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MINI-REVIEW

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Advances in genetic analysis and biotechnology of the cultivated button mushroom, *Agaricus bisporus*

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Abstract During the last decade several major breakthroughs have been achieved in mushroom biotechnology, which greatly enhanced classical mushroom breeding. DNA-based technologies such as restriction fragment length polymorphisms and randomly amplified polydisperse DNA sequences have allowed for a measure of genetic diversity, for the isolation of homokaryons, for the determination of inheritance of nuclear and mitochondrial markers, and for the production of a genetic linkage map. The recent availability of ready-to-use and affordable DNA technologies has resulted in a substantial increase in the number of *Agaricus bisporus* genes that have been identified and characterized. A major breakthrough was achieved in 1996 when the first successful and stable transformation system of *A. bisporus* was reported. Together, the availability of an increasing number of known genes and the possibility to produce transgenic mushrooms will result in a better understanding of the molecular, physiological and biochemical processes that are essential for mushroom production, shelf life and quality aspects such as flavor, texture and disease resistance. Some potential targets for strain improvement are discussed, such as the genes involved in brown discoloration, substrate utilization, carbon and nitrogen metabolism, and fruit body development.

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Introduction

The edible mushroom, *Agaricus bisporus* (Lange) Imbach, represents the most widely cultivated mushroom. Its spawn and fruit body production, trade and processing are of great economic value, especially in the European Community, the United States and China. In 1994 the world production of cultivated mushrooms was almost 5 million metric tons with a total value of U.S. $9.8 billion, and U.S. $3.6 billion for mushroom products (Chang 1996). Other relevant economical data on mushroom breeding and commerce indicate that *A. bisporus*, *A. bitorquis*, *Lentinula edodes*, *Pleurotus* spp., *Auricularia* spp., *Flammulina velutipes* and *Tremella fuciformis* are the most important edible mushrooms (in this order) and that mean production increased by about 40% from 1990 to 1994. In Asia, especially China, the production volume of *Lentinula* and *Pleurotus* (632,000 and 654,000 metric tons, respectively) is about twice that of *Agaricus* and *Auricularia* (359,000 and 385,000 metric tons, respectively) (Chang 1996).

The number of commercial *Agaricus bisporus* strains available to meet specific demands for fresh and processed products is very limited. Despite an enormous effort on the part of mushroom scientists, conventional breeding techniques have resulted in few novel strains. The difficulties of strain improvement of *A. bisporus* can be mainly ascribed to its secondarily homothallic life cycle and unifactorial mating-type system (Raper et al. 1972; Elliot 1985). *A. bisporus* differs from other agarics in that it predominantly produces basidia with binucleate spores, compared to uninucleate basidiospores in other agarics. These binucleate basidiospores contain a haploid nucleus from each of the parental homokaryons (of opposite mating type), are mostly self-fertile, and can germinate to form a heterokaryotic mycelium (Raper et al. 1972; Royse and May 1982; Elliot 1985). Because no monokaryon fusion is required to produce fertile heterokaryotic mycelia, outcrossing is limited which in turn restricts breeding advances (Summerbell et al.

1989). Self-fertile heterokaryons may also show a reduced ability to mate with other isolates, thereby further limiting the intrapopulation gene exchange (Kerrigan et al. 1994). The low level of homokaryotic spores produced during the life cycle of *A. bisporus* (Raper et al. 1972) together with an apparently low frequency of recombination (Kerrigan et al. 1993) has hampered systematic breeding efforts.

From classical mushroom breeding to molecular mushroom breeding

During the 1970s and early 1980s, techniques for breeding *A. bisporus* became more systematic, which led to the introduction of the first hybrid strains (for a review see Fritsche 1991). During the early 1990s, diverse wild type isolates became accessible to breeders through the Agaricus Resource Program (Kerrigan 1991), which will allow further breeding of the mushroom. A promising example is the identification of wild isolates showing a tetrasporic life-cycle with only one nuclear type per spore (Kerrigan et al. 1994). This appears to be a dominant trait, therefore allowing inbreeding into normal bisporic strains.

Since the early 1970s, genetic markers such as allozymes, auxotrophic lesions and morphologic aberrations have been reported in *A. bisporus*, enabling an initial genetic characterization of the cultivated mushroom (Raper et al. 1972; Royse and May 1982; Spear et al. 1983). With the development of molecular biological tools such as restriction fragment length polymorphisms (RFLPs) and randomly amplified polydisperse DNA sequences (RAPDs), DNA markers for *A. bisporus* became available, which allowed a more in-depth study of the genetics (Castle et al. 1987; Williams et al. 1990). These DNA markers have been utilized as indicators of genetic diversity (similarity) by generating "DNA fingerprints" of individual mushroom strains (Loftus et al. 1988; Horgen et al. 1991; Khush et al. 1992) and have clearly indicated that current strains contained limited genetic variation. The availability of isozyme, RFLP and RAPD markers enabled routine analysis and isolation of homokaryons, which are an essential element of mushroom breeding. Other uses were to identify and monitor crosses between homokaryotic isolates (Castle et al. 1987; Horgen et al. 1991), and to determine the inheritance of nuclear and mitochondrial genetic markers (Horgen et al. 1991; Sonnenberg et al. 1988; Summerbell et al. 1989). Furthermore, DNA-markers such as RAPD markers and genes themselves are being used to produce a genetic linkage map in which commercial traits are being linked to well-defined genetic markers (Kerrigan 1991; Kerrigan et al. 1995). The availability of such a genetic linkage map will allow an efficient introduction of commercially important traits from diverse germplasm (Kerrigan 1991) into current commercial strains.

The evolution from classical mushroom breeding to "molecular breeding", a term used to define breeding programs supported by the use of DNA-based technology (Rafalski and Tingey 1993), has already accelerated breeding activities and will greatly benefit the mushroom breeding programs. Mushroom breeding companies have currently invested in these DNA-based technologies and are adapting their breeding strategies with success (Loftus et al. 1995). Besides homokaryon detection methods, marker, assisted selection and linkage maps are now a vital part of strain development programs (Loftus et al. 1995).

Mushroom transformation: development and present state

Transformation of yeast and filamentous (ascomycetous) fungi has now been common practice for many years. Hinnen et al. (1978) were the first to report the complementation of a haploid *Saccharomyces cerevisiae LEU*2 strain. Meanwhile, many yeasts and filamentous fungi appeared transformable with both auxotrophic and dominant markers (for reviews see, for example, Gurr et al. 1987; Fincham 1989; Reiser et al. 1990; Van den Hondel and Punt 1991). Homobasidiomycetes, the class to which many mushrooms belong, including well-known edible mushrooms like *A. bisporus*, *Pleurotus ostreatus* (oyster mushroom) and *Lentinus edodes* (shiitake), generally demonstrated a higher reluctance to genetic transformation. The first successful transformations on homobasidiomycetes were carried out using a tryptophan- auxotrophic (*trp*C) mutant of the wood rotting fungus *Schizophyllum commune*, the homologous *TRP*C gene and polyethylene glycol-mediated DNA entry into protoplasts (Muñoz-Rivas et al. 1986). However, transformation with the *Escherichia coli* hygromycin B phosphotransferase (*hpt*) gene (Gritz and Davies 1983) as a dominant selectable marker appeared to be hampered by heavy methylation of the donor DNA (Mooibroek et al. 1990) and the formation of truncated transcripts (Schuren and Wessels 1998). Best results were reported for *S. commune* using the *Streptoalloteichus hindustanus* phleomycin (*ble*) resistance gene (Schuren and Wessels 1994).

During the past decade, considerable effort has been spent on developing a transformation system for *A. bisporus*. Various attempts at transformation using polyethylene glycol, electroporation and particle bombardment to introduce DNA into protoplasts, mycelia or fruit bodies were unsuccessful (Challen et al. 1991; Royer and Horgen 1991; Li and Horgen 1993; Challen and Elliott 1994). The problems encountered were attributed to the inability to introduce donor DNA into protoplasts, a low level of recombinational activity for integration, methylation of donor DNA sequences, or insufficient expression from heterologous promoters. In 1996, Van de Rhee et al. were successful in establishing an efficient and stable transformation system for an adenine-auxotrophic homokaryotic *A. bisporus* strain (Mooibroek et al. 1996; Van de Rhee et al. 1996a). The

A. bisporus transformation system is based on selection with the *E. coli* hygromycin B resistance (*hpt*) gene, introduced via electroporation into protoplasts and results in about 10–100 transformants per experiment, typically using 10^7–10^8 protoplasts (Van de Rhee et al. 1996a). Occasionally, transformants were also generated from a self-fertile, but quickly degenerating heterokaryon (U1mp10) and from a homokaryon (U1p8), both protoclonal derivatives from commercial strain Horst U1 (ATCC 62462). A 3.2 kb genomic *A. bisporus* fragment comprising two tandem exo-β-1,3-glucanase genes resulted in highly efficient (~80%) homologous integration (Van de Rhee et al. 1996b), in principle opening the prospect for gene disruption. Co-transformation with a β-D-glucuronidase (*GUS*) reporter gene (Jefferson 1987), also driven by the *GPD*2 promoter, resulted in the expression of the GUS enzyme and the appearance of blue stained, intact mycelia after treatment with the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc). This represents the first example of a co-transforming gene expressed in *A. bisporus* transformants (Jennen et al., manuscript submitted).

Many chromosomal segments and mRNAs have currently been identified and sequenced (see below), some of which are most certainly associated with important commercial properties. However, not all sequences obtained can be completely characterized due to the absence of sufficient homology with other known genes in databases. Therefore, it is argued that the primary scientific application of the *A. bisporus* transformation system will be the silencing of genes in order to find out their specific function. This primary goal of gene silencing is complicated by the *A. bisporus* life cycle and by the limitation that until now, using electroporation, only transformation of the adenine-auxotrophic strain (ATCC 24663) has been reproducible. For the production of transgenic heterokaryons and fruit bodies, transformed homokaryons are mated with compatible (non-transformed) homokaryons (Mooibroek et al. 1996) under double selective conditions on minimal medium containing hygromycin, comprising the opposite mating type. The resulting transgenic organism has only one type of its nuclei transformed. This implies that conventional techniques for gene silencing in fungi, like gene disruption, are not applicable to this type of heterokaryons or fruit bodies.

An alternative way to introduce donor DNA into a vast number of organisms, including many plant and fungal species, and even intact tissues, is particle bombardment. This technique is based on gas-driven bombardment with tungsten or gold particles coated with the donor DNA, thus penetrating the recipient tissue. A number of reports describe its use for the introduction of the *GUS* gene into the plant pathogenic fungus *Erysiphe graminis* f. sp. *hordei* (Christiansen et al. 1995), and into urediniospores of *Uromyces appendiculatus* (Li et al. 1993) and for the co-transformation of both the hygromycin B phosphotransferase gene and the *GUS* gene into the ectomycorrhizal fungus *Paxillus involutus* (Bills et al. 1995). For *A. bisporus*, particle bombardment would have the advantage that the laborious and often problematic production and regeneration of protoplasts could be avoided. In our and other laboratories, attempts have been undertaken to introduce hygromycin B resistance or other selectable markers into *A. bisporus* by particle bombardment (M.P. Challen, personal communication). Using fruit body slices for gold particle bombardment, we were able to demonstrate expression in situ of the *GUS* reporter gene driven by the *A. bisporus GPD*2 promoter. However, this technique has not yet resulted in the selection of stable transformants, nor in a generally applicable system.

Recently, a novel transformation protocol for different fungal species, including commercial heterokaryotic *A. bisporus* strain Horst U1, has been described (De Groot et al. 1998a). This is based on infection with the plant-pathogenic bacterium *Agrobacterium tumefaciens* and induction of its virulence (*vir*) gene with the plant hormone acetosyringone. One possible limitation of this *Agrobacterium*-mediated transformation system for *A. bisporus* may be that heterokaryotic sporogenic tissue has to be used and a priori it is not known which of the nuclei will have been transformed.

A list (incomplete) of current patents relevant to mushroom biotechnology is shown in Table 1, demonstrating that the production of novel strains and mutants of *A. bisporus* and a number of other edible mushrooms or even inter- and intraspecies hybrids therefrom has received much attention. Only two patents in Table 1 deal with the transformation of *A. bisporus*, whereas several publications exist concerning transformation of *Pleurotus ostreatus* and *P. florida* (Byun et al. 1989a, b; Peng et al. 1992, 1993a, b; Lemke 1994; Herzog et al. 1995; Yanai et al. 1996). There are none for *Lentinula*, *Volvariella*, *Flammulina* or *Tremella*, indicating how mushroom research is still behind, for example, plant biotechnology. Following the situation for plants, it may be anticipated that within 5–10 years the first generation of transgenic *A. bisporus* and *Pleurotus* spp. mushrooms or mushroom products could penetrate the market.

Identification of possible targets for strain improvement

Improvement of mushroom breeding programs will ultimately depend on the ability to gain more insight into the molecular, biochemical and physiological processes that regulate growth, development, senescence and post-harvest reactions. A better understanding of these important processes will allow breeders to define gene targets for strain improvement. Some of the genes and cDNAs that have been isolated and sequenced to date are shown in Table 2.

Genes involved in brown discoloration

It is generally known that fresh mushrooms are sensitive to rapid brown discoloration. Browning phenomena

Table 1 Some relevant patents on mushroom biotechnology

Subject	Organism	Main inventor	Company/ institute	Reference
Interspecies protoplast fusion	*A. bisporus* *P. ostreatus* *L. edodes* *S. commune* and other mushrooms[a]	Anon.	Meiji-Chem	Japanese patent, J02245179
Interspecies protoplast fusion	*P. ostreatus* *F. japonicus*	Shimai	Piasuaraizu KK	Japanese patent, No. JP 61-158780
Carboxin or benodanil mutant strains	*A. bisporus*	Elliot	National Research Development Corp.	US patent, No. 4,608,775
Interspecies protoplast fusion	*P. ostreatus* *Agrocybe cylindracea*	Morinaga	Nakano Vinegar Company	Japanese patent, JP 02-113884
Interspecies protoplast fusion	*P. ostreatus* *L. edodes*	Anon.	Teikoku Seiyaku, KK	Japanese patent, No. JP 4173034-A
Interspecific strains, protoplast fusion	*A. bisporus* *A. bitorquis*	Dahlberg	Campbell Soup Company	US patent, No. 4,996,390
Homokaryon production, tetrasporic strains	*A. bisporus*	Kerrigan	Sylvan Spawn Laboratory	US patent, No. 5,304,721
Transformation, dominant hybrid production, fingerprinting	*A. bisporus*	Mooibroek	Coöperatieve Nederlandse Champignonkwekersvereniging	PCT-NL94/00164, WO 95/02691
Transformation, etc.	*A. bisporus*	Mooibroek	ATO-DLO	Australian patent, No. AU 682057
New white strains	*A. bisporus*	Loftus	Amycel	PCT-US96/18686, WO 98/21975
Transformation using *A. tumefaciens*	*A. bisporus*, and other fungi[b]	Beijersbergen	Unilever	PCT-EP98/01914, WO 98/45455
Intraspecific hybrids	*A. bisporus*	Moquet	Institut National de Recherche Agronomique	PCT-FR98/00139, WO 98/32327
Intraspecific hybrids	*A. bisporus*	Callac	Institut National de Recherche Agronomique	Australian patent, No. AU 9862187-A
Fruiting-specific gene	*Agrocybe aegerita*	Labarere	Institut National de Recherche Agronomique	PCT-FR98/00732, WO 98/46773

[a] *Pholiota nameko, Flammulina velupites, Tremella fuciformis, Auricularia auricula*-judae, *Volvariella volvaceae, Lenzites betulina, Coriolus versicolor, Ganoderma japonica, Poria cocos*
[b] *Aspergillus awamori, Aspergillus nidulans, Aspergillus niger, Colletotrichum gloeosporoides, Fusarium solani pisi, Neurospora crassa, Trichoderma reesii, P. ostreatus, Fusarium graminearum*

may be induced by (post-harvest) senescence, mechanical injury or by the pathogen *Pseudomonas tolaasii* (Jolivet et al. 1995; Soler-Rivas et al. 1997). Several enzymatic processes have been implicated with melanin formation, e.g. brown discoloration of mushrooms (Burton et al. 1993). The best studied enzyme responsible for browning of mushroom fruit bodies is tyrosinase, a polyphenoloxidase (Burton et al. 1993; Gerritsen et al. 1994; Van Leeuwen and Wichers 1998). Sequences and structural features of plant and fungal tyrosinases have recently been reviewed (Van Gelder et al. 1997). Tyrosinase catalyzes the conversion of the highly stable monophenolic compound tyrosine, to the *o*-diphenol 3,4-dihydroxyphenylanaline (L-DOPA), which is further oxidized by the same enzyme to dopaquinone. The resulting quinones are further converted non-enzymatically into dopachrome (red), 5,6-dihydroxyindole, and 5,6-indolequinone (yellow), which undergo a polymerization process leading to melanochrome (purple) and melanin (black). The wide range of colored compounds from red to black is characteristic of the DOPA melanins. Tyrosinase has been shown to be present in mushrooms in a latent and an active form (Yamagushi et al. 1970). Furthermore, multiple isoforms of tyrosinase have been reported but it is still unclear what role each isoform plays in the browning process (Van Leeuwen and Wichers 1998; Wichers et al. 1996).

Using the *A. bisporus* transformation system, an antisense inhibition strategy was chosen in attempts to silence two tyrosinase genes identified in *A. bisporus* (Mooibroek et al. 1996; Van de Rhee et al. 1996a). For this purpose a large set of transformants was produced containing short (600 bp) inserts from two different *A. bisporus* polyphenoloxidase (tyrosinase) genes (*AbTYR*1 and *AbTYR*2; Ebbelaar et al. 1996; Recourt 1996) cloned in the antisense orientation behind the *A. bisporus* glyceraldehyde-3-phosphate dehydrogenase (*GPD*2) promoter. Also, transformants were selected containing both constructs individually or in larger co-integration vectors (Mooibroek et al. 1996). A Northern blot analysis was performed, using RNA prepared from fruit bodies produced after mating primary homokaryotic transformants with a non-transformed compatible strain (Fig. 1); it shows the occurrence of short (~600 nt) and longer *AbTYR* transcripts plus a variable level of sense tyrosinase mRNAs for different trans-

Table 2 Potential gene targets for strain improvement of *A. bisporus*

Gene function	Gene product	Gene or cDNA	Reference
Brown discoloration	Tyrosinase	Ab*PPO*1 (cDNA)	Ebbelaar et al. 1996
	Tyrosinase	Ab*PPO*2 (cDNA)	Recourt 1996
	Polyphenoloxidase		
	Adenylate cyclase		
Substrate degradation	Laccase	*LCC*1	Perry et al. 1993
	Laccase	*LCC*2 (cDNA)	Perry et al. 1993
	Endo-1,4-β xylanase	*XLN*A	De Groot et al. 1998b
	Cellulase	*CEL*1	Raguz et al. 1992
	Cellulase	*CEL*3	Yagüe et al. 1996
	Cellulase	*CEL*3 (cDNA)	Chow et al. 1994
	1,4-exocellobiohydrolase	*CEL*2 (cDNA)	Yagüe et al. 1997
	Mannosidase	*CEL*4 (cDNA)	Yagüe et al. 1997
Carbon metabolism	NADP-mannitol dehydrogenase	*MtDH* (cDNA)	Stoop and Mooibroek 1998
	Glu-6-P dehydrogenase	*ZWF*A	Schaap et al. 1995
	Glyceraldehyde-3-P dehydrogenase	*GPD* I	Harmsen et al. 1992
	Glyceraldehyde-3-P dehydrogenase	*GPD* II *GPD*A	Harmsen et al. 1992
	3-phosphoglycerate kinase	*PGK*A	Schaap et al. 1995
	Pyruvate kinase	*PKI*	Schaap et al. 1995
	Succinate dehydrogenase		
Nitrogen metabolism	NADP-glutamate dehydrogenase	*GDH*A	Schaap et al. 1996
	Glutamine synthetase	*GLN*A	Kersten et al. 1997
	δ-pyrroline-5-carboxylate dehydrogenase	*PRU*A	Schaap et al. 1997
Fruit body development	Cytochrome P-450	*CYP*A (partial)	De Groot et al. 1997
	Septine	*SEP*A	
	α-subunit of ATP-synthase	*ATP*D	De Groot et al. 1997
	Hydrophobin	*HYP*A	De Groot et al. 1996
Other	Serine protease		
	Hydrophobin	*HYP*C	De Groot et al. 1996
	Hydrophobin	*ABH*1	Lugones et al. 1996
	4-aminobenzoate hydroxylase	4*ABH* (cDNA)	Tsuji et al. 1996
	Aminolaevulinic acid synthase		
	Phenylalanine ammonia lyase		
	Exo-β-1,3-glucanase	Ab*EXG*1	Van de Rhee et al. 1996b
	Exo-β-1,3-glucanase	Ab*EXG*2	Van de Rhee et al. 1996b
	Trifunctional protein		

formants. Transformants were also identified that did not reveal any sense *AbTYR* mRNA at all, suggesting that antisense mRNAs also inhibit the activity of tyrosinase genes in the unaffected nucleus. Therefore the antisense approach in principle works for gene silencing in *A. bisporus* (A. Moller, H. Wichers and H. Mooibroek, unpublished results). However, more work is needed before any firm conclusions can be drawn concerning a possible effect on browning. This is due to the complexity of the tyrosinase system and the occurrence of latent and active forms of the enzymes. Evidence for an antisense effect mediated via the transgenic cytoplasma has been obtained in *Nicotiana benthamiana* plants (Kumagai et al. 1995). It was shown that replication of a viral RNA carrying an antisense carotenoid biosynthetic gene inhibited the expression of its endogenous counterpart. The results found in *A. bisporus* heterokaryons and fruit bodies may also help to elucidate the antisense inhibition mechanism in general.

Recently, the enzyme γ-glutamyl transferase (GGT, EC 2.3.2.2) was characterized (Jolivet et al. 1998). It is involved in the biosynthesis of agaritine (a precursor of potentially toxic aryl diazonium ions) and γ-glutamyl-4-hydroxybenzene (one of the main substrates implicated in mushroom browning). The isolation and application of the corresponding gene may also open perspectives for transformation to control a step in browning phenomena earlier than that performed by tyrosinase.

Genes involved in substrate utilization

Fruit bodies of *A. bisporus* are preferentially cultivated on a specific compost covered with a casing layer. Commercially available compost consists mainly of a lignocellulose fraction and microbial biomass. Basidiomycetes produce a wide range of enzymes to degrade lignocellulosic substrates. These include peroxidases and laccases for lignin degradation and exoglucanases, endoglucanases, β-glucanases and cellobiose dehydrogenase for cellulose degradation (Durrant et al. 1991; Bonnen et al. 1994; Smith et al. 1998).

Genes involved in the breakdown of cellulose have been isolated using differential screening of mycelia grown on cellulose. Three of these cellulose-induced genes (*CEL*1, *CEL*2, *CEL*3) are directly involved in the degradation of cellulose and encode a cellulase (*CEL*1), a cellobiohydrolase (*CEL*2) and a mannanase (*CEL*3) (Chow et al. 1994, Yagüe et al. 1997).

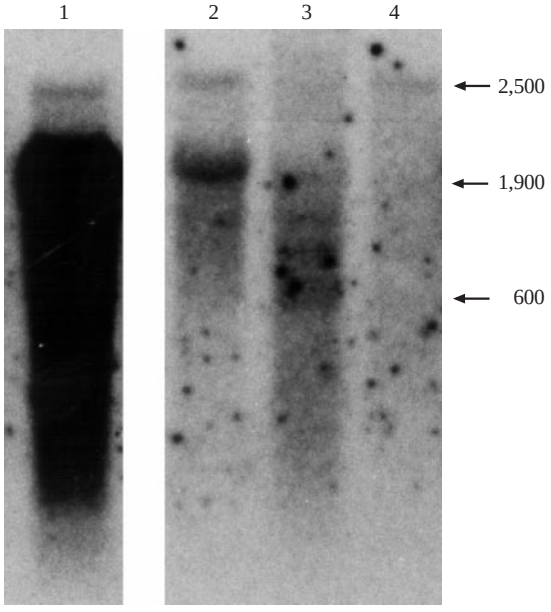

Fig. 1 Northern blot analysis of RNA from selected transgenic fruit bodies co-transformed with *hpt* gene as primary selectable marker and 600 bp *AbPPO*2 antisense cDNA construct, driven by *A. bisporus GPD*2 promoter. Primary ATCC 24663 transformants were mated with a compatible homokaryotic derivative of commercial strain Horst U1, H97, kindly given by Horst Mushroom Experimental Station, Horst, The Netherlands. Fruit bodies were produced as described previously (Van de Rhee et al. 1996a). Blots were hybridized with the *hpt* probe (not shown), with a ribosomal probe to confirm equal RNA loading and integrity of the RNA preparations (not shown) and with the *AbPPO*2 probe (all ^{32}P-labeled). Lanes: *1* control commercial strain Horst U1; *2* control ATCC 24663; *3*, *4* transformed strains tαtyr2-1 and tαtyr2-2, respectively. Note the abundance of *AbPPO*2 mRNA (1,900 nt) in Horst U1 (*lane 1*), the 2,500 nt mRNA corresponding with putative *AbPPO*3 gene (*lanes 1*, *2 and 4*, exclusively hybridizing to *AbPPO*2, but not to *AbPPO*1), the *Abtyr*2-specific bands of variable lengths (≥600 nt), the strong reduction (*lane 3*) and the disappearance (*lane 4*) of the sense *AbPPO*2 mRNA

Two non-allelic laccase genes (*LCC*1 and *LCC*2) with tyrosinase, also belonging to the family of polyphenol-oxidases, have been isolated from *A. bisporus* mycelia and show a highly disparate expression pattern (Smith et al. 1998). The steady-state *LCC*2 mRNA level is approximately 300–7,000 times greater than *LCC*1 mRNA, depending on the mycelial growth conditions. A possible explanation for the difference in mRNA abundance might be that mRNA degradation contributes in part, to the control of gene expression (Sachs 1993).

Besides cellulose and lignin, compost contains hemicellulose which represents a major polysaccharide of plant cell walls. Hemicellulose consists mainly of xylan, a polymer of β-1,4-linked xylosyl residues. Xylan can be degraded into monomeric sugars by the concerted action of several enzymes of which only endo-1,4-β-xylanase has been cloned and characterized (De Groot et al. 1998b). The xylanase-encoding gene *XLN*A is regulated by glucose in a manner similar to *CEL*3 encoding a cellulase, namely induction by low and repression by high levels of glucose (De Groot et al. 1998b).

Genes involved in carbon and nitrogen metabolism

In *A. bisporus*, the six carbon sugar alcohol mannitol is the main storage carbon. It can contribute up to 20% of the mycelium dry weight and up to 50% of the fruit body dry weight (Rast 1965). Sugar alcohols are among the major soluble carbohydrates in fungi. Besides functioning as important metabolites supporting growth and development, sugar alcohols can serve as intracellular osmolytes or compatible solutes (Jennings 1984; Stoop et al. 1996a). Compatible solutes such as glycerol, arabitol and mannitol are believed to accumulate in the fungal cell in response to water or osmotic stress, and prevent inactivation of metabolic processes. Regulation of the metabolic pathways leading to the synthesis and catabolism of polyols may be critical to assure growth and stress tolerance. For example, *A. bisporus* responds to NaCl stress by increasing the synthesis and accumulation of mannitol (Stoop et al. 1996b). The increase in mannitol is correlated with an increased protein synthesis and activity of the NADP-dependent mannitol-synthesizing enzyme, mannitol dehydrogenase (Stoop and Mooibroek 1998). In fungi, mannitol's function as an osmoregulatory compound might also be critical in providing an influx of water from the environment to support turgor and fruit body development (Holtz 1971; Jennings 1984). Recently, the enzyme responsible for mannitol biosynthesis, NADP-dependent mannitol dehydrogenase (MtDH, EC 1.1.1.138), was purified to homogeneity and *MtDH* cDNA was cloned, sequenced and characterized (Stoop and Mooibroek 1998). Salt-stressed fruit bodies showed an overall increase in mannitol biosynthesis as was evident from the increase in MtDH activity, MtDH abundance and *MtDH* mRNA accumulation. Increased steady-state *MtDH* mRNA levels were also observed when mycelia were grown on defined media supplemented with NaCl concentrations up to 200 mM (Fig. 2). Besides being regulated by salt, the *MtDH* transcript level seems to be under developmental control, as *MtDH* RNA accumulated during maturation of the fruit body (Stoop and Mooibroek 1998).

Other genes involved in carbon metabolism (*PGK*, *PKI*A, *ZWF*A, Table 2) have been cloned by heterolo-

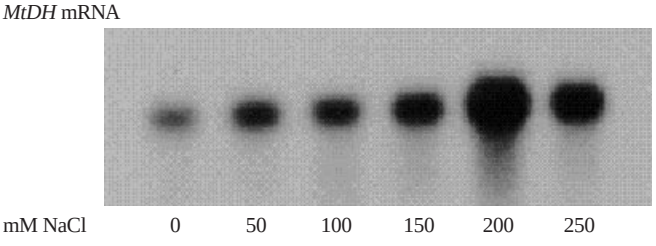

Fig. 2 Effect of NaCl on *MtDH* mRNA accumulation in mycelia of *A. bisporus*. Mycelia were grown on MMP agar media supplemented with either 0, 50, 100, 150, 200 or 250 mM NaCl. Each treatment was replicated four times. Total RNA was extracted from each treatment and relative amounts of *MtDH* transcript (1,000 nt) were determined by RNA blot analysis, hybridized to a partial ^{32}P-labeled *MtDH* fragment. Equal loading of lanes and integrity of mRNAs were confirmed by hybridization to a ribosomal DNA probe (not shown)

gous hybridization with probes from other organisms such as yeast and *Aspergillus nidulans* (Schaap et al. 1995).

Besides consisting of complex carbons, the compost on which the common white button mushroom is grown is rich in organic nitrogen sources and low in free ammonium which can inhibit growth. *A. bisporus* can incorporate ammonium into the amino acids glutamate or glutamine, through reactions catalyzed by the enzymes NADP-dependent glutamate dehydrogenase (NADP-GDH) and glutamine synthetase (GS), respectively. The genes encoding NADP-GDH and GS, *GDH*A and *GLN*A respectively, have been characterized (Schaap et al. 1996; Kersten et al. 1997). The transcription of both genes is repressed upon addition of ammonium to the culture medium. As was the case for NADP-mannitol dehydrogenase of *A. bisporus* (Stoop and Mooibroek 1998), no direct relationship between enzyme activity and mRNA expression level has been observed (Kersten et al. 1997). When mycelia were grown on protein as nitrogen source a high *GDH*A mRNA level was observed, but only low enzyme activity, while GS activity and mRNA levels did not always correlate (Kersten et al. 1997).

Genes involved in fruit body development

Identification of the genes specifically expressed during fruiting or whose transcriptional activities are induced during fruiting, has met with little success. Only two fruit specific genes (*HYP*A and pDG47) have been identified using differential screening techniques. Individual screening of 100 cDNA clones of a fruit body cDNA expression library resulted in the identification of nine cDNA clones, of which only three (*ATP*D, *SEP*A and *CYP*A) showed significant homology with known sequences (De Groot et al. 1996, 1997). However, the *ATP*D, *SEP*A and *CYP*A genes are not specific for fruit body development, as they are also expressed during the vegetative phase of the mushroom life cycle.

Genes as molecular markers

Cloned genes can also serve as markers in the construction of a genomic map of *A. bisporus* (Schaap et al. 1995) and to gain more information on the gene structure. A cDNA library constructed from emergent fruit bodies was screened for abundantly expressed genes. In this way, several genes, such as that encoding S15a, a small subunit protein of the ribosome, were identified by comparison with the deduced amino acid sequence (Schaap et al. 1995). Comparison of the characterized genes with those in the EMBL/Genbank databases revealed that further refinement of the screening method is needed to distinguish between fruit body induced and fruit body specific genes. Clones that showed an increased steady-state mRNA level in fruit bodies as compared to mycelium were identified in order to study the process of phase transition from vegetative to reproductive growth and fruit body development (De Groot et al. 1997).

Trends and future prospects

Biological tools, such as the mushroom transformation system, can be instrumental in elucidating the role of potential gene targets by either over-expressing or down-regulating the gene of interest and evaluating its effect on the quality and production efficiency of mushrooms. The work described in this paper concerning the silencing of tyrosinase genes by the antisense approach is the first example of attempts to down-regulate *A. bisporus* genes by transformation, although the phenotypic effect still has to be established. Also, the use of the *GUS* reporter gene and its expression in *A. bisporus* described here represents the first example of a heterologous bacterial gene expressed from co-transforming donor DNA. These two accomplishments represent a solid basis for further work, which will certainly include the application of genes determining commercial characteristics. By over-expression or down-regulation of the *MtDH* gene (Stoop and Mooibroek 1998), a basic insight can be generated into its possible function in a number of metabolic activities, especially fruit body development and cap opening. This will also have a commercial spin off. For the coming years, the challenge will be to design the best metabolic engineering strategies. In this respect it may be significant to note that current mushroom cultivation occurs under highly optimized, artificial conditions. This implies that naturally occurring threats may not be relevant under current cultivation conditions and, vice versa, pathogens affecting mushroom cultivation may not be relevant to wild populations. It is therefore questionable whether wild populations have developed strategies to survive threats during their evolution, strategies which only operate under artificial cultivation conditions. In designing metabolic engineering strategies, a clear distinction should be made between active resistance and fortuitous insensitivity towards pathogens. This insensitivity may be the addition of a number of properties that in themselves are not directly related to controlling the pathogen. If these considerations are correct, classical breeding strategies aiming at the introduction of pathogen resistance derived from wild populations, which may not be determined by a single gene, will be inefficient and novel molecular techniques will be the ultimate choice. In this case, applicable heterologous genes from non-related species may be better candidates for strain improvement than homologous genes from wild isolates. A clear example for this approach in plants is the application of the *Bacillus thuringiensis* Bt toxin gene.

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