Superoxide Dismutase - Mushrooms under Stress!

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Abstract: Competitiveness of the UK mushroom industry is reliant on high-quality mushroom production. Currently, there is a lack of information on the physiological changes and biochemical reactions contributing to quality loss post-harvest and their points of regulation. Recently, a library of genes up-regulated during senescence has been produced and a stress-adaptation genes identified. Both the cDNA and the genomic DNA for superoxide dismutase have been fully sequenced and comparisons of the derived protein sequence with databases confirm its identity with very high probability. Expression studies have been carried out to examine levels of gene transcripts over time (post-harvest) and in different tissues. Here we discuss the advantage to a harvested mushroom of having greatly elevated transcript levels of superoxide dismutase is to be able to potentially able to respond rapidly to potentially lethal levels of superoxide radicals and the significance of these observations for improved post-harvest quality.

Key words: *Agaricus bisporus*, superoxide dismutase, oxidative stress, post-harvest quality

1 Introduction

Stress affects all organisms and responses to stress have been shown to be controlled at the molecular level. In *Agaricus bisporus*, detachment of the mushroom and post-harvest storage are likely to induce stress. Following harvest, the mushroom continues to develop though is subject to a number of stresses besides wounding including nutritional and water deprivation. This response to water and nutrient limitation in the harvested sporophore has been termed a "post harvest stress disorder".[1, 2] Post-harvest conditions are associated with a number of physiological, molecular and biochemical changes which affect consumer quality. Harvesting is itself a wounding event that is accompanied by a massive disruption in metabolism. The isolated sporophore continues to develop similarly to the non-harvested fruit body.[3] As the mushroom matures during storage, the cap expands, gill formation occurs and spores are produced and released.[4] Detached sporophores continue to respire following harvest.[5] During this time, there are compositional changes in resources form the stipe to the cap and gill tissues.[6] Consumer expectation of high quality mushrooms can be defined by a white colour, firm texture, uniform maturity and good flavour.[7] In order to maintain or improve on any or all of these key characteristics, it essential to fully understand the biochemistry and molecular biology of the harvested sporohore.

Superoxide dismutase is associated with stress tolerance and the gene encoding this sequence has been shown to be up-regulated in post-harvest sporophores.[8] It catalyses the reaction of superoxide radicals with protons to produce hydrogen peroxide which is highly reactive and potentially damaging.[9] The superoxide dismutase gene family is a broad family, all requiring metal cofactors for activity. There are two evolutionary distinct superoxide gene families - the manganese or iron type, and the copper/zinc family.[10] The different sub-types predominate in different compartments of the cell. In *A. bisporus*, superoxide dismutase belongs to the iron/manganese family and is usually located in the mitochondria.[11] Superoxide dismutase is involved in the enzymic dismutation of superoxide in which the superoxide radical combines with two hydrogen ions to give oxygen and hydrogen peroxide. Superoxide radicals are highly reactive, non-reduced forms of oxygen that have been shown to cause severe damage to DNA, lipids and proteins.[12] It has been suggested that superoxide

dismutase is required to protect aerobic organisms from the damaging and lethal effects of reactive oxygen species, superoxide, which is produced in large amounts during oxidative stress. Superoxide dismutases have been reported in a number of aerobic organisms.[13] Differential screening and targeted gene cloning in *A. bisporous* has identified 20 genes with increased transcription levels two days post-harvest compared to freshly harvested mushroom.[8, 14] Both the cDNA and the genomic DNA for superoxide dismutase has been fully sequenced and comparisons of the derived protein sequence with databases confirm its identity with very high probability. Initial studies examining gene expression by Northern analysis suggest that this gene may be up-regulated after harvest. The aim of this study is to further characterise the spatial and temporal expression profile of this gene post-harvest and to examine whether differences exist in transcription levels between different tissues of the mushroom. Northern analysis will be used to study expression levels of superoxide dismutase over time and in different tissues of the mushroom.

2 Materials and Methods

2.1 Strains

A. bisporus strain A15 (Sylvan, UK), was grown at Warwick HRI, Wellesbourne, according to commercial practice. During cropping, sporophores are produced in synchronous weekly flushes; mushrooms used in experiments were harvested from the second flush.
Harvested mushrooms were either placed directly in liquid nitrogen (termed day 0), or stored for up to 5 days in a controlled environment at 18°C and 90-95% relative humidity, before freezing under liquid nitrogen. Samples were taken from stored mushrooms at a) three hourly intervals for 24 hours, b) 24 hourly intervals for 5 days, and c) 24 hourly intervals for 2 days, where mushrooms were also dissected into stipe, cap and gill tissue. Frozen samples were stored at -80°C.

2.2 RNA isolation

Mushroom tissues were ground to a fine powder with a pestle and mortar under liquid nitrogen. RNA from ground tissues was isolated using a phenol / chloroform extraction protocol.[15] Absorbance measurements at 260 and 280 nm were used to assess RNA concentration and purity. RNA integrity was determined by formaldehyde agarose gel electrophoresis.[16] For reverse transcriptase and Q-PCR (TaqMan™), RNA samples were treated with RNAse-free DNAse enzyme according to the manufacturer's instructions (Promega, UK).

2.3 Northern analysis

Total RNA (20 µg) from each sample was separated by agarose gel electrophoresis prior to Northern blot analysis.[15] Following electrophoresis, RNA was transferred onto Hybond-NT nitrocellulose membrane (Amersham International, UK) using capillary blotting according to standard techniques.[15] Hybridisation was carried out with ^{32}P-labelled cDNA gene from superoxide dismutase. *Agaricus bisporus* 28SrRNA gene was used as a loading control as described previously.[8]

3 Results

Northern analysis of gene expression profiles in *A. bisporus* has suggested that the superoxide dismutase gene was being switched on post-harvest. Transcript levels in mushroom samples and tissues have been examined at various time points post-harvest to further discriminate between changes occurring over time:

3.1 Transcript analysis

3.1.1 Gene expression of superoxide dismutase over 5 days post-harvest

Figure 1 shows the pattern of expression observed for superoxide dismutase over five days after harvest. Transcript levels of superoxide dismutase showed increased expression one day after harvest. Transcript levels appear to decline after two days of storage.

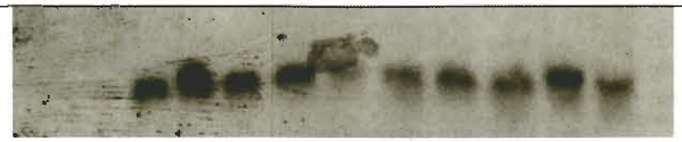
Gene	Days Following harvest
	0 0 1 1 2 2 3 3 4 4 5 5
Superoxide dismutase	

Figure 1. Northern expression analysis of the superoxide dismutase gene over a five-day period post-harvest

3.1.2 Detection of up-regulated gene expression of superoxide dismutase in harvested mushroom at 3 hourly intervals for 24 hrs

In Figure 2, superoxide dismutase transcript levels were very low at hour 0, levels increased slightly between hours 3, 6, 9 and 12, but then at hour 15 a larger increase in expression was observed, after which the level of expression remained constant.

Gene	Hours Following harvest
	0 3 6 9 12 15 18 21 24
Superoxide dismutase	

Figure 2. Northern expression analysis of the superoxide dismutase gene over a 24 hour period post harvest, with 3 hourly sampling regime

3.1.3 Expression of superoxide dismutase in stipe, cap and gill tissues over a 48 hour period post- harvest

As expression of superoxide dismutase gene was detected in mushrooms after 48 hrs, followed by a reduction or very slight increase in signal, analysis of gene expression in the different mushroom tissues focussed on days 0, 1 and 2. The results are shown in Figure 3 where transcription levels of superoxide dismutase increased in all harvested tissues of the mushroom following 48 hours post-harvest.
In stipe tissue, high levels of superoxide dismutase gene transcript levels were detected. Superoxide dismutase gene was up regulated following harvest. In cap tissue, expression of superoxide dismutase was at low levels in day 0 but increased one day following post-harvest, remaining constant at day 2. Transcript levels of the superoxide dismutase gene were detected equally between day 0 and day 1 in gill tissue with possibly a slight increase of signal at day 2 (Figure 3).

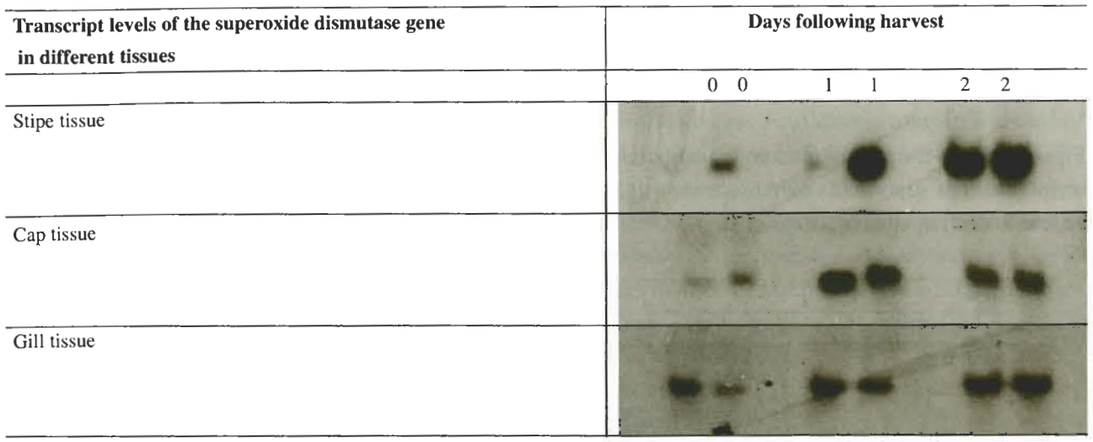


Figure 3. Northern expression analysis of the superoxide dismutase gene in different tissues of the mushroom over a 48 hour period post-harvest

4 Discussion

Post-harvest, the superoxide dismutase gene is up-regulated in *A. bisporus* sporophores. The role of superoxide dismutase is to act as a free radical scavenger preventing oxidative damage. Stresses facing plant and fungi can be classed as abiotic or biotic. Abiotic stresses include heat, chilling, freezing, light, salinity, drought and ozone whereas biotic stresses are those imposed by wounding, pests and pathogens. Most abiotic and biotic stresses lead directly or indirectly to the production of free radicals and reactive oxygen species. This can be classed as oxidative stress. The secondary effects of abiotic stress is the production of reactive oxygen species. Superoxide dismutase is involved in the dismutation of superoxide radicals to produce reactive oxygen species which can be damaging to cellular function and membranes. *A. bisporus* possesses an iron/manganese-type superoxide dismutase gene which is up-regulated following harvest.

Analysis of transcript levels of superoxide dismutase showed that they are present in all sporophore tissue over long-term post-harvest storage, with levels peaking at day 1. Thereafter, levels of superoxide dismutase decrease. When examining transcript levels for the superoxide dismutase gene with a finer time discrimination, further trends occur. Three hours after sporophore harvest, the transcript levels increase. Transcription increased again between 12 and 15 hours. However, transcripts for superoxide dismutase were found in all tissues of the sporophore. Expression levels were greater in stipe tissue than in cap or gill tissue 48 hours post-harvest. There is an advantage to a harvested mushroom of having greatly elevated transcript levels of superoxide dismutase in that it is able to respond rapidly to a potentially lethal level of superoxide radicals. Overexpression of Mn-superoxide dismutase and Fe-superoxide dismutase in transgenic alfalfa has been shown to significantly increase yield and tolerance to abiotic stress.[17, 18] Forty eight hours post-harvest the mushroom is still developing though deprived of nutrients and water. Indeed de Grey[19] has reported substantial extension of maximum life span in nematodes where levels of antioxidant enzymes have been increased.

Further work of particular interest will be the effect on expression of superoxide dismutase under certain stress conditions such as freezing, high temperature, water stress and damage. Transcription levels of the superoxide dismutase gene will be analysed using Quantitative Polymerase Chain Reaction (Q-PCR), a powerful and sensitive gene analysis technique that can provide more detailed information than that from conventional Northern analysis. The method involves monitoring fluorescence from a fluorochrome when intercalated into double-stranded DNA.[20] This technique can provide more detailed information on changes in transcription otherwise undetectable by conventional Northern analysis and will be used to study expression levels of superoxide dismutase over time and in different tissues of the mushroom subjected to a variety of stresses. Understanding

the molecular response to stress will contribute important information leading to improved post-harvest quality in the edible mushroom.

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