reported for the transformation of *A. bisporus* UImp10 (van de Rhee *et al.,* 1996a). Furthermore the hybridisation patterns for individual transformants always remained the same and we never observed a disappearance of inte-

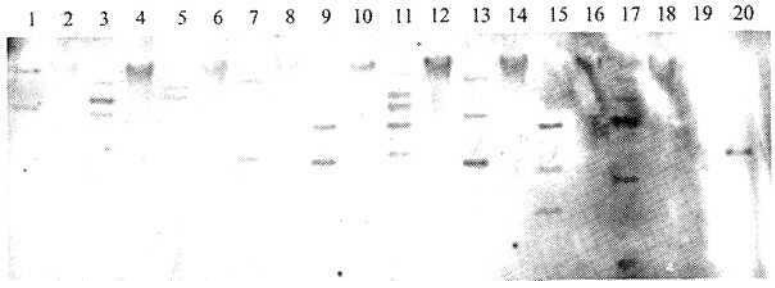

Figure 1: Southern blot hybridised with a hyg^R probe. DNA of transformants digested with *Bgl II* (odd numbers) or uncut (even numbers). Controls lane 20: hyg^R gene, lane 19: *A. bisporus* Horst* Ul digested with *Bgl II.*

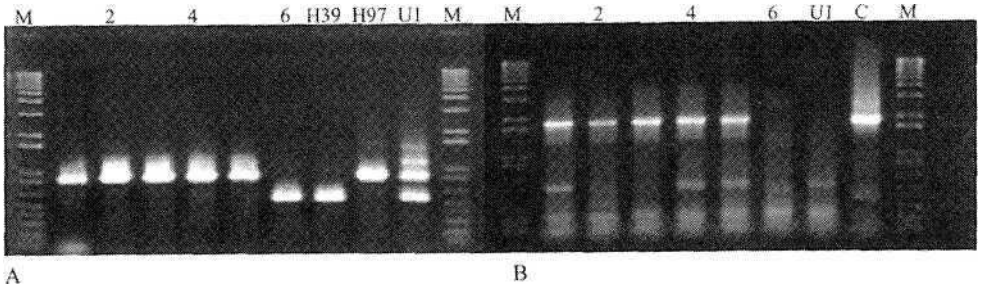

Figure 2: Ethidium bromide-stained PCR products. Panel A PCR scorable marker linked to the mating type. Lanes 1 to 6 single spore isolates. H39, H97 and Ul homokaryotic and heterokaryotic controls. Panel B amplification of hyg^R gene. Lanes 1 to 6 same single spore isolates as in panel A. Ul: negative control and C: hyg^R positive control. M: DNA size marker, Ul: *A. bisporus* Ul, H39, H97: constituent homokaryons of U1.

grated T-DNA or the appearance of additional hybridisation fragments (data not shown).

To investigate the meiotic stability of integrated T-DNA copies a heterokaryotic transformant (BAT 13) was chosen for lab-scale fruiting body production and isolation of single spore cultures. The fruit bodies produced by this recombinant clone showed normal morphology and spore production. In order to obtain single-spore isolates, spore prints were plated in different dilutions on non-selective medium and slow growing colonies were transferred to fresh non-selective medium in order to isolate homokaryotic offspring. PCR amplification was used to verify the presence of the hyg^R gene (Fig. 2), and single-spore isolates have been transferred to selective medium to investigate the expression of the hyg gene. As expected, all single spore isolates that bear the hyg^R gene did grow on selective medium, proving that the hyg^R expression cassette is inherited and that expression is not necessarily hampered by gene silencing or DNA rearrangements during meiosis.

3.1.3 *Nuclear distribution and inheritance of the hygromycin resistance gene*

To test the genomic position of the T-DNA copies in the heterokaryotic transformant BAT-13, both nuclear constituents were recovered as homokaryons by protoplasting. Southern analysis of the resulting BAT 13-4 and BAT 13-17 indicates that both nuclear types are transformed with the former containing 3 copies and the latter one copy of the T-DNA (Fig. 3). CHEF analysis (Fig. 4a) shows that the three copies of T-DNA in BAT 13-4 are located on chromosome I whereas the single copy in BAT 13-17 is located on a different chromosome (probably VIII).

To analyse the distribution of the three T-DNA copies in BAT 13-4 on chromosome I, genomic DNA was digested with *BamHl* that cuts only once in the recombinant T-DNA. Southern analysis indicates that two copies are integrated in tandem (Orr-Weaver *et al.,* 1981) and that the third copy is integrated in a different location on chromosome I (Fig. 4b).

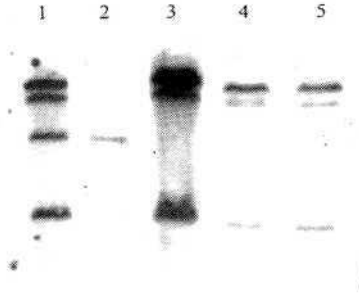

Figure 3: Southern blot hybridised with a hyg^{11} probe.
Lane 1: BAT13, lane 2: BAT13-17, lane 3: Batl3-4
lane 4 and 5: single spore isolates of BAT 13 (SI
and S2 respectively). All DNA digested with *Bgl II.*

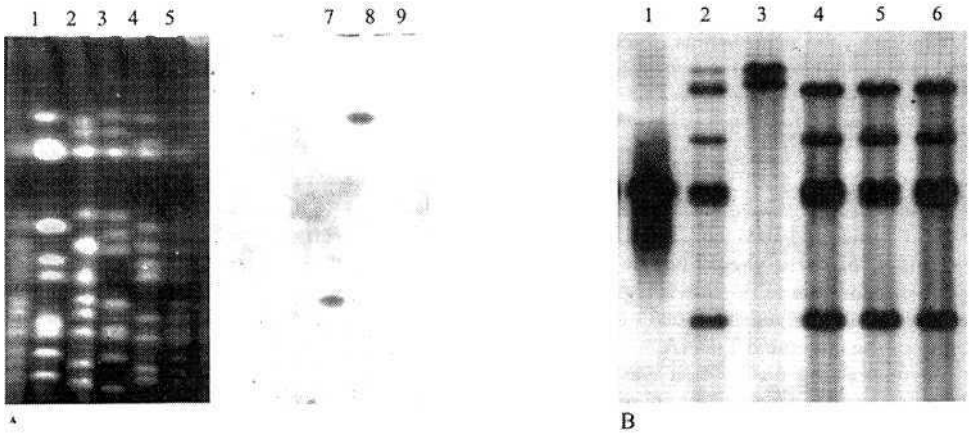

Figure 4: Panel A CHEF analysis, ethidium bromide-stained gel (left) and southern hybridisation with hyg^R probe (right). Panel B Southern blot hybridised with hyg^R probe. Panel A: lane 1: H39, lane 2: H97, lane 3: BAT13-17, lane 4: BAT13-4, lane 5: BAT13. Lanes 7 to 9 same as 3 to 5 respectivly. Panel B: lane 1: T-DNA from left to right border used as size marker to identify tandem integrations, Iane2: BAT13, lane 3: BAT13-17, lane 4: BAT13-4, lane 5: BAT13-S1, lane 6: BAT13-S2. Chromosomal DNA digested with *Bam HI.*

These results show that both karyotypes can be transformed at once and that there is no preferential site for integration. Southern analysis of two hyg^R single spore cultures (BAT 13-S1 and BAT 13-S2) derived from BAT 13 that have inherited chromosome I of BAT 13-4 show a parental type of banding pattern (Fig. 3 and 4b). This indicates that there has been no cross-over between the tandem and single T-DNA copy in the two single spore isolates. Moreover, this shows the intact inheritance of a chromosomal region containing three copies of the T-DNA underlining the meiotic stability of the transformants.

3.2 *Transformation of vegetative mycelium of A. bisporus Horst[9] Ul*

In order to investigate the possibility to use the system for the transformation of vegetative mycelium of *A. bisporus* Horst® U1 we cocultivated *A. tumefaciens* with mycelium fragments generated from vegetative cultures of the basidiomycete. Hygromycin-resistant colonies have been repeatedly transferred to fresh selective medium to verify integration and expression of the hyg^R marker.

Although the data are still limited for this transformation strategy we can conclude that transformation of vegetative mycelium of either heterokaryotic mycelium of *A. bisporus* Horst® Ul or the two homokaryotic strains H39 and H97 which make up *A. bisporus* Horst® Ul should be possible with the *A. tumefaciens* system. This allows in principle, the transformation of commercial lines and homokaryotic strains. At the moment of submitting this paper, we are cultivating mycelium of putative transformants in order to test whether and how T-DNA has integrated.

4 DISCUSSION

In 1998, De Groot *et al.* described that the *Agrobacterium tumefaciens* mediated plant transformation system could be used to transform fungi as well. For some ascomycetous fungi, this system proved to be even more efficient than the so far used PEG or electroporation mediated introduction of recombinant plasmids into protoplasts. Even more remarkable, however, was that this system could also be used to genetically transform the more recalcitrant basidiomycetes *A. bisporus* and *Pleurotus ostreatus.* Although the number of transformants that were obtained by cocultivating *A. tumefaciens* with germinated basidiospores of *A. bisporus* was rather low,

the results were promising and, therefore, adapted by our lab.

Similar to the experiments described by De Groot and colleagues, we used germinating basidiospores in the first transformation experiments. An optimisation showed that sufficient transformants of *A. bisporus* could be obtained in this way. A number of these transformants were further analysed to investigate the incorporation into the genome and the stability of the integrated DNA. This analysis showed that most transformants have integrated multiple copies of the hyg^R gene at different genomic location and that tandem integration can occur. This resembles the integration patterns found in other filamentous fungi transformed with plasmid DNA (Turner and Balance, 1985). It indicates that the use of *A. tumefaciens* for the transformation of *A. bisporus* should not be limited by a peculiar integration behaviour of the T-DNA. Southern analysis of vegetatively propagated transformants on non-selective medium and single spore isolates derived from one of the transformants clearly shows the mitotic and meiotic stability of the integrated T-DNA.

An interesting observation was that both nuclei in heterokaryotic mycelium can be transformed simultaneously. This might be important for a heterokaryotic mycelium in which each cell contains a variable number of nuclei of both mating-types. It is very likely that not all nuclei in the primary transformant contain one ore more copies of the T-DNA. Growth on a selective medium probably eliminates all non-transformed cells and/or nuclei. Protoplasts derived from a transformant showed no difference in regeneration frequency on selective and non-selective medium, which supports the absence of non-transformed nuclei (data not shown).

Preliminary experiments showed that hyphal fragments could also be transformed. The numerous hygromycin-resistant colonies that were obtained of Horst® Ul indicate that fragmented mycelium would be even a better target for *A. tumefaciens* mediated transformation. Although these putative transformants await further analysis, this would allow a directed transformation of existing commercial lines or their constituent homokaryons.

Next to the advantage of eliminating the more elaborate protoplasting technique, the presented transformation system also reduces the amount of non-host DNA that is incorporated in the transformants. Contrary to bacterial vectors that are used in the classical transformation system, only the T-DNA containing the selection marker is incorporated into the host genome. We are constructing now vectors containing *A. bisporus* sequences in the T-DNA to test whether sequence homology between the vector and the host genome can target the integration. This would allow the targeted disruption of genes and, therefore, be a major tool to study gene function.

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