BREEDING A **BETTER MUSHROOM**

Ever since Gregor Mendel started growing sweet peas in the monastery garden in 1857, we have come to understand that the traits of parents are combined in their offspring. This is the basis of breeding programs for everything from apples to zucchini. Creating a hybrid is relatively easy to manage when cross pollinating flowers; you simply add the pollen of one flower to the stigma (female part) of the other. But how do you 'cross pollinate' a mushroom?

Dr Jenny Ekman investigates.

Breeding new mushroom varieties 101

Breeding mushrooms presents significant challenges. Improvements in quality and productivity have largely been achieved through better growing techniques, with breeding playing only a minor role.

Agaricus are a member of the basidiomycetes. One of this family's characteristics is that every mycelial cell contains two different nuclei. Each of these nuclei are haploid, meaning that they contain only half of the total fungal genetic information. The result is a self-fertile "**hetero**karyote" capable of forming harvestable mushrooms.

Structures called basidia form within the gills of the fruiting body. In a process called meiosis, the two separate nuclei fuse inside the gill cells and then divide to form two copies of each set of genetic material. These four sets of genetic material are distributed to individual spores that bud off from the basidia.

In plant cells, when the nuclei fuse during meiosis the DNA mixes substantially. In Agaricus, however, this mixing is highly restricted so that the parental traits remain largely intact. This means that when the spores germinate, most produce heterokaryotic mycelia (Figure 1), that are virtually genetically identical to the parent material.

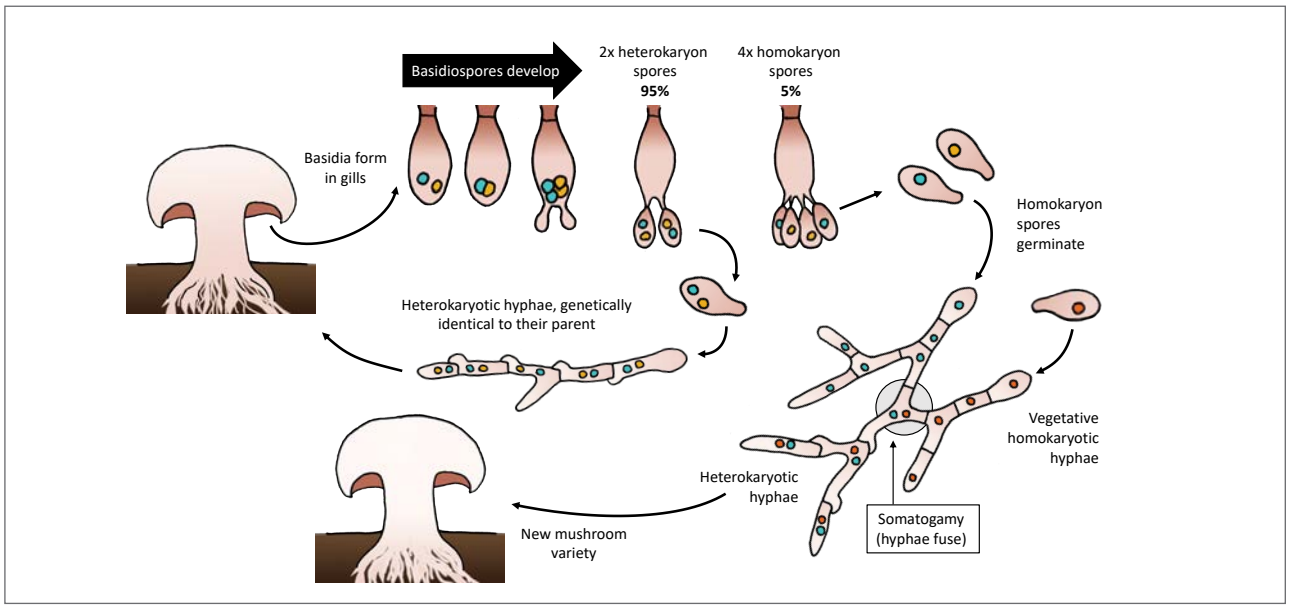

Figure 1. Method used to breed new mushroom varieties.

However, about 5% of basidia (and perhaps less) form (**homo**karyon) spores that contain only a single nucleus[1].

When **homo**karyon spores germinate they form vegetative hyphae with only half the fungus's normal genetic material. These hyphae are relatively slow growing and may be short lived, being unable to generate fruiting bodies.

When two genetically different types of **homo**karyotic hyphae contact each other they can fuse. This process is called somatogamy.

This is the process breeders conventionally use to develop new mushroom cultivars; they generate cultures from single spores, identify those which are **homo**karyons, then grow them with other cultures in the hope they will combine, forming new, **hetero**karyotic hyphae with two nuclei in each cell. Adding to the difficulty, not all homokaryon strains are compatible and able to combine.

Homokaryons can sometimes be identified by examining variation in growth habit and appearance from the original strain. Others need to be confirmed through molecular techniques.

As this brief explanation suggests, breeding new strains of *Agaricus* using conventional methods is extremely time consuming and difficult. For example, a research team in India raised 1,642 single spore isolates from parental strains, of which only 36 were homokaryons. They used these to make 253 crosses, of which only 7 were compatible[2].

One way to improve the frequency of fusion between homokaryons is through protoplasting. Protoplasts are created using enzymes that dissolve the walls of individual homokaryon cells, but leaving the cell membrane and contents intact. Protoplasted cells from two different lines can be placed in a pulsed electric field, increasing the likelihood that they will fuse. With time and care, the resulting heterokaryotic cells can re-form their cell wall, and develop normally into hyphae[3].

Is there a better way to breed mushrooms?

The development of CRISPR (clustered regularly interspaced short palindromic repeats) is currently revolutionising plant breeding. CRISPR is a tool for biotech breeding that differs from older style Genetically Modified Organisms (GMOs) in that no new genes are introduced. Instead, CRISPR uses a combination of a protein and an RNA that together act like molecular scissors. These can be targeted to precise locations in the DNA, selectively removing or altering parts of the genome.

Essentially, CRISPR allows the breeder to use their knowledge of the mushroom genome and the function of the encoded proteins to achieve the same results as conventional breeding. That is, to improve the characteristics of mushrooms.

The Australian Gene Technology Regulator has determined that novel varieties produced using CRISPR where no new DNA is inserted resemble plants or fungi produced using the traditional breeding techniques that rely on natural or induced mutations. CRISPR can, therefore, be used to produce improved plants and mushrooms that are NOT considered "GMOs".

In contrast, plant varieties like "Roundup Ready" soybeans have had a new section of DNA inserted into the genome to reduce the effects of the herbicide. Such GMO varieties are regulated and must be produced under licence.

In 2016 it was announced that CRISPR had been used to produce a new, non-browning mushroom variety. Professor Yinong Yang, a plant pathologist at Penn State's College of Agricultural Sciences, claimed to have successfully deleted genes responsible for browning. The result was stated to be a mushroom that could potentially be mechanically harvested and had increased shelf life.

Unfortunately, the work has never been published in the scientific literature and no commercial variety has yet emerged. Further investigation has suggested that Professor Yang's approach resulted in strains that lacked important commercial attributes.

However, new work now being undertaken at the University of Sydney could change this.

Australian CRISPRs

Associate Professor Brian Jones is an expert in the use of CRISPR technology in plants and fungi. A molecular biologist who has spent much of his career in Europe, he is now based at Sydney University, where he is focused on finding efficient ways to increase the productivity of food plants and reduce food waste.

Professor Jones has already conducted trials using

CRISPR to develop a non-browning mushrooms. "To tell the truth, it is much more difficult than I initially expected," he comments. "This is because mushrooms are in some ways far more complex than plants."

In order for CRISPR to modify the DNA it first needs to be inserted into the target cells. The first barrier is the cell wall. In plants and fungi, the cell wall is a cross-linked lattice of biomolecules which blocks entry for particles larger than 5 to 20 nm (0.000005 to 0.00002 mm) across. In fungi, most pores are thought to be less than 4 nm across, although some may be up to 30 nm. The delivery vehicle for the CRISPR complex needs to find a way through this unimaginably tiny space to reach the inside of the cell.

The second issue relates to the number of nuclei. Plant and animal cells normally have a single nucleus which holds all of their DNA. In fungi, the two genetically distinct haploid nuclei both need to be reached by the CRISPR complex. Added to this, a single hyphal cell can contain 20 to 40 nuclei, all of which should be transformed for the desired genetic traits to be reliably passed on to new cells.

According to Professor Jones, the answer to this conundrum lies in using nanoparticles. "Positively charged nanoparticles that bind the CRISPR complex can transport it through the cell walls and into the cells where it can reach the nuclei" he explains. "Nanoparticle-CRISPR complexes are now used commonly in animal cells, and we are developing their application to plant and fungal cells."

A third issue, specifically for the postharvest browning trait, is the whole mechanism of browning. Browning occurs when cells are damaged, allowing polyphenol oxidase (PPO) in the cytoplasm to react with phenolic

Changes in browning potential of mushroom skin, stem and gills during maturation, expressed as activity of polyphenol oxidase (PPO) enzymes 2, 3 and 4. Activity ranges from dark green (zero) to dark orange (intense). Adapted from Weijn et al., 2013[4]. Mushroom growth stages from Hammond and Nichols, 1976[5].

compounds normally contained in the cell vacuole. The reaction forms the brown compound melanin. Mushrooms contain no less than six different PPOs, as well as a PPO coenzyme. The main enzymes involved in browning are PPO3 and PPO4 (Figure 2).

And yet, problems still remain: "Unfortunately, deleting the main PPO genes (PPO3 and PPO4) appears have negative effects on mycelium growth. So, we are targeting sequences that will leave these genes intact, but greatly reduce their potential to cause postharvest browning."

So, what happens next?

Developing the tools and techniques to use CRISPR routinely in *Agaricus* breeding is clearly challenging, so a team is needed.

Here we introduce University of Sydney PhD candidate Samali Welgamage. Ms Welgamage recently commenced a PhD through the University of Sydney, focussed on improving mushroom quality and storage life.

She is planning to work on progressing CRISPR transformation of mushrooms as part of her studies. Samali plans to use nanomaterials to deliver either pre-synthesised CRISPR ribonucleoprotein (RNP) complexes or non-integrative CRISPR vectors to fungal cells. She will then test the extent to which CIRSPR has altered the targeted genes, and whether the trait is uniformly and reliably integrated into the novel mycelium and fruiting bodies.

Mushroom mycelia, as viewed using confocal laser scanning microscopy. Individual cells are visible, as well as the nucleii inside them. Image by S. Welgamage.

Hort Innovation Strategic levy investment | MUSHROOM FUND

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1 Sonnenberg A. et al. 2016. A detailed analysis of the recombination landscape of the button mushroom *Agaricus bisporus* var. *bisporus*. Fungal Genetics and Biology. 93:35-45.

2 Manju S, Suman BC, Dharmesh G. 2017. Development of *Agaricus bisporus* hybrids and their evaluation for higher yield. Indian J. Hort. 73:550-556.

3 Sonnenburg ASM, Wessels JGH. 1987. Heterokaryon formation in the basidiomycete Schizophyllum commune by electrofusion of protoplasts. Theor. Appl. Genetics. 74:654-658.

4 Weijn A et al. 2013. Melanin biosynthesis pathway in Agaricus bisporus mushrooms. Fungal Genetic Biology. 55:42-53.

5 Hammond JBW, Nichols RY. 1976. Carbohydrte metabolism in Agaricus bisporus (Lange) Sing: Changes in soluble carbohydrates during growth of mycelium and sporophore. Microbiology. 93: 309-320.

PhD candidate Samali Welgamage operating the CLS microscope at the University of Sydney

Profile - Samali Welgamage

Originally from Sri Lanka, Ms Welgamage has settled in Australia and already achieved permanent residency. With a great drive to succeed in her chosen field of postharvest horticulture, she has worked at both the University of Sydney and Applied Horticultural Research over the last 12 months. At AHR Ms Welgamage conducted molecular tests for disease on hundreds of samples of mushrooms, compost, and even straw – you may have seen her in a previous issue of this journal, hard at work on our PCR machine.

This sparked an interest in all things mushroom. She has now taken on the major challenge of a PhD. Titled "Strategies to reduce browning and extend shelf life of *Agaricus* bisporus mushrooms", the project is a wide-ranging examination of the technologies and techniques proposed as ways to reduce browning and senescence.

Not wanting to rely on one strategy alone, Ms Welgamage has an ambitious, multifaceted research plan for the next three years.

In addition to the CRISPR research already described, her tasks include:

Developing methods to increase calcium content of mushrooms

Irrigation with water containing calcium chloride ($CaCl_2$) has been demonstrated to improve firmness and shelf life of button mushrooms. However, $CaCl_2$ is not a registered treatment for mushrooms. Moreover, it increases salt deposits that can block irrigation nozzles. Ms Welgamage will examine other ways of increasing calcium levels in mushrooms, including supplementation of compost and casing and alternative forms of calcium fertigation.

Examining the effect of ethylene on mushroom quality

Mushrooms produce only minor amounts of ethylene and are not usually considered very ethylene sensitive. Despite this, there have been published reports that the ethylene blocker 1-methylcyclopropene (1-MCP, marketed as SmartFresh) can delay senescence and browning. 1-MCP is already widely used on fruit and vegetables and can be applied by fumigating overnight or including a sachet in packed cartons. Ms Welgamage plans to investigate whether treating with 1-MCP can increase mushroom storage life and quality.

Increasing anti-oxidant compounds in mushrooms

Mushrooms contain natural anti-oxidant compounds that reduce browning. This may be one reason first flush mushrooms often resist bruising better than second and third flush. Ms Welgamage plans to test a range of plant defence elicitors as well as products that will increase calcium content, to determine their effects on postharvest browning.

Microscopy

What better way to understand what is happening during mushroom senescence than looking inside the cells? Ms Welgamage recently trained in the use of confocal laser scanning microscopy (CLSM) at the Australian Centre for Microscopy and Microanalysis located at the University of Sydney. CLSM is an optical imaging technology that uses lasers to capture two-dimensional images on the surface of or deep within a tissue sample. Ms Welgamageis currently using the CLSM to examine the structure and cell contents of different types of mycelium. She can then compare what is happening inside the cells with changes we can see on the outside.