Transgenic Breeding of *Agaricus bisporus*: The Next Frontier

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Abstract: The advent of a tractable method for DNA transfer in *Agaricus bisporus* has created new vistas for the genetic enhancement of this crop, and now enables this mushroom species to be explored as a biofactory for the production of high-value pharmaceutical proteins. Early transgenic breeding achievements with *A. bisporus* will probably involve those input traits meeting commercial success in crop plants, such as resistance to viruses and insects. However, improvements in output traits that directly benefit the consumer, including bruising, shelf life, flavor components, and health constituents, would serve to hasten the acceptance of biotech mushrooms. Compared to other crops, the mushroom represents an intriguing platform for the production of biopharmaceuticals, as an extraordinarily high biomass can be produced in a short time under high security and containment. Moreover, unlike therapeutic proteins derived from animal-based systems nowadays, mushroom-made drugs would not carry the risk of human pathogenic contaminants.

Key words: Transgenic breeding, *Agaricus bisporus,* genetic enhancement, biofactories

1 Introduction

A scientific milestone in the field of genetic engineering occurred during the 1990's with the discovery that *Agrobacterium tumefaciens*, the bacterial workhorse for gene transfer in plants, could be used to shuttle genes into fungi.[1,2] *Agrobacterium*-mediated genetic transformation (Agro-transformation) in fungi was first demonstrated for baker's yeast, *Saccharomyces cerevisiae*, and subsequently extended to numerous filamentous examples ranging from the common *Aspergillus* black mold to the cultivated button mushroom, *Agaricus bisporus*. Today, Agro-transformation is proving highly effective for a rapidly growing number of fungal species, many of which have been recalcitrant to more conventional gene transfer techniques (i.e. direct uptake by protoplasts, biolistics). Moreover, the inter-kingdom delivery of genes by *Agrobacterium* is not limited to fungi, since the mechanism was shown to operate with HeLa cells.[3] Thus, A transformation methodology will likely be applicable to most, if not all, fungi. The availability of a facile gene transfer system will allow a diversity of genetically enhanced fungi to play prominent roles in a wide variety of new and novel industrial processes and applications.[4] The original Agro-transformation method reported by de Groot et al.[2] proved too inefficient to be applied to the breeding and scientific investigation of *A. bisporus*. Thereafter, we described an Agro-transformation procedure holding the promise of a practical tool for the genetic manipulation of this important mushroom species.[5] Herein, we outline our Agro-transformation protocol and discuss several of its potential applications in *A. bisporus*.

2 Materials and Methods

2.1 Source of tissue

Fruiting bodies of commercial hybrid strains were grown at the Penn State experimental mushroom research facilities as described previously[6] except Phase I composting was carried out in a bunker and Phase II in a tunnel.

2.2 Plasmid vectors and bacterial strain

Agaricus bisporus was transformed using *A. tumefaciens* strain AGL-1 and either plasmid vector pBGgHg[5] or pBHg.[7] Each vector contained the *Escherichia coli* hygromycin B phosphotransferase gene controlled by the *A. bisporus* glyceraldehyde 3-phosphate dehydrogenase promoter and *Cauliflower mosaic virus* 35S terminator, all of which were flanked by the *Agrobacterium* T-DNA border sequences.

2.3 Genetic transformation

The fruiting body Agro-transformation protocol as outlined by Chen et al.[5] was followed. Transformation efficiency was expressed as a percentage of the fruiting body explants generating hygromycin-resistant colonies.

3 Results and Discussion

3.1 Adapting Agro-transformation for *Agaricus bisporus*

Tissues comprising the fruiting body of *A. bisporus*, particularly the gill, were found to be highly receptive to DNA transfer mediated by *A. tumefaciens*.[5] The steps in the fruiting body Agro-transformation method are depicted in Fig. 1. The use of the gill as the recipient tissue had a dramatic effect on the rate of transformation, overcoming the low efficiency or poor reproducibility observed with the use of basidiospores and vegetative mycelium.[2, 8, 9, 10]

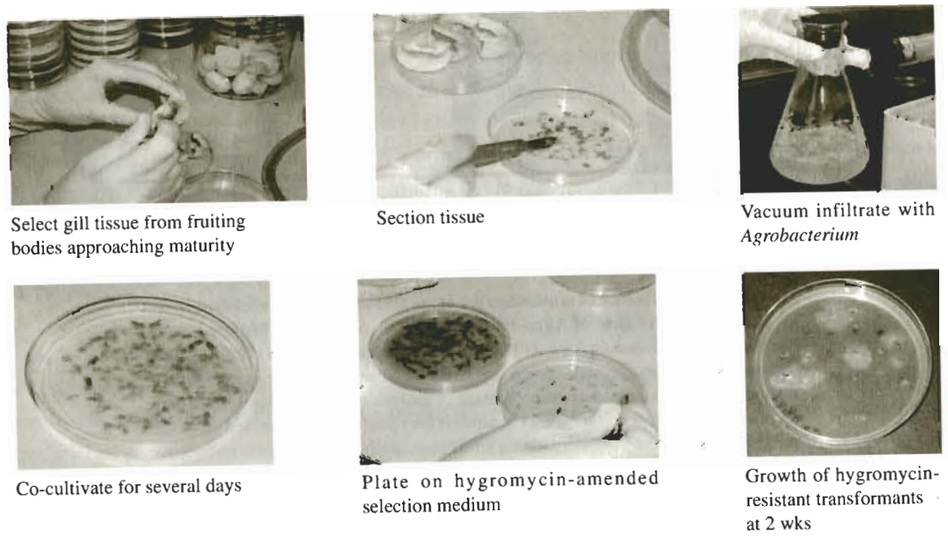

Figure 1. The fruiting body Agro-transformation method for *Agaricus bisporus*

In their original report on Agro-transformation of filamentous fungi, de Groot et al.[2] using basidiospores obtained a 0.00003% transformation efficiency for *A. bisporus*, while Chen et al.[5] using gill explants achieved rates in excess of 30%; an apparent seven orders of magnitude increase. However, these efficiencies are not directly comparable, because the unit of transformation differs (i.e. basidiospore vs. cell cluster). For example, simply increasing the size of the gill explant increases the apparent transformation efficiency. Nonetheless, the method offers a high "experimental efficiency"or perhaps "effectiveness". Acquiring large populations of transformants for research or breeding purposes can now be achieved with ease. The method has proven suffi

ciently robust to be reproduced by other research groups.[10-13] Also, our vector pBGgHg has been employed to transform other fungi, including *Suillus bovines*,[14] *Hebeloma cylindrosporum*,[15] *Helminthosporium turcicum* [16] and *Beauveria bassiana*.[17] Using the fruiting body Agro-transformation protocol, high rates of transformation were obtained for three commercial hybrid off-white strains of *A. bisporus* as well as S1901, a proprietary sporeless strain of Sylvan Inc. (Fig. 2). The method was equally effective for brown strains (portabellas) as well (Romaine, unpubl.). Both the gills and the fleshy context of the cap and stem could serve as the recipient tissue for DNA transfer, although gill tissue tended to produce higher rates of transformation and a more rapid regeneration of transformants. In studies comparing differentiated and undifferentiated gill explants, similarly high rates of transformation were observed. Thus, any portion of the fruiting body at probably any developmental stage can serve as a source of the recipient tissue.

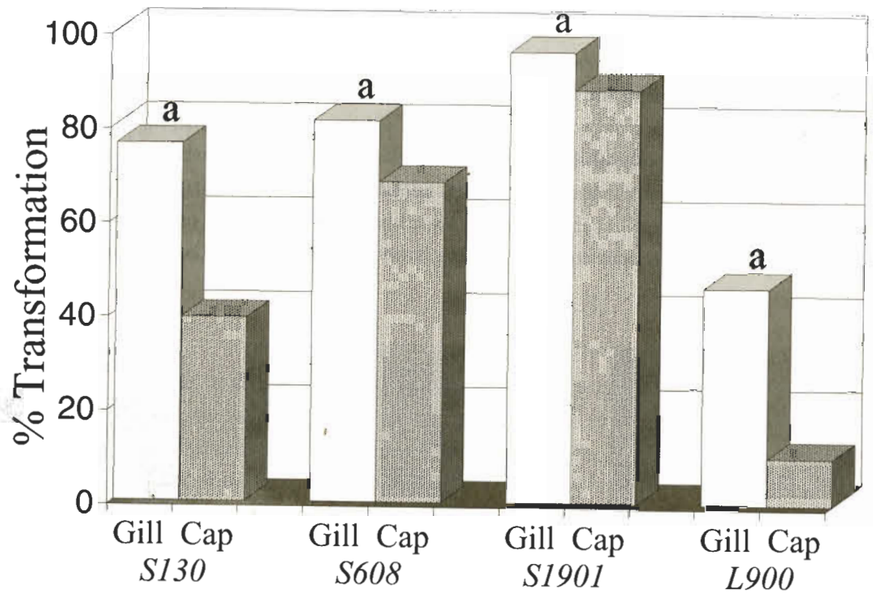

Figure 2. Influence of mushroom genotype and source of the recipient tissue on the rate of Agro-transformation in *Agaricus bisporus*
For each of four mushroom lines, S130 (Sylvan hybrid), S608 (Sylvan hybrid), S1901 (Sylvan's proprietary sporeless strain), and L900 (Lambert hybrid), the rate of transformation, which is expressed as the percentage of the fruiting body explants generating hygromycin-resistant colonies, was compared for recipient tissue derived from the gill (Gill) or cap (Cap). Means within a mushroom strain followed by the same letter are not significantly defferent according to the stuednt's *t*-test at P=0.05.

Spores on the gills are not the target for DNA uptake in our method for the following reasons: i) substituting spores alone for gill tissue failed to yield transformants, ii) gills of a sporeless mushroom strain were highly receptive to DNA uptake, iii) undifferentiated gill tissue lacking spores was amendable to *Agrobacterium*-mediated DNA transfer, and iv) appreciably high transformation rates could be obtained with cap and stem explants.

Evidently, the fruiting body is composed of highly specialized tissues that are not merely aggregates of vegetative mycelium. Indeed, major differences in the composition of the cell wall exist between the vegetative mycelium and fruiting body.[18-20] Whether these differences explain the high receptivity of fruiting body tissues

is not known, but binding of *A. tumefaciens* to the cell wall is a requisite for DNA transfer in plants.[21]

3.2 Crop improvement

The overwhelming popularity of the Horst 'U1' hybrid off-white mushroom strain introduced during the 1980's [22, 23] has created a near monoculture in the commercial mushroom industries in the Americas and Europe. This situation is precarious from the standpoint of disease and pest susceptibility, and has limited the choice of production characteristics. For more than two decades, no notable advances have been made in breeding strains with strikingly improved features. This is due largely to the cumbersome genetics of *A. bisporus* and what was widely held as a scarcity of commercially desirable traits. It has recently come to light, however, that a collection of wild isolates of *A. bisporus* could represent a vast and unexploited resource of valuable characteristics.[24] Even so, the difficulty encountered in breeding *A. bisporus* by traditional methods suggests that the largest strides in crop improvement will be realized by transgenic approaches.

A major dilemma in the transgenic breeding of plants, which also applies to *A. bisporus*, has been the greater technical challenge in incorporating output traits that benefit the consumer compared to input traits favoring the seed dealer and grower. Most of the transgenic accomplishments with crop plants to date have revolved around herbicide tolerance and disease and insect resistance. Farmers have embraced molecular biotechnology with open arms, because transgenic crops have reduced their workload or increased profit. But few, if any, incentives exist for the consumer to choose biotech over non-biotech produce in the marketplace. Consequently, transgenic breeding programs have now been redirected towards output traits affording greater consumer appeal, as for example, improved shelf life, appearance, color, flavor, nutrition, hypoallergenicity, etc. When and only when biotech food presents a clear advantage to the consumer over its conventional counterpart can it hope to gain public acceptance.

Despite the need to emphasize output traits for greater consumer appeal, early transgenic breeding achievements with *A. bisporus* will likely shadow those in crop plants, such as resistance to pathogens, insects, and pesticides. With the mapping of the mushroom genome and a detailed understanding of the molecular control over complex traits, genetic feats might entail improvements in yield, size, color, shelf life, tolerance to heat and water stress, food and health constituents, fruiting cycle regulation, strain stability, and substrate utilization (see [8] for a detailed review of the topic). Several of the more obvious transgenic breeding targets follow.

3.2.1 Virus resistance

One of the most broadly successful applications of transgenics in plants has been for viral resistance. Of the various strategies enabling plants to defend themselves against viral attack, those based on the principle of "pathogen-derived resistance" (PDR)[25] have shown the greatest utility. In a majority of the examples where a PDR strategy has proven effective, homology-dependent gene silencing[26] have been found to be the underlying mechanism.

A PDR strategy for viral resistance was successfully applied to double-stranded RNA (dsRNA) viruses in *S. cerevisiae*. Expression of the gag-pol fusion protein of L-A virus[27] and N-terminal fragments of the capsid polypeptides of ScVL1 and ScVLa viruses[28] completely cured the fungal host of infection. Though it was not determined if gene silencing mediated resistance in these two examples, it should be noted that the mechanism has been described in fungi, including *Neurospora crassa*[29], *Schizophyllum commune*[30], *Magnaporthe oryzae* [31] and *Venturia inaequalis*.[32]

La France disease is among the major infectious pathologies on cultivated *A. bisporus* worldwide[33], although in recent years it has become more of a sporadic nuisance. Symptoms of the disease include aberrant mycelial growth, reduced yield, and malformation of the fruiting bodies (Fig 3). All cultivated varieties of *A. bisporus* succumb to the disease, which makes transgenic resistance an alluring prospect.

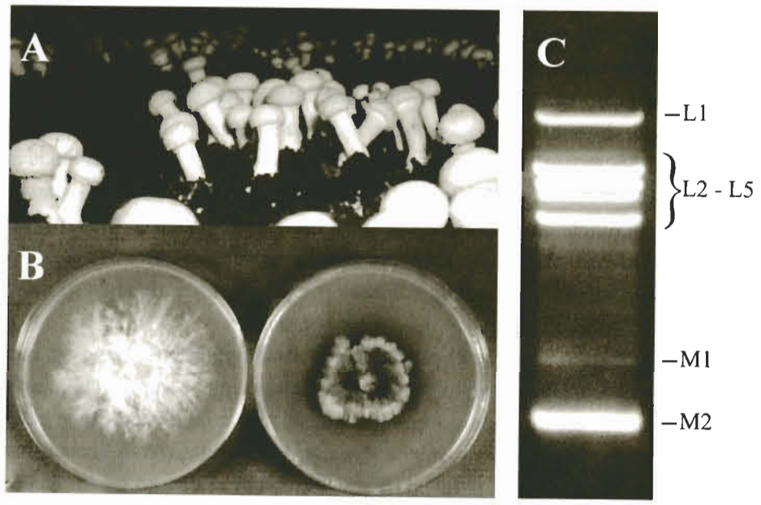

Figure 3. La France disease in *Agaricus bisporus*
A) crop affected by the disease showing the "drumstick" syndrome in which the fruiting bodies have small deformed caps and elongated stems; **B**) healthy vegetative culture of *A. bisporus* (left) and diseased culture (right) exhibitng reduced vigor, appressed mycelium, and an increased brown discoloration; **C**) dsRNAs (L1-L5, M1, and M2) of La France isometric virus, the implicated causal agent.

A wealth of evidence implicates a dsRNA virus (LIV, La France isometric virus) as the causal agent of the disease.[33] LIV is a 36-nm isometric particle containing six major genomic dsRNAs (L1-L5 and M2) of 3.8 to 1.3 kbp (Fig. 3). In a study underway in our laboratory, we are transforming *A. bisporus* with 11 different constructs representing three LIV gene sequences in an effort to evaluate their effect on viral replication. A similar PDR approach could be taken for mushroom virus X disease, a highly destructive malady of cultivated mushrooms in Europe that is associated with a distinct dsRNA complex.[33-35]

3.2.2 Fly resistance

The major insect pest of *A. bisporus* throughout much of the world is the fly *Lycoriella mali* (Diptera:Sciaridae). Larvae feed actively on developing mushroom primordia and significantly reduce the production of mature fruiting bodies. Production can be reduced 85-90% in the absence of larvicides and adulticides, and up to 20% with pesticide applications.[36] Effective fly management also improves the control of bacterial blotch, green mold, and dry bubble diseases, because adults are the primary vectors of the causative pathogens[37, 38] (Keil, pers. comm.).
A promising alternative to chemical pesticides for the control of flies is the biocontrol bacterium *Bacillus thuringiensis (Bt) var. israelensis (Bti)*.[36, 39] Suspensions of spores and toxin crystals of this bacterium can provide excellent control of flies without crop damage. However, application of liquid formulations to the compost requires substantial amounts of water to penetrate the substrate and achieve an even distribution. The excess water can retard crop development and result in the growth of unwanted competitor fungi. Also, the cost of *Bti* formulations is significantly higher than competing larvicides. Hence, the development of transgenic mushroom strains expressing *Bt* toxins could possibly offer a practical and safe means of fly control.
Bti is a thermophilic spore former that produces a parasporal inclusion body. This inclusion body is a complex,

amorphous crystal comprised of at least four toxin proteins: Cry4a, Cry4b, Cry11a, and Cyt1a. The Cry toxins are released from the crystal in the alkaline environment of the insect gut and bind to receptors in the gut membrane.[40] Except for the Cyt1a toxin having cytolytic properties, all of the Cry toxins plus Cry11b from *Bt jegathesan* are active against dipterous larvae and would be appropriate candidates for incorporation into *A. bisporus*.
An obvious experimental approach to transgenic fly resistance in *A. bisporus* would entail the expression of the Diptera-active Cry toxins under the control of an *A. bisporus* tissue-specific promoter. Targeting expression of the insecticidal protein gene to the vegetative mycelium would place the toxin at the initial site of insect activity where is it is likely to be most effective. Since the *Bt* genes are well characterized, and have been a focus in transgenic plants, their movement into mushrooms would be a logical first step in the application of gene transfer technology to a critical pest management problem faced by growers.

3.2.3 Bruising resistance

Postharvest browning is a major factor contributing to the rapid loss of quality in *A. bisporus* fruiting bodies destined for fresh market sale. An important cause of browning is bruising, which results from mechanical damage ("slip-shear") to the mushroom skin during harvest and handling.[41] This damage to the tissues leads to a loss of cellular integrity and the enzymatic oxidation of phenolic compounds forming brown-colored quinones and melanins. [42]
The enzyme tyrosinase is the major polyphenol oxidase associated with the fruiting body of *A. bisporus* and is purported to play a principal role in the enzymatic browning reaction.[42, 43] One possible approach to breeding mushroom strains with increased bruising resistance would involve the homology-dependent silencing of the two known tyrosinase genes.[44, 45]

3.2.4 Reduced agaritine content

Agaritine is a phenylhydrazine found in high concentrations (0.2-0.8% dry wt.) in the fruiting bodies of *A. bisporus*.[46] While most hydrazines are carcinogenic,[47] there is no evidence for agaritine per se having such an activity, although there is some question as to its analogs or derivatives having this potential.[47, 48] Though conclusive data is wholly lacking for the involvement of agaritine in human cancer, the availability of low-agaritine mushroom strains would permit more informative clinical studies to be carried out. Here, the transgenic approach would call for disrupting (*Agrobacterium*-mediated insertional mutagenesis) or homology-dependent silencing of genes encoding key enzymes in the agaritine biosynthetic pathway.

3.3 Mushroom-made bioproducts

Aside from transgenic manipulations aimed at crop improvement for mushrooms grown as food, there are those genetic alterations directed at transforming *A. bisporus* into a factory for the commercial-scale manufacture of valuable bioproducts. Compared to biotech food, this application of transgenic breeding is less subject to public opposition, because many of the potential end products would improve the quality of human life. Equipping the mushroom with entirely new genetic apparatuses or modifying the existing apparatus could allow for the *de novo* or enhanced synthesis of a variety of bioproducts having industrial and medical applications, as for example, i) homologous proteins (e.g. commercial *A. bisporus* polyphenol oxidase and lectin), ii) carbohydrates (e.g. beta glucans), iii) glycolipids (e.g. glycoinositol-phosphoceramides), and iv) heterologous proteins (e.g. insulin), just to name a few. And, in the short time since a practical gene transfer technique has become available, researchers have begun to characterize heterologous protein production in *A. bisporus* (Table 1).
Harnessing the mushroom for the commercial-scale synthesis of biopharmaceuticals (a.k.a protein-based drugs,

Table 1. Heterologous proteins expressed experimentally in *Agaricus bisporus*

Protein	Reference
Alpha-galactosidase	[12]
Human growth hormone	
Chymosin	
Bacillus thuringiensis Cry1Ac	[13]
Cyanovirin-N	
Endostatin	

biologics) is the most compelling direction for future scientific exploration and development. In year 2000, global sales of biopharmaceuticals, which includes enzymes, monoclonal antibodies (Mabs), and hormones, amounted to more than U.S. $20 billion[49] (Table 2). Although protein drugs represent only 5% of the total pharmaceutical market, as a sector they have experienced the greatest growth, generating global sales of U.S. $40 billion in 2003 and a predicted U.S. $70 billion in 2008.[50]

Table 2. Global value of biopharmaceuticals by category for the year 2000. Estimate excludes 167 recombinant protein vaccines

Category	Year 2000 Value (Billions U.S. $)
Structural Proteins	
Human serum albumin	1.3
Collagen	0.4
Fibrinogen	0.3
Blood Products	
Insulin	3.0
Coagulant factors VIII & IX	1.4
Complex Proteins	
Interferons	3.0
EPO	4.5
Enzymes	
Alpha-glucosidase	3.0
Cerazyme/Ceredase	0.5
Monoclonal Antibodies/Other	
Enbrel	0.7
Rituxan	0.4
Herceptin	0.3
Others	2.1

Source: Reference [49]

The aging demographics in many countries around the world and the human genome project are two major forces contributing to the pharmaceutical market boom. With a shift towards an aged population comes an increase in the incidence of age-related illnesses-osteoporosis, arthritis, diabetes, and Alzheimer's and Parkinson's diseases.[51] Also, advances in human genomics are expected to increase the number of treatable disorders and illnesses from the existing 500 or so to more than 10,000, spurring the discovery of not only protein-based drugs, but also nucleic acid-based and conventional drugs.[50] At the same time, the quest will continue for improved treatments for the leading killers-cardiovascular disease, cancer, and respiratory disorders and the

epidemic infectious diseases-influenza, herpes, and AIDS.

There is a sense of urgency in the pharmaceutical industry today, because it recognizes the looming need to produce therapeutic proteins, especially therapeutic Mabs and vaccines, in unprecedented quantity and variety in order to meet the surging market demand, but it is somewhat undecided as to which biosynthetic platform (e. g. bacteria, yeast, animal cells, multicellular organisms) will enable both simple and complex recombinant proteins to be manufactured with the greatest economy and safety.

Presently, biopharmaceuticals are obtained by either extraction from animal sources or from bacteria, yeast, and animal (e.g. Chinese hamster) cells nurtured in large bioreactors. However, both of these production schemes have inherent shortcomings. An animal-based platform, be it brute extraction from animal carcasses or sophisticated Chinese hamster ovary cell culture, carries the risk of contamination by human pathogenic viruses and prions.[52, 53] Further, cell culture is an expensive proposition, with U.S. $300-500 million cost and a 5- to 7-year construction period for a single bioreactor facility, as well as the need for expensive fetal bovine serum-based growth medium for animal hosts.[54] An alarming aside here is that 75% of the currently available bioreactor capacity is devoted to production of just four protein-based drugs!

The exorbitant capital expenditures required to construct and maintain bioreactor facilities and the threat of pathogen contamination with animal systems have driven pharmaceutical companies to explore other biosynthetic platforms. In what is often referred to as "pharming", a melding of the words pharmaceutical and farming, transgenic crop plants (e.g. corn and tobacco) and animals (e.g. cows and goats) are being piloted for heterologous protein production. One clear advantage of pharming over bioreactor technology is a 10- to 50-fold greater economy.[54] Pharming in plants shows greater promise than in animals for the reason discussed early regarding human pathogens. Containment issues, however, jeopardize plant systems. The threat of transgenic pharm crops commingling with their counterpart food crops has already been realized.[55] Several features of the commercial cultivation scheme and of *A. bisporus* itself render mushrooms a uniquely alluring platform for pharming.[4, 7, 13, 56] First, mushrooms are cultivated indoors in either aboveground, controlled environment structures or belowground in abandoned mines. This feature of mushroom culture would allow year-round production of biopharmaceuticals to take place without geographic restriction. The ability to produce mushrooms in such extreme seclusion, which could be enhanced even further by recruiting a sporeless mushroom strain as host, would solve the containment problem confounding pharming in field crops. Second, *A. bisporus* can produce an enormous biomass of tissue in relatively short time, an output unrivaled by any plant model. For instance, Zhang *et al.*[13] estimated the biomass output of *A. bisporus* to be 5.5-fold greater than that of tobacco. Third, mushrooms are vegetatively propagated, which, unlike plant models, would obviate the need to produce seed for scale up. Here, pharm mushroom strains could be cryopreserved for long-term storage and retrieved on demand for unlimited vegetative increase. Fourth, the relatively short ca. 30-day production cycle from spawning to harvesting would offer the flexibility and responsiveness desired in a commercial production scheme. This cycle could be reduced to ca. 16 days by using "grain-fed mushrooms" in which fructification occurs directly on the grain spawn with other enhancing amendments (Romaine et al., unpubl.). The cost of a grain-based substrate is far too impractical for mushrooms grown as food, but a cost analysis might prove otherwise where biopharmaceuticals are concerned. Moreover, circumventing the environmental regulatory and public concern issues besieging mushroom composting might be well worth the added cost. Fifth, a facile gene transfer system is available that can generate new pharm mushroom strains in a matter of a few months. Unlike plants, transgenesis in the mushroom does not require an elaborate and lengthy tissue differentiation scheme. Sixth, a mushroom platform would offer high human safety with regard to pathogenic contaminants and allergens. Finally, there is every reason to expect proteins manufactured in mushrooms to be more humanlike than those produced in plants.[57] This is relevant as some proteins of pharmaceutical interest normally undergo co- or post-translationally decoration with sugar moieties (glycosylation). If the glycosylation pattern is not sufficiently humanlike, then the protein will be cleared rapidly from the bloodstream with little therapeutic benefit. Though the glycosylation patterns of mushroom-made proteins are still unknown, they are predicted to resemble those

of animals, given the close proximity of fungi and animals evolutionarily[58] and what is already known about fungal proteins.[57, 59] There also exists the possibility of humanizing the glycosylation pathway in *A. bisporus* as has been done in the yeast *Pichia pastoris*[60] or glycosylating recombinant proteins *in vitro*.[61]
In the future, biotech mushroom strains with genuinely novel and obviously improved traits will change the landscape of the commercial industry with new options for solving problems, simplifying tasks, and increasing the efficiency of production. Additionally, *A. bisporus* shows enormous potential as a platform for the production of cheaper and safer, and quite possibly more potent, therapeutic proteins. As Agro-transformation becomes routine for a greater number of fungi, mushroom species as a group, owing to their extraordinarily high biomass capacities and versatile metabolisms, will come to occupy pivotal positions in an array of industrial applications and processes.

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