Unravelling the bruising-discoloration of *Agaricus bisporus*, the button mushroom

Amrah Weijn

Thesis committee

Promotor
Prof. dr. H.J. Wichers
Professor of Immunomodulation by Food
Wageningen University

Co-promotor
Dr. J.J. Mes
Senior Scientist at Food & Biobased Research
Wageningen University

Other members
Prof. dr. W.J.H. van Berkel, Wageningen University
Dr. M.P. Wach, Sylvan Research, Kittanning, USA
Prof. dr. H.A.B. Wösten, Utrecht University
Prof. dr. ing. E.J. Woltering, Wageningen University

This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

Unravelling the bruising-discoloration of *Agaricus bisporus*, the button mushroom

Amrah Weijn

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr. M.J. Kropff
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 14 June 2013
at 1.30 p.m. in the Aula.

Amrah Weijn
Unravelling the bruising-discoloration of *Agaricus bisporus*, the button mushroom

PhD Thesis, Wageningen University, Wageningen, NL (2013)
With references, with summaries in English and Dutch

ISBN 978-94-6173-563-8

Contents

Chapter 1

General Introduction

1 Introduction

The Netherlands are the fourth producer of the white button mushroom over the world (Ministerie van Landbouw, 2010). China is the largest producer of mushrooms with an average production of approximately 2.7 million ton per year from 1992 until 2010, followed by the USA (0.37 million ton) and the Netherlands (0.24 million ton) (mushroom and truffles, http://faostat3.fao.org/home/index.html #VISUALIZE). In 2012 there were 131 mushroom producing companies in the Netherlands, this is 70 % less then in 2000 (546 companies) (http://www.cbs.nl/nl-NL/menu/themas/landbouw/publicaties/artikelen/archief/2012/2012-champignons-2012-art.htm). Also the cultivation area decreased since 2000 with 30 % to 665,000 m^2 in 2012. Due to production of mushrooms in European countries with lower labour costs the mushroom market in the Netherlands is challenged. In 2010 it was calculated that the production costs for a mushroom grower for the fresh market were 1.31 euro per kg mushrooms in the Netherlands, in the United Kingdom this was 1.14 euro per kg and in Poland 0.97 euro per kg (Visie Nederlandse Champignon Versmarkt, 2010). Labour costs, which are part of the production costs, can be lowered by automatic harvesting. A full automation of harvest is technologically feasible but requires adaptation of the present production system. Next to this, the critical success factor is the availability of strains that are less sensitive to mechanical damage. Contact-based discoloration, or bruising, is caused by a mechanical process known as ‘slip-shear’ (Burton, 2004), a downwards force and a sideways movement. This bruising based discoloration of mushrooms can occur during picking by hand or by robotic picking equipment and leads to lowering of quality. Mechanical harvesting is more cost-efficient than picking by hand, but cannot be applied yet to serve the fresh market, as commercial strains are too sensitive to bruising. When strains with less sensitivity to bruising are available an increase in quality for both the fresh and the preserved mushroom industry is possible.

2 Button mushroom cultivation and harvesting

The button mushroom *Agaricus bisporus* is grown indoors in large tunnels. Six steps are involved in mushroom growth (Royse and Beelman, n.d.). The first step is the production of mushroom compost which lasts around 6-14 days. The substrate for the cultivation of mushrooms is horse manure compost, which consists of a mixture of horse manure, some broiler chicken manure, and water, to which gypsum is added for structural stability and for stabilizing the pH. Alternatively, synthetic compost can be made which is based on wheat straw and broiler chicken manure (van Griensven and van Roestel, 2004). The second step of composting is pasteurization to kill any insects, nematodes, pest fungi, or other pests that may be present in the compost. At the same time it is necessary to condition the compost and remove the ammonia that was formed during the first step. The third step in mushroom growing is mixing the compost with spawn. Spawn consists of hydrated and sterilized grains colonized with a pure culture of mushroom mycelium. Mycelium is thin thread-like cells which can grow vegetatively from germinated spores. It requires around 13-20 days to obtain fully colonized compost. At step four, a top soil layer, called casing, is applied to the spawn-run compost. On top of the casing layer, mushrooms will form. Step five of mushroom growing is the pinning phase, in which tiny mushrooms are formed starting from a pin. The sixth step is called cropping or harvesting. Mushrooms are harvested over a 2-4 day period in a 7-10 day cycle called flushes or breaks (Beyer, 2003).

The complete life cycle of *A. bisporus* was unravelled in the early seventies of the previous century (Figure 1) (Sonnenberg *et al.*, 2011). After an apparently normal meiosis, predominantly bisporic basidia are produced, each containing two non-sister post meiotic nuclei. Upon germination these spores generate heterokaryotic mycelia. Only spores from the rare four spored basidia contain one haploid nucleus and can be used to generate hybrids in breeding programs. This

four-spored variety was found in the Sonoran desert of California and is named *Agaricus bisporus* var. *burnettii* (Callac *et al.*, 1993).

Button mushrooms consist of different tissue types, which develop during the growth of the mushroom (Figure 2 and 3). The outer layer of the cap is the skin, which covers the flesh. The gills contain the spores and are protected by the velum in early developmental stages. The mushroom cap is formed on a stem, which is connected via the mycelium to the compost. Figure 3 shows the different developmental stages of the button mushroom defined from stage 1 until stage 7 (Hammond and Nichols, 1975; Hammond and Nichols, 1976). The button mushroom is usually harvested for the market at developmental stage 2 or 3, while the larger Portobello's are harvested at a more mature stage.

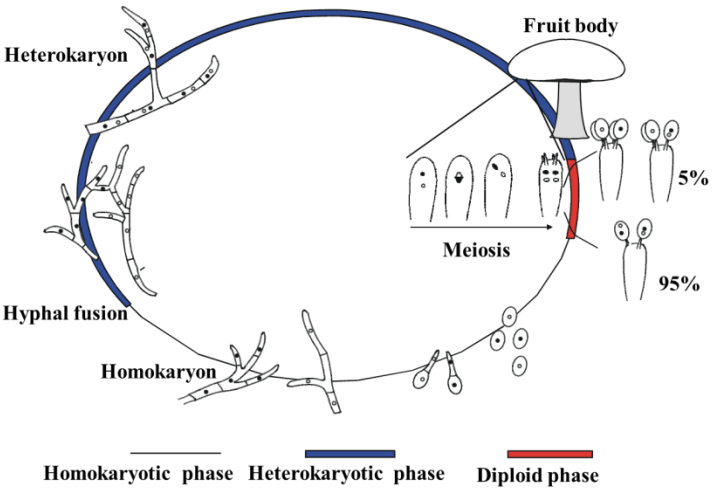


Figure 1. Life cycle of *Agaricus bisporus* Retrieved from Sonnenberg *et al.* (2011).

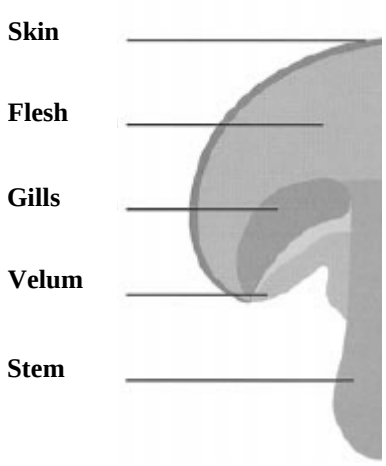


Figure 2. Tissues of button mushroom *Agaricus bisporus*.
Adapted from van Leeuwen and Wichers (1999).

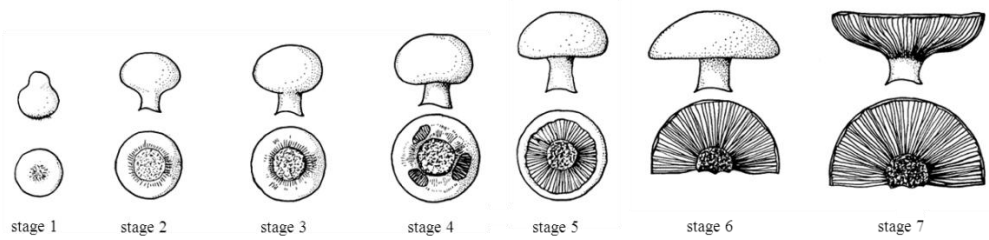


Figure 3. Development stages of button mushroom Adapted from Hammond and Nichols (1975; 1976).
Stage 1: pinhead, is characterized by undifferentiated velum (diameter of the cap is < 5 mm).
Stage 2: button, is characterized by visible and intact but not stretched velum (diameter of the cap is 20-30 mm).
Stage 3: closed cup, the velum is stretched but still intact (diameter is 30-40 mm).
Stage 4: cup, velum starting to tear (diameter 30-40 mm).
Stage 5: cup, velum torn, cap still cup shaped, gills clearly visible (diameter 30-50 mm).
Stage 6: flat, gill surface flat or slightly concave (diameter is 40-60 mm).
Stage 7: flat, the gill surface curving upwards (diameter is 50-70 mm).

3 Mushroom bruising and discoloration

3.1 Methods to analyse discoloration and bruising-sensitivity

Discoloration can be measured with a chromameter, which is a tool for precise and objective assessment of surface colour (Muizzuddin *et al.*, 1990). The chromameter colour measurements are given in the CIE L*a*b* colour space (http://www.hunterlab.com/appnotes/an07_96a.pdf). L* stands for lightness, which is measured from 0 (black) to 100 (white). Positive a* values stand for red colour, negative a* values for green. Positive b* values stand for yellow colour, negative b* values for blue.

Several studies used a mechanical shaker to mimic mechanical damage. Burton and Noble (1993) used a polystyrene shaking box which oscillated horizontally to bruise mushrooms. Bruising of mushrooms was caused largely by rolling along the base of the box, but also by collisions with the sides of the box or with other mushrooms. The bruising treatment applied caused discoloration of the mushrooms which was equivalent to approximately seven days of storage at 5 °C or two days of storage at 18 °C without bruising. Noble *et al.* (1997) used the same technique as Burton and Noble (1993) to bruise mushrooms and found that the sides of the mushrooms showed a higher degree of discoloration than on the top of the mushroom cap. A Gyrotory G2 shaker (New Brunswick Scientifiuc Co. USA) was used at 300 rpm to damage mushroom during a controlled period of time by Esquerre *et al.* (2009), Gaston *et al.* (2010) and O'Gorman *et al.* (2010). Gaston *et al*. (2010) stated that a shaking period of 10 min led to loss of 6 units of lightness (L*) and a shaking period of 20 min led to a loss of 12 units of L*. Mushrooms can be bruised on the top of the cap with the bruisometer developed by Burton (2004) (Figure 4). The damage exerted on the top of the cap is controlled by the bruisometer. To use the bruisometer the mushroom has to be dissected into three parts, this probably can already trigger metabolic pathways in the mushroom and is laborious when analysing many mushrooms for bruising-sensitivity.

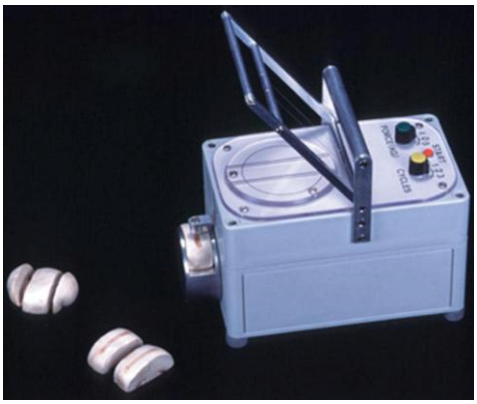


Figure 4. Bruisometer.
Retrieved from http://www.teagasc.ie/publications/2011/1056/Kerry_Burton.pdf

3.2 Factors that influence bruising-sensitivity

A. bisporus button mushroom quality is determined by colour, texture, cleanliness, maturity, flush number and flavour. Of these, colour is the most important parameter because it is first perceived by consumers and discoloration decreases the commercial value (Burton, 2004). Mushrooms are prone to develop brown discoloration due to bruising of the mushrooms as a result of mechanical damage during harvest, through fruit senescence or by microbial infections (Jolivet *et al.*, 1998). It has been shown that enzymatic browning of mushrooms is caused by polyphenol oxidases (PPOs: tyrosinases and laccases) and peroxidases through an enzyme-catalysed oxidation of phenolic substrates into quinones (Jolivet *et al.*, 1998). The quinones undergo further oxidative polymerization reactions leading to melanin: high molecular mass dark brown or black pigments (Fogarty and Tobin, 1996).

Mushroom quality is influenced by many factors including the strain used, flush number, compost, and environmental factors. The choice of strain can have a major effect on bruising-related discoloration (Burton, 2004). Research includes most of the time only one or several strains, but differences in discoloration

between strains are found (see further on in the introduction and this thesis). Bartley *et al*. (1991) found that mushrooms from the second flush, prior to harvest and during postharvest storage, are in general whiter than first flush mushrooms. These first flush mushrooms in turn discolour more slowly than third flush mushrooms. Burton and Noble (1993) concluded the same for mushrooms stored at 5 °C, which were bruised in a polystyrene shaking box which oscillated horizontally. Mohapatra *et al*. (2010) analysed the discoloration of the mushrooms upon storage and found that mushrooms stored at 5 °C for five days produced a higher browning index different from the control at 3.5 °C.

Burton (2004) tested discoloration of mushrooms grown on three different types of compost. The mushrooms grown on the less degraded, straw-like compost were slightly but significantly less discoloured. Burton concluded that the major factors influencing mushroom bruising relate to water such as casing water, humidity and to a lesser extent calcium chloride. To reduce bruising-related discoloration, mushrooms should be grown wet (casing water and humidity) and allowed to dry out somewhat towards the end of the crop harvest. If bruising is a frequent problem, then calcium chloride irrigation (0.3-0.5 %) can effectively reduce the problem. The compost parameters 'degree of straw degradation' and 'amount of sugar beet lime in the casing' had a minor effect on bruising-related discoloration. Miklus and Beelman (1996) also tested the effect on cap colour by the addition of 28 mM calcium chloride to tap water for the irrigation of button mushrooms. This calcium chloride addition significantly improved the colour of mushrooms at harvest without reducing crop yield. When compared to Burton (2004), 28 mM calcium chloride (0.31 % w/v) is in the same dosing range. Noble *et al*. (1997) analysed the effect of casing layer depth on the sensitivity of mushrooms to bruising. Mushroom grown on a casing layer of 25 mm deep showed more discoloration as a result of mechanical damage than mushrooms grown on 40 or 55 mm casing, which gave similar results.

3.3 Biochemical changes involved in mushroom discoloration

Discoloration of button mushrooms is a common phenomenon which decreases their commercial value. Due to picking, handling and storage, discoloration reactions are initiated, which are considered to be mediated by enzyme-catalysed oxidation of phenolic substrates into quinones, leading to the formation of brown coloured melanin (Burton, 2004; Jolivet *et al.*, 1998). Microbial infections can also cause brown discoloration and lead to decreased yield and economic losses of the crop. For instance, *Pseudomonas tolaasii* is the causal agent of brown blotch disease (Jolivet *et al.*, 1998), and *Lecanicillium fungicola* is a fungal pathogen that can cause dry bubble disease on button mushrooms (Foulongne-Oriol *et al.*, 2012). In both diseases, brown discoloration is part of the symptoms.

Different fungal taxonomic classes form melanin from different mono- and diphenolic precursors. DOPA-melanin is formed from tyrosine via 3,4-dihydroxyphenylalanine (L-DOPA) as intermediate phenolic compound. In the group of basidiomycotina, melanin is mostly derived from γ-L-glutaminyl-3,4-dihydroxybenzene (GDHB) or catechol, as the immediate phenolic precursor of the melanin polymer (Bell and Wheeler, 1986). Melanin in cell walls of basidiospores of *A. bisporus* is synthesized from γ-L-glutaminyl-4-hydroxybenzene (GHB) (Bell and Wheeler, 1986). Catechol melanin is formed by the oxidation of catechol by PPO or through free radicals or quinone-catechol adducts (Bell and Wheeler, 1986). Catechol has been identified in mushroom cap tissue (Paranjpe *et al.*, 1978). In ascomycotina and related deuteromycotina melanins are generally synthesized from the pentaketide pathway in which 1,8-dihydroxynaphthalene (DHN) melanin is formed, starting from the precursors acetyl-CoA or malonyl-CoA (Eisenman and Casadevall, 2012). Another type of melanin that can be formed is PAP-melanin occurring from *p*-aminophenol (PAP) (Jolivet *et al.*, 1998).

The melanin forming pathway for *A. bisporus* starts from chorismate, derived from the shikimate pathway (Dewick, 1998). Different enzymes convert

chorismate into the different mono- and diphenolic compounds like the above mentioned GHB, PAP and L-DOPA. Subsequent enzymatic and spontaneous oxidative polymerization steps convert these substrates in GHB-, PAP- and DOPA-melanin, respectively (Jolivet *et al*., 1995). The melanin biosynthesis pathway can also result in other compounds than melanin as L-phenylalanine can branch to the formation of compounds such a flavonoids (Calla *et al.*, 2009).

GHB, a phenolic amino acid characteristic of the *Agaricus* genus, was first discovered in *Agaricus hortensis* (Jolivet *et al*., 1999). GHB is formed from shikimic acid. Shikimate becomes aminated at the 4-position during *p*-aminobenzoic acid biosynthesis (Tsuji *et al.*, 1981). The latter metabolite then undergoes decarboxylation and hydroxylation by a FAD-dependent monooxygenase, 4-aminobenzoate hydroxylase (4ABH), to form *p*-aminophenol. Subsequently, γ-glutamyltransferase (GGT, EC 2.3.2.2) transfers the glutamyl part to *p*-aminophenol to give GHB. In the presence of PPO and oxygen, GHB is readily oxidized into the corresponding diphenol (GDHB) and *o*-quinone (GBQ), which polymerizes into melanins (Weaver *et al.*, 1971). Several major phenolic amino acids (tyrosine, GHB, L-DOPA, and GDHB) were isolated and characterized in mushrooms. GBH was found, in every part of the fruiting bodies, at higher concentrations than other phenolic amino acids (Choi and Sapers, 1994). Choi and Sapers (1994) found that the contents of GHB plus tyrosine and GDHB plus L-DOPA (it was impossible to isolate and quantify the compounds separately, due to the overlapping peaks for GHB and tyrosine and for GDHB and L-DOPA) was highest in the gills of dry-packed mushrooms, followed by skin, stipe and cap. Another amino acid specific for the *Agaricus* genus is agaritine (β-N-(γ-L(+)-glutamyl-4-hydroxymethylphenylhydrazine), a naturally occurring phenyl-hydrazine derivative (Roupas *et al.*, 2010). The likely site of agaritine synthesis is the vegetative hyphae in contact with the wheat straw compost (Baumgartner *et al.*, 1998).

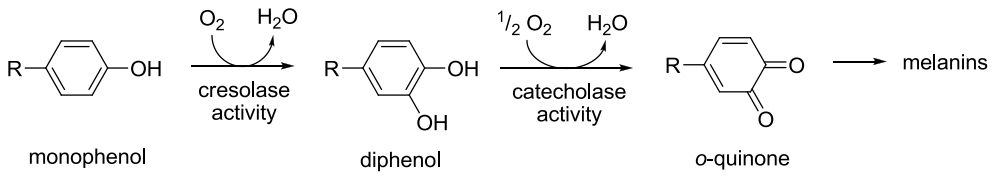


Figure 5. The oxidation of phenolic compounds catalysed by polyphenol oxidase, leading to the production of dark-coloured melanins. Adapted from Marusek *et al.* (2006).

3.4 Tyrosinase (Polyphenol oxidase)

The best studied enzyme from the melanin pathway is tyrosinase, an enzyme involved in the conversion of phenolic compounds (Figure 5). Tyrosinases are copper containing metalloproteins and essential enzymes in melanin biosynthesis (Selinheimo *et al.*, 2007). Tyrosinases are bifunctional enzymes (Selinheimo *et al.*, 2007) and are especially responsible for the first steps of melanin synthesis from L-tyrosine leading to the formation of L-dopaquinone and L-dopachrome (Sanchez-Ferrer *et al.*, 1995). Characteristic for tyrosinases is the catalysis, via *o*-hydroxylation, of monophenols (cresolase or monophenolase activity) and the subsequent oxidation of the resulting *o*-diphenols into reactive *o*-quinones (catecholase or diphenolase activity). Both reactions use molecular oxygen (Figure 5). Tyrosinases can thus accept both mono- and diphenols as substrates (Selinheimo *et al.*, 2007). Subsequently, the *o*-quinones self-polymerize or react with other substances to form, via non-enzymatic reactions, high molecular weight brown or black melanins (Marusek *et al.*, 2006). Enzyme nomenclature differentiates between monophenol oxidase (tyrosinase, EC 1.14.18.1) and catechol oxidase or *o*-diphenol: oxygen oxidoreductase (EC 1.10.3.2), but most often the general term polyphenol oxidase (PPO) is used (Mayer, 2006).

Tyrosinases contain two copper atoms (CuA and CuB) which are coordinated by three conserved histidine residues (Jolivet *et al.*, 1998). Marusek *et al.* (2006) showed that many of the important structural features of the N-terminal domains of

the sweet potato *Ipomoea batatas* catechol oxidase and giant octopus *Octopus dolfeini* hemocyanin appear to be present in PPOs from other plants and fungi. These structural features (Figure 6) are the copper-ligating histidine residues, the thioether bridge (a covalent bridge between the second histidine residue in CuA and a cysteine residue), the gate residue (an amino acid located above the active site of tyrosinase, partially blocking its entrance), the tyrosine motif (Y/FxY/F residues sequence located downstream the CuB site) and the YG motif (conserved residues in fungal tyrosinases). A conserved arginine and aspartate are found both in plant and fungi, which are both bound to the tyrosine motif (Marusek *et al.*, 2006).

```
  1  msliatvgpt ggvknRlniv dfvknekfft lyvrslellq akeqhdyssf fqlagiHglp
 61  ftewakerps mnlykagyCt HgqvlfptwH rtylsvfeqi lqgaaievan kftsnqtdwi
121  qaaqdlrqpy wdwgfelmpp devikneevn itnydgkkis vknpilryhf hpidpsfkpy
181  gdfatwrttv rnpdrnrred ipglikkmrl eegqirekty nmlkfndawe rfsnhgisdd
241  qhanslesvH ddiHvmvgyg kieghmdhFf faafdpifwl hHtnvDrlls lwkainpdvw
301  vtsgrnrdgt mgiapnaqin detplepfyq sedkvwtsas ladtarlgYs Ypdfdklvgg
361  tkelirdaid dliderYGsk pssgarntaf dlladfkgit kehkedlkmy dwtihvafkk
421  felkesfsll fyfasdggdy dqencfvgsi nafrgttpet cancqdnenl iqegfihlnh
481  ylardlesfe pqdvhkflke kglsyklysr edksltslsv kiegrplhlp pgehrpkydh
541  tqdrvvfddv avhvin
```

Figure 6. Structural features of tyrosinase. The amino acid sequence shown is of PPO_2 from JGI (http://genome.jgi-psf.org/Agabi_varbisH97_2/Agabi_varbisH97_2.home.html, version 3.0). The arginine in yellow is involved in the pi-cation interaction with the tyrosine motif, which is shown in red. Histidines in green are of CuA. The thioether bridge is formed between the cysteine in blue and the second histindine residue of CuA. Histidines in pink are of CuB. The gate residue is shown in purple. The aspartate in light brown is involved in a hydrogen bond to the tyrosine motif (red). The YG-motif is shown in grey.

Plant PPOs are synthesized as preproteins and contain an N-terminal transit peptide directing the preprotein to the chloroplast thylakoid lumen (Steffens *et al.*, 1994). Within the lumen of the chloroplast thylakoid, PPOs are soluble or loosely associated with the thylakoid membrane (Marusek *et al.*, 2006). Fungal PPOs are in most cases cytosolic enzymes (Halaouli *et al.*, 2006). Rast *et al.* (2003) found that PPO can be both a membrane-bound and a soluble enzyme. Cell wall extracts were made of *A. bisporus* sporocarps and PPO was identified as being a Class-I (bound via hydrogen or weak ionic bonding), Class-III (bound via strong electrostatic forces) and Class-IV (bound via covalent linkages) wall-associated enzyme. Both plant and fungal PPOs exist as latent (inactive) enzymes and undergo proteolytic cleavage to yield an active form of the enzyme (van Gelder *et al.*, 1997; Whitaker, 1995). Flurkey and Inlow (2008) analysed the proteolytic processing of PPO from plants and fungi. They concluded that processing of plant PPOs is consistent with one of the following scenarios: i) a form of the enzyme from which only the N-terminal transit peptide has been cleaved (this form is likely to be latent) or ii) a form of the enzyme from which the N-terminal transit peptide and a larger C-terminal fragment have been cleaved, the C-terminal fragment being cleaved at a site shortly after the conserved tyrosine motif. Processing of fungal PPOs is consistent with one of the following scenarios: i) a form of the enzyme which has not undergone proteolysis at either end of the polypeptide chain (this form is likely to be latent) or ii) a form of the enzyme from which the C-terminal fragment has been cleaved at a site shortly after the tyrosine motif. In *A. bisporus*, the latent form represented 98-99 % of the total tyrosinase activity (van Leeuwen and Wichers, 1999).

Mushroom tyrosinase is commonly found as a tetrametric protein (H_2L_2) with a molecular mass of 120 kDa, composed of two subunits of 43 kDa (heavy chain) and two subunits of 14 kDa (light chain) (Strothkamp *et al.*, 1976). Ismaya *et al.* (2011) unravelled the crystal structure of *A. bisporus* tyrosinase. They discovered

that the tetramer H_2L_2 is made up of two H-chains of PPO_3 and two L-chains (PPO_3 and the L-chain will be discussed in detail later in the thesis). Other crystal PPO-structures are available e.g. the catechol oxidase from the sweet potato *Ipomoea batatas* (Klabunde *et al.*, 1998) and a bacterial tyrosinase from *Streptomyces castaneoglobisporus* (Matoba *et al.*, 2006). The core structure of the H-chain of *A. bisporus* (PPO_3 amino acid residues 2-392) is similar to the sweet potato catechol oxidase (189 matched residues) and to the tyrosinase domain of *S. castaneoglobisporus* (211 matched residues) (Ismaya *et al.*, 2011). The crystal structure of *S. castaneoglobisporus* is made up from tyrosinase and a so called "caddie" protein. The L-chain of *A. bisporus* does not show any sequence or structural similarity to this caddie protein of *S. castaneoglobisporus*. The function of the L-chain has so far remained unknown.

At the start of this research two polyphenol oxidase genes were already known for *A. bisporus*, namely *PPO_1* and *PPO_2* (Wichers *et al.*, 2003). Wu *et al.* (2010) identified *PPO_3* and *PPO_4* and Li *et al.* (2011) also reported on these two new PPO genes in *A. bisporus*. Based on the full genome sequence of *A. bisporus*, we have identified two additional PPO-encoding sequences, which are named *PPO_5* and *PPO_6*. *PPO_6* has two starting codons and was initially annotated as two different tyrosinases. The PPO gene family is discussed in more detail in Chapters 4 and 6.

3.5 Other enzyme classes involved in discoloration

Phenylalanine ammonia-lyase (EC 4.3.1.5, PAL) is an enzyme that is involved in phenyl propanoid metabolism. PAL is generally known as a wound-induced enzyme, its activity increases due to cell injury provoked during minimal processing, low temperature, or pathogen attack in plants (Dixon and Paiva, 1995). An increase in PAL provokes an increase in the concentration of phenolic compounds, which are substrates for oxidase enzymes such as PPO and peroxidase.

PAL activity increased after wounding in potato cultivars, which is a typical behaviour of the enzyme PAL as a response to this stress (Cantos *et al.*, 2002).

Another important class of enzymes probably involved in brown discoloration are peroxidases (EC 1.11.1.7). Peroxidases are enzymes that use hydrogen peroxide to catalyse the oxidation of a variety of compounds (Conesa *et al.*, 2002; Hofrichter *et al.*, 2010). Peroxidases are classified into three classes: Class-I are intracellular peroxidases, Class-II are extracellular fungal peroxidases, Class-III are extracellular plant peroxidases (Conesa *et al.*, 2002). Peroxidases that do not fit within this classification are the haloperoxidases. Haloperoxidases catalyse the oxidation of halides by H_2O_2 resulting in the halogenation of organic compounds. Known peroxidases of *A. bisporus* are manganese peroxidase (Lankinen *et al.*, 2005) and chloroperoxidase (GenBank ID: 9796009). Manganese peroxidase is an extracellular peroxidase involved in lignin degradation. Chloroperoxidase is a haloperoxidase that catalyses the oxidation of halides with peroxides such as hydrogen peroxide resulting in the halogenation of organic compounds (Hofrichter *et al.*, 2010).

3.6 Inhibitors of discoloration

Discoloration of food products as well fresh, minimally processed or fully processed is a major problem in the food industry. To prevent browning food additives can be used, such as reducing agents and enzyme inhibitors (Loizzo *et al.*, 2012). The food industry frequently uses ascorbic acid and various forms of sulphite-containing compounds as antibrowning agents. Alternative methods to prevent browning are autoclaving, blanching, irradiation, pulsed electric fields, and microwave energy. Loizzo *et al.* (2012) give an overview and update about the latest tyrosinase inhibitors from natural and synthetic sources. Many plant extracts show tyrosinase inhibitory activity and the largest group of natural tyrosinase inhibitors are flavonoids.

Three possible mechanisms for browning inhibition by sulphite have been suggested: 1) irreversible inhibition of PPO, 2) reduction of *o*-quinones, thereby reversing the enzymatic reaction, and 3) formation of addition products between sulphite and *o*-quinones, preventing them from reacting further into melanin (Kuijpers *et al.*, 2012). Kuijpers *et al.* (2012) found that sodium bisulphite ($NaHSO_3$) has a dual inhibitory effect in PPO catalysed browning. Their research shows that different sulphur-containing compounds can inhibit *in vitro* browning of chlorogenic acid by mushroom PPO in two different ways (Figure 7). $NaHSO_3$ and DTT give inhibition of the enzymatic activity. $NaHSO_3$, cysteine and GSH give inhibition by the formation of colourless adducts with enzymatically formed *o*-quinones. This shows that both enzymes (PPO) and phenolic compounds can be the rate limiting step in brown discoloration.

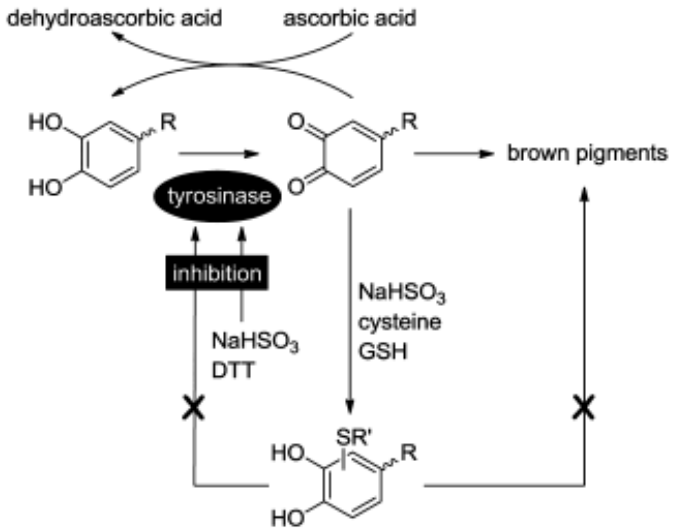


Figure 7. Schematic representation of the action of tyrosinase on chlorogenic acid, with a possible mechanism of inhibition of browning. Retrieved from Kuijpers *et al.* (2012). DTT = dithioteitol, GSH = glutathione. R = side group of chlorogenic acid. R' = side group of sulphur-containing compound.

4 Genetic and molecular pathway of discoloration

4.1 Function of melanins

Melanins function as a defence system in all organisms (Bell and Wheeler, 1986). Melanins in fungi are important to confer resistance to microbial attack. On the other hand pathogenic fungi synthesize melanin to increase their virulence (Fogarty and Tobin, 1996; Butler and Day, 1998). Fungi produce appressoria, structures that penetrate plant tissue, allowing the organisms to invade the host. Melanin in the cell wall of these structures provides mechanical strength to the appressoria that aids in tissue penetration (Eisenman and Casadevall, 2012). Melanins can protect the fungi to survive under environmental stress (Fogarty and Tobin, 1996), such as for instance protection against irradiation and UV light, desiccation, and extreme temperatures (Bell and Wheeler, 1986). Melanin can protect certain fungi against lysis in natural soils (Butler and Day, 1998) possibly via a mechanism in which melanin inhibits various enzymes used by the antagonists to digest the fungal cell wall (Bell and Wheeler, 1986). Plants can use PPO as a reaction to infection or wounding of the plant tissue (Yoruk and Marshall, 2003). The reaction products on disease resistance can functionally be classified into three groups: 1) oxidative polymerization of quinones from insoluble melanin over wounds, sealing off infected tissues, 2) bactericidal and fungicidal effect of hydroxyphenolics and quinones to assaulting microorganisms and conferring toxic effects of the polymerized phenolics on invading viruses in injured tissues or 3) covalent modification of proteins by quinones as an antinutritive defence mechanism. The quinones generated by reaction of PPO with a variety of phenolic substrates can modify dietary proteins by reaction with amino, sulfhydryl, phenolic and imidazole groups, reducing their nutritive value to insect herbivores (see review of Yoruk and Marshall, 2003, for a complete overview).

4.2 Phenolic compounds involved in brown discoloration in A. bisporus

Beaulieu *et al.* (1999) analysed phenolic compounds in *A. bisporus* var. *albidus* mushrooms during seven days of storage at 15 °C. During storage the mushrooms showed a decrease in whiteness (measured as the L* value), so brown discoloration occurred upon storage. Ethanol extractions were made and prephenate (±8 - ±35 mg/g) and tyrosine (±7.5 - ±12.5 mg/g) were found to be the two major phenolic compounds. During seven days of storage chorismate (±4 - ±11 mg/g), prephenate (±8 - ±35 mg/g) and *p*-aminobenzoic acid (±0.8 - ±1.8 mg/g) increased significantly. The lowest amount was found for *p*-aminophenol (±0.3 - ±0.5 mg/g), which is the direct precursor of GHB. Unfortunately, Beaulieu *et al.* (1999) did not measure the amount of GHB in the mushroom samples. This makes it difficult to conclude if *p*-aminophenol is converted to GHB and accumulated or whether it was transformed into other compounds and probably eventually into melanin. Rast *et al.* (1979) did measure the amount of GHB in *A. bisporus* strain A-6 (ATCC 382581) mushrooms. The amount of GHB increased in the whole fruit body during maturation, from 2.7 mg/g dry weight in 3 mm cap diameter mushrooms till 6.4 mg/g dry weight in 120 mm cap diameter mushrooms. The highest amount of GHB was found in the lamellae (21.3 mg/g dry weight) of a 120 mm cap diameter mushroom. Jolivet *et al.* (1995) analysed two *A. bisporus* strains, B62 and S609 having high and low sensitivity to browning, respectively. The content of tyrosine, GHB and GDHB was nearly sevenfold higher in the B62 than in the S609 strain. Mamoun *et al.* (1999) analysed five *A. bisporus* strains and found that GHB content did not significantly differ among wild strains (Bs70D, Bs78F, and Bs247 from INRA-CTC, France) which did differ in natural colour. A significant difference was found for the GHB content between wild and commercial strains (C45 and X25 from Le Lion, France).

Tsai *et al.* (2007) analysed L-phenylalanine and L-tyrosine in *A. bisporus* mushrooms (MS strain from France, grown in Taiwan) which were harvested at different developmental stages. The amount of both compounds was highest in stage 3 mushrooms, 1.96 g/kg dry weight L-phenylalanine and 1.62 g/kg dry weight L-tyrosine. Tseng and Mau (1999) also analysed the amount of L-tyrosine and L-phenylalanine in button mushrooms with HPLC. They used the strain Tainung 3 which was also grown in Taiwan. Mushrooms of 25-40 mm were used with maturity in the button stage, of which the veil was still intact and tight. Mushrooms were stored at 12 °C for twelve days. At day 0 the L-phenylalanine content was 6.92 g/kg dry weight and the amount of L-tyrosine was 1.11 g/kg dry weight. An increase was found for both amino acids during storage until day nine, at day twelve the level decreased. Sommer *et al.* (2010) found 2.60 g/kg dry weight for L-phenylalanine and 1.65 g/kg dry weight for tyrosine in *A. bisporus* mushroom of the *Don Juan Selection* (Hungary) from the same cultivation and harvesting stage (veil intact). The difference between the amount of L-phenylalanine and L-tyrosine found by Tsai *et al*. (2007), Tseng and Mau (1999), and Sommer *et al.* (2010) is probably influenced by different factors as growth conditions, stage used, storage temperature, enzyme activity, and the strain that was used.

Sharman *et al*. (1990) analysed the amount of agaritine in two button mushrooms cultivated in the United Kingdom. HPLC analysis showed agaritine levels to range between 100-250 mg/kg fresh weight for strain 1 and between 80-190 mg/kg fresh weight for strain 2. The amount of agaritine was found to be different between button, cup and open cup mushrooms and was different between flushes. The findings of Sharman *et al.* (1990) were not consistent with levels previously found in literature in which the highest agaritine levels were found in the lowest developmental stage mushrooms. Fisher *et al.* (1984) did observe a decrease in the amount of agaritine during maturation for both a white and a brown strain. Sommer *et al*. (2009) found 1.47-1.59 g/kg dry weight agaritine in *A.*

bisporus mushroom of the *Don Juan Selection* (Hungary), mushrooms were used from the same cultivation and harvest. The difference in amount of Sharman *et al.* (1990) and Sommer *et al.* (2009) is probably due to the analysis in fresh and dried mushrooms, respectively.

Kim *et al.* (2008) found that the phenolics in methanolic extracts of *A. bisporus* mushrooms from Korea were gallic acid, pyrogallol, protocatechuic acid, naringin, and myricetin. Palacios *et al.* (2011) investigated the phenolic compounds in methanolic extracts of *A. bisporus* mushrooms from Spain and found caffeic acid, catechin, chlorogenic acid, *p*-coumaric acid, ferulic acid, gallic acid, *p*-hydroxybenzoic acid, homogentisic acid, myricetin, procatechuic acid and pyrogallol as the main phenolic compounds. Liu *et al.* (2013) identified phenolics in ethanolic extracts of *A. bisporus* mushrooms from China and found gallic acid, protocatechuic acid, catechin, caffeic acid, ferulic acid, and myricetin. The phenolic compounds identified by all three studies are protocatechuic acid and myricetin. Protocatechuic acid is a dihydroxybenzoic acid and myricetin is a flavonoid, both compounds can be formed via the shikimate pathway. Palacios *et al.* (2011) identified homogentisic acid to be the main phenolic compounds in their extract of button mushrooms. Kim *et al.* (2008) also used homogentisic acid as a standard phenolic compound, but did not identify it in their extract of button mushrooms. The phenolic compounds isolated from *A. bisporus* show a major diversity, which is probably mainly dependent on the type of mushroom strain used, and can also be influenced by environmental factors, harvesting conditions, extraction solvents and detection methods (Rispail *et al.*, 2005).

4.3 Comparison with discoloration of other type of mushrooms

Kanda *et al.* (1996) analysed the relationship between tyrosinase activity and gill browning during the preservation of Shiitake (*Lentinula edodes*) fruit bodies at 25 °C for five days. They found a correlation between increasing tyrosinase

activity and increase of gill browning. Changes were found in latent- and active-tyrosinase content during gill browning and indicated the possibility of a *de novo* synthesis of latent-tyrosinase. Nagai *et al.* (2003) showed that both a tyrosinase and a laccase have a role in melanin synthesis in the gills of *L. edodes*.

Smith *et al.* (1993) compared three white mushroom strains for postharvest quality and discoloration during storage at 18 °C. The strains analysed are a commercial *A. bisporus* strain (Somycel U3) and two tropical *A. bitorquis* strains (ATCC 32675 and AGC W20). At harvest the tops of the cap of the *A. bitorquis strains* mushrooms were significantly whiter than the *A. bisporus* strain. Besides that, Smith *et al.* (1993) identified that the *A. bitorquis* strains, ATCC 32675 showed a very slow maturation during storage. This indicates that when this genetic trait can be explored mushrooms with a longer shelf-life and whiter colour might be developed.

Another edible mushroom species is *Pleurotus*, which also can form melanin pigments. Villaescusa and Gil (2003) analysed the quality of *Pleurotus ostreatus*, the oyster mushroom. Based on visual colour inspection, judged by a trained panel and by a chromameter, it was shown that *Pleurotus* stored at 0 °C maintained the initial intense colour, whereas a moderate discoloration to yellow was observed at 4 and 7 °C during storage.

4.4 Tyrosinase reactions in fruit and vegetables

Newman *et al.* (1993) identified a PPO gene family consisting of seven genes in tomato (cultivar VFNT cherry). The seven genes are named PPO A, A', B, C, D, E and F and are all located on chromosome eight. Thipyapong *et al.* (1997a) analysed the PPO gene family in tomato during vegetative and reproductive development. They found that PPO expression is primarily confined to early stages of development and the gene mRNA levels showed complex patterns of spatial and temporal regulation in vegetative and reproductive organs of the tomato plant. PPO

B is the most abundant PPO mRNA in young tomato leaves, the levels subsequently declined during maturation of the leaves. Thipyapong *et al.* (1997b) found that only PPO F is transcriptionally and differentially activated in response to mechanical wounding and by fungal and bacterial pathogens. PPO activity was downregulated by introducing antisense potato PPO cDNA in tomato (Thipyapong *et al.*, 2004). All members of the tomato PPO gene family were down-regulated, this did not effected growth, development, or reproduction of the plant. However, susceptibility to *Pseudomonas syringae* was increased. On the other hand, Li and Steffens (2002) showed that overexpression of a potato PPO cDNA in tomato enhanced resistance to *P. syringae*. It was shown that a higher potential for phenolic oxidation correlated with substantially higher levels of resistance to the pathogen. So, PPO-mediated phenolic oxidation is important in limiting plant disease development.

Thygesen *et al.* (1995) identified five PPO genes in potato tuber, next to the two PPO genes found before by Hunt *et al.* (1993). Thipyapong *et al.* (1995) analysed wound responsive expression of PPOs in five-week old potato plants and found that it is regulated at the level of transcriptional activity or mRNA stability, and results in increased accumulation of PPOs and PPO activity. Unfortunately, it is not clear from this research which PPO genes of potato are increased. Cantos *et al.* (2002) studied browning development in fresh cut potatoes of five different cultivars. PPO, peroxidase, hydrogen peroxide, ascorbic acid content and initial phenolic content as well as total and individual phenolic compound accumulation were analysed, but no significant correlation was found between either rate or degree of browning and any other biochemical and physiological attribute investigated. Cantos *et al.* (2001) also did not find a clear correlation between browning of lettuce and PPO, PAL and peroxidase activities, total and individual phenols accumulation, and ascorbic acid content.

The browning reaction in apples is a complex process involving several factors. Substrate levels, enzyme activity, presence of ascorbic acid and other inhibitors or promoters influence the browning reaction to a certain extent. Coseteng and Lee (1986) analysed several apple cultivars to examine the relationship between changes in PPO and polyphenol concentrations to the degree of browning. The cultivars *Classic Delicious*, *RI Greening*, *Cortland*, and *McIntosh* showed a direct correlation between PPO activity and degree of browning upon cold storage with a correlation coefficient (R^2) of 0.827. On the other hand the cultivars *Empire*, *Gold Delicious*, and *Rome* showed a correlation between total phenolics and degree of browning of 0.986 (R^2). No correlation was found between the type or concentration of phenolic compounds and degree of browning among cultivars. Murata *et al.* (1995) found that an immature *Fuji* apple turned brown quicker, then in mature fruit which correlated with both polyphenol content and PPO activity. The active PPO was mainly localized near the core in the mature apple, while it was uniformly distributed in immature apple. Boss *et al.* (1995) found enhanced expression of homologous PPO (pAPO5) six hours after wounding of *Granny Smith* apples. They suggest that induction of PPO occurs in cells adjacent to the wound. Kim *et al.* (2001) identified two PPOs in *Fuji* apples (pAPO5 and pMD-PPO2). Both genes are differently expressed in apple tissues and developmental stages. Upon wounding, *pAPO5* was significantly induced in leaves and fruits; whereas the level of *MD-PPO2* mRNA was not affected by mechanical damage. This is in agreement with the findings of Boss *et al.* (1995).

Recent mapping of apple's genome revealed that PPO is genetically encoded in a diverse, multi-gene family. Apples have at least eight PPO genes, in three main PPO gene families (http://www.okspecialtyfruits.com/arctic-apples/browning-and-nonbrowning-science). Okanagan Specialty Fruits Inc. (http://www.okspecialtyfruits.com/) has developed non-browning apples, termed Arctic apples. Gene silencing, based on the use of PPO genes from apples, resulted in less than 10 % of

the PPO amount produced in the original cultivars. This low level of PPO is not enough to get brown apples and clearly indicate that PPO is one of the major factors causing browning of apple flesh. Okanagan Specialty Fruits Inc. claim that a targeted and specific gene modification is used that silences the PPO enzyme but does not change any other aspect of the cultivar.

Considerable effort has been spent on developing a transformation system for *A. bisporus*, but so far transformation is not very efficient and is difficult to perform. Stoop and Mooibroek (1999) describe in their review the advances in genetic analysis and biotechnology of *A. bisporus* until the end of the nineties of the previous century. While many other transformation techniques are not very reliable or stable, the use of the soil bacterium, *Agrobacterium tumefaciens*, for transformation yielded stable transformants for *A. bisporus* (Mikosch *et al.*, 2001). The *Agrobacterium* system allows transformation of both homokaryons and heterokaryons.

5 Aim and outline of the thesis

The future of the Dutch mushroom industry requires an innovative production platform where high quality mushrooms can be produced at considerable lower costs. This research intends to generate knowledge and tools needed to breed new mushroom strains with low sensitivity to bruising and thus suitable for mechanical harvesting systems (i.e. robot picking). This allows a strong reduction in production costs. The goal of this research is to identify the key genes or other key rate-limiting factors in brown discoloration in button mushrooms upon bruising. After identifying key genes, several strategies can be used to come to bruising tolerant mushroom varieties or products. This can be via conventional breeding strategies (Sonnenberg *et al.*, 2006), mutagenesis based strategies or via transgenic approaches. These approaches will be most successful when these key factors have been identified and the functions and regulations of these key genes have been

studied in depth. The genetic variance of wild button mushrooms strains can be used to identifying the key genes. For that research which will be presented in this thesis shall form an important basis.

In **Chapter 2** the development of a bruising device and image analysis system to quantify mushroom bruising sensitivity is described. In order to develop a reliable and reproducible method, several parameters were studied such as the influence of flush, the effects of the developmental stage of the mushrooms, the time between harvest and applying the bruise, and the time between bruising and analysing discoloration. Finally, a collection of *A. bisporus* strains was screened for their bruising sensitivity in order to analyse the phenotypic variation among strains.

In **Chapter 3** a correlation between different discoloration methods is described for several mushroom strains. The developed bruising method and discoloration caused by a conveyor belt and discoloration after seven days storage at 4 °C or 8 °C at 90 % RH were compared.

In **Chapter 4** the most relevant genes in the genome of *A. bisporus* which are likely involved in the melanin biosynthetic pathway are listed and characterized. Known gene sequences of *A. bisporus* or other fungi were used to identify new genes in the genome of *A. bisporus.* The automatic annotation made it possible to search directly in the KEGG and KOG databases that were based on the *A. bisporus* genome. For almost all individual genes, even when several homologs were identified in the genome, RT-qPCR analyses were performed to determine their expression, tissue specificity and to study gene expression in different developmental stages of the button mushroom.

In **Chapter 5** a UHPLC-PDA-MS method is described to analyse the phenolic compounds of the melanin biosynthesis pathway. For the analysis a selection of bruising-tolerant and bruising-sensitive button mushroom strains were used. Compounds were identified that show differences between bruising-tolerant and sensitive strains.

In **Chapter 6** a comparison is made between genomic sequences and gene expression between bruising-tolerant and bruising-sensitive strains. DNA sequence comparison was performed to identify possible gene mutations that might be related to bruising sensitivity. The mutations were analysed with RT-qPCR in order to analyse the link between the presence of the mutation and the bruising-sensitivity of several *A. bisporus* strains. Gene expression analyses were dedicated to determine differential expression among bruising-sensitive and tolerant *A. bisporus* strains. The same strains are used as in Chapter 5 in order to correlate gene expression with phenolic substrates.

In **Chapter 7** a population was made by selecting homokaryons after meiosis of a cross between two parental lines recovered from two bruising-tolerant strains. The homokaryons were crossed with another parental line to produce mushrooms. The population was screened for bruising sensitivity using the method developed in Chapter 2. A selection of most tolerant and most sensitive strains of the population was made. This selection was used to analyse gene expression (like described in Chapter 4 and 6) and phenolic compound availability (Chapter 5) in non-bruised and bruised skin tissue.

Our hypothesis is that these tools, together with enzymatic methods, will support us in determining the key parameters for bruising tolerance of *A. bisporus* strains. This will support dedicated breeding and selection strategies to develop mushroom strains suitable for mechanical harvesting.

References

Bartley, C.E., Beelman, R.B., Winnett, J.R. (1991). Factors affecting colour of cultivated mushrooms (*Agaricus bisporus*) prior to harvest and during postharvest storage. *Mushroom Science, XIII*, 689-694.

Baumgartner, D., Hoesch, L., Rast, D.M. (1998). The biogenesis of beta-N-(gamma-glutamyl)-4-hydroxymethylphenylhydrazine (agaritine) in *Agaricus bisporus*. *Phytochemistry*, *49*(2), 465-474.

Beaulieu, M., Beliveau, M., D'Aprano, G., Lacroix, M. (1999). Dose rate effect of gamma irradiation on phenolic compounds, polyphenol oxidase, and browning of mushrooms (*Agaricus bisporus*). *Journal of Agricultural and Food Chemistry*, *47*(7), 2537-2543.

Bell, A.A., Wheeler, M. H. (1986). Biosynthesis and functions of fungal melanins. *Annual Review of Phytopathology*, *24*, 411-451.

Beyer, D.M. (2003). Basic procedures for *Agaricus* mushroom growning. Retrieved from http://www.americanmushroom.org/agaricus.pdf on December 10th 2012.

Boss, P.K., Gardner, R.C., Janssen, B.J., Ross, G.S. (1995). An apple polyphenol oxidase cDNA is up-regulated in wounded tissues. *Plant molecular biology*, *27*(2), 429-433.

Burton, K.S. (2004). Cultural factors affecting mushroom quality - cause and control of bruising. *Proceedings of the XVIth International Congress of Science and Cultivation of Edible and Medicinal Fungi* (pp. 397-402). Maimi.

Burton, K.S., Noble, R. (1993). The influence of flush number, bruising and storage temperature on mushroom quality. *Postharvest Biology and Technology*, *3*(1), 39-47.

Butler, M.J., Day, A.W. (1998). Fungal melanins: a review. *Canadian Journal of Microbiology*, *44*(12), 1115-1136.

Calla, B., Vuong, T., Radwan, O., Hartman, G.L., Clough, S.J. (2009). Gene expression profiling soybean stem tissue early response to *Sclerotinia sclerotiorum* and in silico mapping in relation to resistance markers. *The Plant Genome Journal*, *2*(2), 149-166.

Callac, P., Billette, C., Imbernon, M., Kerrigan, R.W. (1993). Morphological, genetic and infertility analyses reveal a novel, tetrasporic variety of *Agaricus bisporus* from the Sonoran desert of California. *Mycologia*, *85*, 835-851.

Cantos, E., Espín, J.C., Tomás-Barberán, F. A (2001). Effect of wounding on phenolic enzymes in six minimally processed lettuce cultivars upon storage. *Journal of Agricultural and Food Chemistry*, *49*(1), 322-30.

Cantos, E., Tudela, J.A., Gil, M.I., Espin, J.C. (2002). Phenolic compounds and related enzymes are not rate-limiting in browning development of fresh-cut potatoes. *Journal of Agricultural and Food Chemistry*, *50*(10), 3015-3023.

Choi, S.W., Sapers, G.M. (1994). Effects of washing on polyphenols and polyphenol oxidase in commercial mushrooms (*Agaricus bisporus*). *Journal of Agricultural and Food Chemistry*, *42*(10), 2286-2290.

Conesa, A., Punt, P.J., Van den Hondel, C.A.M.J.J. (2002). Fungal peroxidases: molecular aspects and applications. *Journal of Biotechnology*, *93*(2), 143-58.

Coseteng, M.Y., Lee, C.Y. (1987). Changes in apple polyphenoloxidase and polyphenol concentrations in relation todegree of browning. *Journal of Food Science*, *52*(4), 985-989.

Dewick, P. M. (1998). The biosynthesis of shikimate metabolites. *Natural Product Reports*, 17-58.

Dixon, R.A., Paiva, N.L. (1995). Stress-induced phenylpropanoid metabolism. *The Plant Cell*, *7*(7), 1085-1097.

Eisenman, H.C., Casadevall, A. (2012). Synthesis and assembly of fungal melanin. *Applied Microbiology and Biotechnology*, *93*(3), 931-40.

Esquerre, C., Gowen, A.A., O'Donnell, C.P., Downey, G. (2009). Initial studies on the quantitation of bruise damage and freshness in mushrooms using visible-near-infrared spectroscopy. *Journal of Agricultural and Food Chemistry*, *57*(5), 1903-1907.

Fischer, B., Luthy, J., Schlatter, C. (1984). Gehaltsbestimmung von agaritin im Zuchtchampignon (*Agaricus bisporus*) mittels hochleitungsflussigchromatographie (HPLC). *Zeitschrift fuer Lebensmittel-untersuchung und -forschung*, *179*, 218-223.

Flurkey, W.H., Inlow, J.K. (2008). Proteolytic processing of polyphenol oxidase from plants and fungi. *Journal of Inorganic Biochemistry*, *102*(12), 2160-70.

Fogarty, R.V., Tobin, J.M. (1996). Fungal melanins and their interactions with metals. *Enzyme and Microbial Technology*, *19*(4), 311-317.

Foulongne-Oriol, M., Rodier, A., Savoie, J. (2012). Relationship between yield components and partial resistance to *Lecaniculium fungicola* in the button mushroom, *Agaricus bisporus*, assessed by quantitative trait locus mapping. *Applied Environmental Microbiology*, *78*(7), 2435-2442.

Gaston, E., Frías, J.M., Cullen, P.J., O'Donnell, C.P., Gowen, A.A. (2010). Prediction of polyphenol oxidase activity using visible near-infrared hyperspectral imaging on mushroom (*Agaricus bisporus*) caps. *Journal of Agricultural and Food Chemistry*, *58*(10), 6226-6233.

Halaouli, S., Asther, M., Sigoillot, J.-C., Hamdi, M., Lomascolo, A. (2006). Fungal tyrosinases: new prospects in molecular characteristics, bioengineering and biotechnological applications. *Journal of Applied Microbiology*, *100*(2), 219-32.

Hammond, J.B.W., Nichols, R. (1975). Changes in respiration and soluble carbohydrates during the post-harvest storage of mushrooms (*Agaricus bisporus*). *Journal of the Science of Food and Agriculture*, *26*(6), 835-842.

Hammond, J.B.W., Nichols, R. (1976). Carbohydrate metabolism in *Agaricus bisporus* (Lange) Sing: changes in soluble carbohydrates during growth of mycelium and sporophore. *Journal of General Microbiology*, *93*(2), 309-320.

Hofrichter, M., Ullrich, R., Pecyna, M.J., Liers, C., Lundell, T. (2010). New and classic families of secreted fungal heme peroxidases. *Applied Microbiology and Biotechnology*, *87*(3), 871-897.

Hunt, M.D., Eannetta, N.T., Yu, H., Newman, S.M., Steffens, J.C. (1993). cDNA cloning and expression of potato polyphenol oxidase. *Plant molecular biology*, *21*(1), 59-68.

Ismaya, W.T., Rozeboom, J., Weijn, A., Mes, J.J., Fusetti, F., Wichers, H.J., Dijkstra, B.W. (2011). Crystal structure of *Agaricus bisporus* mushroom tyrosinase: identity of the tetramer subunits and interaction with tropolone. *Biochemistry*, *50*(24), 5477-5486.

Jolivet, S., Arpin, N., Wichers, H.J., Pellon, G. (1998). *Agaricus bisporus* browning : a review. *Mycological Research*, *102*(12), 1459-1483.

Jolivet, S., Pellon, G., Gelhausen, M., Arpin, N. (1999). GHB: biosynthesis and metabolic fate after applying on *Agaricus bisporus*. *Phytochemistry*, *50*(4), 581-587.

Kanda, K., Sato, T., Suzuki, K., Ishi, S., Ejiri, S. (1996). Relationships between tyrosinase activity and gill browning during preservation of *Lentinus edodes* fruit-bodies. *Bioscience Biotechnolgy and Biochemistry*, *60*(3), 479-480.

Kim, J.Y., Seo, Y.S., Kim, J.E., Sung, S.-K., Song, K.J., An, G., Kim, W. T. (2001). Two polyphenol oxidases are differentially expressed during vegetative and reproductive development and in response to wounding in the Fuji apple. *Plant Science*, *161*(6), 1145-1152.

Kim, M.-Y., Seguin, P., Ahn, J.-K., Kim, J.-J., Chun, S.-C., Kim, E.-H., Seo, S.-H., Kang, E.-Y., Kim, S.-L., Park, E.-H., Ro, H.-M., Chung, I.-M. (2008). Phenolic compound concentration and antioxidant activities of edible and medicinal mushrooms from Korea. *Journal of Agricultural and Food Chemistry*, *56*(16), 7265-7270.

Klabunde, T., Eicken, C., Sacchettini, J.C., Krebs, B. (1998). Crystal structure of a plant catechol oxidase containing a dicopper center. *Nature Structural Biology*, *5*(12), 1084-1090.

Kuijpers, T.F.M., Narváez-Cuenca, C.-E., Vincken, J.-P., Verloop, A.J.W., Van Berkel, W.J.H., Gruppen, H. (2012). Inhibition of enzymatic browning of chlorogenic acid by sulfur-containing compounds. *Journal of Agricultural and Food Chemistry*, *60*(13), 3507-3514.

Lankinen, P., Hilden, K., Aro, N., Salkinoja-Salonen, M., Hatakka, A. (2005). Manganese peroxidase of *Agaricus bisporus*: grain bran-promoted production and gene characterization. *Applied Microbiology and Biotechnology*, *66*, 401-407.

Li, L., Steffens, J.C. (2002). Overexpression of polyphenol oxidase in transgenic tomato plants results in enhanced bacterial disease resistance. *Planta*, *215*(2), 239-247.

Li, N.-Y., Cai, W.-M., Jin, Q.-L., Qin, Q.-P., Ran, F.-L. (2011). Molecular cloning and expression of polyphenoloxidase genes from the mushroom, *Agaricus bisporus*. *Agricultural Sciences in China*, *10*(2), 185-194.

Liu, J., Jia, L., Kan, J., Jin, C.-H. (2013). In vitro and in vivo antioxidant activity of ethanolic extract of white button mushroom (*Agaricus bisporus*). *Food and Chemical Toxicology*, *51*, 310-316.

Loizzo, M.R., Tundis, R., Menichini, F. (2012). Natural and synthetic tyrosinase inhibitors as antibrowning agents: an update. *Comprehensive Reviews in Food Science and Food Safety*, *11*(4), 378-398.

Mamoun, M., Moquet, F., Savoie, J.-M., Devesse, C., Ramos-Guedes-Lafargue, M., Olivier, J.M., Arpin, N. (1999). *Agaricus bisporus* susceptibility to bacterial blotch in relation to environment: biochemical studies. *FEMS microbiology letters*, *181*(1), 131-136.

Marusek, C.M., Trobaugh, N.M., Flurkey, W.H., Inlow, J.K. (2006). Comparative analysis of polyphenol oxidase from plant and fungal species. *Journal of Inorganic Biochemistry*, *100*(1), 108-123.

Matoba, Y., Kumagai, T., Yamamoto, A., Yoshitsu, H., Sugiyama, M. (2006). Crystallographic evidence that the dinuclear copper center of tyrosinase is flexible during catalysis. *The Journal of Biological Chemistry*, *281*(13), 8981-8990.

Mayer, A.M. (2006). Polyphenol oxidases in plants and fungi: going places? A review. *Phytochemistry*, *67*(21), 2318-2331.

Miklus, M.B., Beelman, R.B. (1996). $CaCl_2$ treated irrigation water applied to mushroom crops (*Agaricus bisporus*) increases Ca concentration and improves postharvest quality and shelf life. *Mycologia*, *88*(3), 403-409.

Mikosch, T.S.P., Lavrijssen B., Sonnenberg, A.S.M., van Griensven, L.J.L.D. (2001). Transformation of the cultivated mushroom *Agaricus bisporus* (Lange) using T-DNA from *Agrobacterium tumefaciens*. *Current Genetics*, *39*(1), 35-39.

Ministerie van Landbouw, N. en V. (2010). Sector Special Champignons. *Berichten Buitenland Special Champignons*, *36*(3), 1-29.

Mohapatra, D., Bira, Z.M., Kerry, J.P., Frías, J.M., Rodrigues, F.A. (2010). Postharvest hardness and color evolution of white button mushrooms (*Agaricus bisporus*). *Journal of Food Science*, *75*(3), E146–E152.

Muizzuddin, N., Marenus, K., Maes, D., Smith, W.P. (1990). Use of a chromameter in assessing the efficacy of anti-irritants and tanning accelerators. *Journal of the Society of Cosmetic Chemists*, *41*, 369-378.

Murata, M., Tsurutani, M., Tomita, M., Homma, S., Kaneko, K. (1995). Relationship between apple ripening and browning: changes in polyphenol content and polyphenol oxidase. *Journal of Agricultural and Food Chemistry*, *43*(5), 1115-1121.

Nagai, M., Kawata, M., Watanabe, H., Ogawa, M., Saito, K., Takesawa, T., Kanda, K., Sato, T. (2003). Important role of fungal intracellular laccase for melanin synthesis: purification and characterization of an intracellular laccase from *Lentinula edodes* fruit bodies. *Microbiology*, *149*, 2455-2462.

Newman, S.M., Eannetta, N.T., Yu, H., Prince, J.P., De Vicente, M.C., Tanksley, S.D., Steffens, J.C. (1993). Organisation of the tomato polyphenol oxidase gene family. *Plant Molecular Biology*, *21*(6), 1035-1051.

Noble, R., Rama, T., Miles, S., Burton, K.S., Stephens, T.M., Reed, J.N. (1997). Influences of compost and casing layer depths on the mechanical properties of mushrooms. *Annual Applied Biology*, *131*, 79-90.

O'Gorman, A., Downey, G., Gowen, A.A., Barry-Ryan, C., Frias, J.M. (2010). Use of Fourier transform infrared spectroscopy and chemometric data analysis to evaluate damage and age in mushrooms (*Agaricus bisporus*) grown in Ireland. *Journal of Agricultural and Food Chemistry*, *58*(13), 7770-7776.

Palacios, I., Lozano, M., Moro, C., D'Arrigo, M., Rostagno, M.A., Martínez, J.A., García-Lafuente, A., Guillamón, E., Villares, A. (2011). Antioxidant properties of phenolic compounds occurring in edible mushrooms. *Food Chemistry*, *128*(3), 674-678.

Paranjpe, M.S., Chen, P.K., Jong, S.C. (1978). Phenolic and other organic compounds in morphologically different tissues of *Agaricus bisporus*. *Transactions of the Mycoligical Society of Japan*, *19*, 169-180.

Rast, D.M., Baumgartner, D., Mayer, C., Hollenstein, G.O. (2003) Cell wall-associated enzymes in fungi. *Phytochemistry*, *64*(2), 339-366.

Rast, D., Stussi, H., Zobrist, P. (1979). Self-inhibition of the *Agaricus bisporus* spore by CO_2 and / or y-glutaminyl-4-hydroxybenzene and y-glutaminyl-3,4-benzoquinone: a biochemical analysis. *Physiologia Planetarum*, *46*(3), 227-234.

Rispail, N., Morris, P., Webb, K.J. (2005). Phenolic compounds: extraction and analysis. In A. J. Marquez (Ed.), *Lotus Japonicus handbook* (pp. 349-355).

Roupas, P., Keogh, J., Noakes, M., Margetts, C., Taylor, P. (2010). Mushrooms and agaritine: a mini-review. *Journal of Functional Foods*, *2*, 91-98.

Royse, D.J., Beelman, R.B. (n.d.). Six steps to mushroom farming. Retrieved from http://extension.psu.edu/mushroom-grower-info/publications/guides/SixSteps.pdf on December 7th 2012.

Sanchez-Ferrer, A., Rodriguez-Lopez, J.N., Garcia-Canovas, F., Garcia-Carmona, F. (1995). Tyrosinase: a comprehensive review of its mechanism. *Biochimica et Biophysica Acta*, *1247*, 1-11.

Selinheimo, E., NiEidhin, D., Steffensen, C., Nielsen, J., Lomascolo, A., Halaouli, S., Record, E., O'Beirne, D., Buchert, J., Kruus, K. (2007). Comparison of the characteristics of fungal and plant tyrosinases. *Journal of Biotechnology*, *130*(4), 471-480.

Sharman, M., Patey, A.L., Gilbert, J. (1990). A survey of the occurrence of agaritine in U.K. cultivated mushrooms and processed mushroom products. *Food Addtives and Contaminants*, *7*(5), 649-656.

Smith, J.F., Love, M.E., Burton, K.S. (1993). Comparative studies of the quality of fresh and stored mushrooms of *Agaricus bisporus* with two tropical *Agaricus bitorquis* strains. *Annual Applied Biology*, *122*, 593-603.

Soler-Rivas, C., Arpin, N., Oliver, J.-M., Wichers, H.J. (1999). The effects of tolaasin, the toxin produced by *Pseudomonas tolaasii* on tyrosinase activities and the induction of browning in *Agaricus bisporus* fruiting bodies. *Physiological and Molecular Plant Pathology*, *55*, 21-28.

Sommer, I., Schwartz, H., Solar, S., Sontag, G. (2009). Effect of gamma-irradiation on agaritine, gamma-glutaminyl-4-hydroxybenzene (GHB), antioxidant capacity, and total phenolic content of mushrooms (*Agaricus bisporus*). *Journal of Agricultural and Food Chemistry*, *57*(13), 5790-5794.

Sommer, I., Schwartz, H., Solar, S., Sontag, G. (2010). Effect of gamma-irradiation on flavour 5′-nucleotides, tyrosine, and phenylalanine in mushrooms (*Agaricus bisporus*). *Food Chemistry*, *123*(1), 171-174.

Sonnenberg, A.S.M., Baars, J.J.P., Hendrickx, P.M., Kerrigan, R.W. (2006). Breeding mushrooms: state of the art. *Proceedings of the 5th International Conference on Mushroom Biology and Mushroom Products*, *12*, Shanghai, 163-173.

Sonnenberg, A.S.M., Baars, J.J.P., Hendrickx, P.M., Lavrijssen, B., Gao, W., Weijn, A., Mes, J.J. (2011). Breeding and strain protection in the button mushroom *Agaricus bisporus*. *Proceedings of the 7th International Conference on Mushroom Biology and Mushroom Products*, *14*, INRA Bordeaux, 7-15.

Steffens, J.C., Harel, E., Hunt, M.D. (1994). Recent advances in phytochemistry, genetic engineering of plant secondary metabolism. In B.E. Ellis, G.W. Kuroki, H.A. Stafford (Eds.), *Recent advances in phytochemistry, genetic engineering of plant secondary metabolism* (pp. 275-311). New York: Plenum Press.

Stoop, J.M.H., Mooibroek, H. (1999). Advances in genetic analysis and biotechnology of the cultivated button mushroom, *Agaricus bisporus*. *Applied Microbiology and Biotechnology*, *52*(4), 474-483.

Strothkamp, K.G., Jolley, R.L., Mason, H.S. (1976). Quartenary structure of mushroom tyrosinase. *Biochemistry and Biophysics research Communications*, *70*, 519-524.

Thipyapong, P., Hunt, M.D., Steffens, J.C. (1995). Systemic wound induction of potato (*Solanum tuberosum*) polyphenol oxidase. *Phytochemistry*, *40*(3), 673-676.

Thipyapong, P., Hunt, M.D., Steffens, J.C. (2004). Antisense downregulation of polyphenol oxidase results in enhanced disease susceptibility. *Planta*, *220*(1), 105-17.

Thipyapong, P., Joel, D.M., Steffens, J.C. (1997a). Differential expression and turnover of the tomato polyphenol oxidase gene family during vegetative and reproductive development. *Plant Physiology*, *113*(3), 707-718.

Thipyapong, P., Steffens, J.C. (1997b). Tomato polyphenol oxidase: differential response of the polyphenol oxidase F promoter to injuries and wound signals. *Plant Physiology*, *115*(2), 409-418.

Thygesen, P. W., Dry, I. B., Robinson, S. P. (1995). Polyphenol oxidase in potato: a multigene family that exhibits differential expression patterns. *Plant physiology*, *109*(2), 525-531.

Tsai, S.-Y., Wu, T.-P., Huang, S.-J., Mau, J.-L. (2007). Nonvolatile taste components of *Agaricus bisporus* harvested at different stages of maturity. *Food Chemistry*, *103*(4), 1457-1464.

Tseng, Y.-H., Mau, J.-L. (1999). Contents of sugars, free amino acids and free 5'-nucleotides in mushrooms, *Agaricus bisporus*, during post-harvest storage. *Journal of the Science of Food and Agriculture*, *79*, 1519-1523.

Tsuji, H., Bando, N., Ogawa, T., Sasaoka, K. (1981). Studies on the biosynthesis of N-(gamma-L-glutamyl)-4-hydroxyaniline in *Agagricus bisporus* identification of the position in shikimic acid at which the amination occurs. *Biochimica et Biophysica Acta*, *677*, 326-329.

van Gelder, C.W., Flurkey, W.H., Wichers, H J. (1997). Sequence and structural features of plant and fungal tyrosinases. *Phytochemistry*, *45* (7), 1309-1323.

van Griensven, L.J.L.D., van Roestel, A. J. J. (2004). The cultivation of the button mushroom, *Agaricus bisporus*, in the Netherlands: a successful industry. *Revista Mexicana de Micologia*, *19*, 95-102.

van Leeuwen, J., Wichers, H.J. (1999). Tyrosinase activity and isoform composition in separate tissues during development of *Agaricus bisporus* fruit bodies. *Mycological Research*, *103*(4), 413-418.

Villaescusa, R., Gil, M.I. (2003). Quality improvement of *Pleurotus* mushrooms by modified atmosphere packaging and moisture absorbers. *Postharvest Biology and Technology*, *28*(1), 169-179.

Visie Nederlanse Champignon Versmarkt, published by Accenture, 17 juni 2010.

Weaver, R.F., Rajagopalan, K.V., Handler, P., Byrne, W.L. (1971). y-L-Glutaminyl-3,4-benzoquinone structural studies and enzymatic synthesis. *The Journal of Biological Chemistry*, *246*(7), 2015-2020.

Whitaker, J.R. (1995). Polyphenol oxidase. In D. W. S. Wong (Ed.), *Food Enzymes Structure and Mechanism* (pp. 271-307). New York: Chapman Hall.

Wichers, H.J., Recourt, K., Hendriks, M., Ebbelaar, C.E.M., Biancone, G., Hoeberichts, F.A., Mooibroek, H., Soler-Rivas, C. (2003). Cloning, expression and characterisation of two tyrosinase cDNAs from *Agaricus bisporus*. *Applied Microbiology and Biotechnology*, *61*(4), 336-341.

Wu, J., Chen, H., Goa, J., Liu, X., Cheng, W., Ma, X. (2010). Cloning, characterization and expression of two new polyphenol oxidase cDNAs from *Agaricus bisporus*. *Biotechnology Letters*, *32*(10), 1439-1447.

Yoruk, R., Marshall, M.R. (2003). Physicochemical properties and function of plant polyphenol oxidase: a review. *Journal of Food Biochemistry*, *27*, 361-422.

Chapter 2

A new method to apply and quantify bruising sensitivity of button mushrooms

A. Weijn[a,b], M.M.M. Tomassen[a], S. Bastiaan-Net[a], M.L.I. Wigham[a], E.P.J. Boer[a,d], E.A.H.J. Hendrix[c], J.J.P. Baars[c], A.S.M. Sonnenberg[c], H.J. Wichers[a,b] and J.J. Mes[a]

a Food & Biobased Research, Wageningen University and Research Centre, Bornse Weilanden 9, 6708 WG Wageningen, The Netherlands
b Laboratory of Food Chemistry, Wageningen University and Research Centre, Bomenweg 2, 6703 HD Wageningen, The Netherlands
c Plant Research International – Mushrooms, Wageningen University and Research Centre, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands
d Biometris, Wageningen University and Research Centre, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands

LWT – Food Science and Technology 2012 (47) 308-314

Abstract

Mushrooms are prone to develop brown discoloration due to bruising caused by mechanical damage during harvest, which leads to reduced quality of the mushrooms. In order to study the mechanism behind discoloration and to breed for bruise-related browning tolerant strains, a high throughput bruise application method and reliable quantification of bruising-sensitivity is essential. In this study a new bruising device was developed to bruise mushrooms in a fast and reproducible way. The bruising device can apply damage to the cap tissue of button mushrooms by a slip-shear sliding process. The severity of the bruise was quantified with a computer imaging system combined with a newly developed software programme. A protocol was developed to obtain the most reliable and reproducible method to compare bruising-sensitivity of different *Agaricus bisporus* strains. For this purpose, the extent of damage applied, time between harvest and bruising, developmental stage of the mushrooms and flushes were taken into account. The new method of bruising and image based quantification was subsequently applied to a collection of wild, commercial and hybrid *Agaricus bisporus* strains and a distinction was made between bruising-sensitive mushrooms and tolerant mushrooms.

1 Introduction

Agaricus bisporus button mushroom quality is determined by colour, texture, cleanliness, maturity, flush number and flavour. Of these, colour is the most important parameter because it is first perceived by consumers and discoloration decreases the commercial value (Burton, 2004). Mushrooms are prone to develop brown discoloration due to bruising of the mushrooms as a result of mechanical damage during harvest, through fruit senescence or by microbial infections (Jolivet *et al.*, 1998). Contact-based discoloration, or bruising, is caused by a mechanical process known as 'slip-shear' (Burton, 2004), a downwards force and a sideways movement, which can occur during picking by hand or by robotic picking equipment with a suction cup device.

Mechanical harvesting is more cost efficient than picking by hand, but cannot be applied yet to serve the fresh market, as commercial strains are too sensitive to bruising. It has been shown that enzymatic browning of mushrooms is caused by polyphenol oxidases (PPOs: tyrosinases and laccases) and peroxidases through an enzyme-catalysed oxidation of phenolic substrates into quinones (Jolivet *et al.*, 1998). These products undergo subsequent reactions leading to the formation of the brown pigment melanin. In order to breed for bruising-tolerant lines and to study the molecular and biochemical processes in depth it will be necessary to determine the bruise-related browning sensitivity in a reproducible way. Burton (2004) has developed a device to apply a slip-shear stress on mushroom slices. However, the damage applied was much stronger than in practice occurs and the method was too laborious to apply to a large number of samples.

Flush number is a factor considered to have a major influence on quality. Bartley *et al.* (1991) found that mushrooms prior to harvest and during postharvest storage of the first flush are in general whiter than second flush mushrooms. These second flush mushrooms in turn discolour more slowly than third flush mushrooms. Burton and Noble (1993) concluded the same for mushrooms stored at

5 °C, which were bruised in a polystyrene shaking box which oscillated horizontally through a distance of 40 mm at a frequency of 2 Hz.

Here, a newly developed bruising device and image analysis system to quantify bruising-sensitivity is described. In order to develop a reliable and reproducible method, several parameters were studied such as the influence of flush, the effects of the developmental stage of the mushrooms, the time between harvest and applying the bruise, and the time between bruising and analysing discoloration. Finally, a collection of *A. bisporus* strains was screened for their bruising sensitivity in order to analyse the phenotypic variation among strains. This method identified the genetic variation of bruising sensitivity among strains and can now support unravelling the molecular and biochemical basis for this trait.

2 Material and Methods

2.1 Mushroom strains

For the first experiment 50 different *A. bisporus* strains were grown in two replicates. In the second experiment, 10 *A. bisporus* strains (Table 1) were grown in ten replicates. In the third experiment 46 different *A. bisporus* strains were grown in two replicates (Tables 2 and 3). In each experiment the strains were randomly distributed over the growing room. Strains used in this research originated either from the department of Plant Breeding at Wageningen UR (old and present day cultivars) or from the *Agaricus* resource program (ARP) culture collection (wild collected strains, Kerrigan, 1996).

2.2 Mushroom growth

Spawn was prepared by boiling sorghum grain (*Sorghum bicolor*) for 20 min in water. After draining of water, gypsum (2.4 % w/w) and chalk (0.7 % w/w) were added before sterilizing. After sterilizing and cooling, grains were transferred to "full-gas microboxes" (Combiness, Gent Belgium) and inoculated with a pure

culture of an *A. bisporus* strain grown on agar (1 % malt extract w/w, 0.5 % mycological peptone w/w, 5 mM MOPS pH 7). The colonization was completed in approximately two weeks with occasional shaking of the boxes to distribute colonized grains. Cultivation was performed in boxes (56 x 36 x 20 cm) filled with 16 kg of phase II compost (Van Gils, 1988). Each box was inoculated with 110 ml of spawn. After a spawn run period of fourteen days (air temperature at 21–23 °C; RH 95 %; 3500 ppm CO_2) casing soil was applied. After colonisation of the casing soil for ten days at 21-23 °C, the casing layer was ruffled. Three days after ruffling the boxes were vented at a rate of 0.075 °C/h towards 18 °C air temperature. At the same time CO_2 was lowered at 35 ppm/h to a value of 1000 ppm and RH was set at 90 – 92 %. Depending on the strain, pins appeared between three and ten days after onset of venting. Harvest of the fastest strains started seven days after venting and majority of the strains produced 12-13 days after venting.

Table 1. Ten white *A. bisporus* strains used to optimize the bruising method.

Number	Strain	Classification
1	Sinden A61	sensitive
2	Somycel X135	sensitive
3	Bisp 051	sensitive
4	Claron A3.2	moderate
5	Les Miz 36	moderate
6	Royal 21A	tolerant
7	Royal 23A	tolerant
8	Le Lion X20	tolerant
9	Horst U1	tolerant
10	Darlington 735	tolerant

2.3 Bruising device

A new mushroom bruising device was constructed, which is able to apply damage to the cap tissue by a slip-shear sliding process using a spatula on a moving wheel (Figure 1). Ten mushrooms were fixed on a tray and bruised in

series. The force with which the spatula presses on the mushroom caps can be adjusted by the weights placed on the horizontal bar on top of the machine. After initial experiments the total weight of the spatula was set to 40 g. Mushrooms were kept at 20 °C at all time after harvest. After a recorded period of time, the mushrooms were placed into an illuminated cupboard (four Philips Fluotone TLD on each side of the tray) equipped with a photo-camera (JVC KY-F30E colour video camera with a JVC TV UM lens) and pictures were taken.

2.4 Computer image analysis

The developed quantitative image analysis system can measure various parameters from colour images of mushrooms and gives a quantitative value of discoloration. All images of a single experiment were calibrated using the standard Gretag-Macbeth colour checker (MSCCC) in order to compensate for small, unintentional variations in colour and illumination during the course of an experiment. A sheet of white paper was used to calibrate for the brightness and to compensate for variations in illumination over the cupboard. The bruising-discoloration was analysed using specially developed software (based on van Loon *et al.*, 1995; 1996) according to the CIE L*a*b* colour system (Robertson, 1990). Pictures taken give RGB colour space values which first need to be transformed to CIE XYZ values, for which the following transformation matrix is used (Fairman *et al.*, 1997),

0.49	0.31	0.20
0.17697	0.81240	0.01063
0.00	0.01	0.99.

The CIE XYZ values can then be transformed to CIE La*b* values (Schanda, 2007), with the following formulas,

$$L^* = 116 \int (Y/Y_n) - 16$$

$$a^* = 500 [\int (X/X_n) - \int (Y/Y_n)]$$

$$b^* = 200 [\int (Y/Y_n) - \int (Z/Z_n)].$$

Parameters were calculated for each mushroom individually and performed in the L, a*, b* colour space (Robertson, 1990). However, it must be noted that the software is not calibrated to the standard L, a*, b* colour space and as a consequence the outcome cannot be compared to results of other instruments. Images can only be compared mutually.

The position of each of the 10 mushrooms on the tray is set by the position calibrator, which identifies and numbers the locations of every mushroom in the tray. The measurement area (Figure 2) is a circle on the centre of the mushroom, whose radius is established such that 50 % of the cap area is selected to exclude shadow effects. To calculate the bruising parameters, three distinct areas of the mushroom are defined; the control area, bruise boundary and bruised area (Figure 2). The control area contains only undamaged tissue. The bruise boundary indicates the boundary of the area which is bruised. The bruised area contains only bruised tissue. The bruising parameters used in this research are the whiteness index (WI) and the whiteness index difference (WI difference). WI is calculated as L-(3xb*), as defined by Hunter (2008). The WI difference is the difference between the average WI of the bruised area and the average WI of the control area.

2.5 Outlier test

After calculating the bruising parameters from the normalised pictures, the data were analysed with Matlab. This program calculated the most deviant values (outliers) for each ten mushrooms on a tray. This calculation is based on an analysis of variances of the trays. Mushrooms within a tray with a standardized residual of more than 2.3 were rejected and not used in further analysis.

2.6 Statistical analysis

Statistical analysis was performed using Genstat (Edition 13th). The linear mixed model (REML) was used to calculate significant differences between strains

with a level of significance of 0.05, this was done with the least significant difference (LSD).

Table 2. Result of bruising-sensitivity of 24 white *A. bisporus* strains after 60 min.
Least significant difference (LSD) shown at 0.05 level of significance.

Number	Strain	WI diff	Stdev	LSD
1	Commercial hybrid 1	10.81	7.70	a
2	Traditional off-white strain 1	13.80	4.46	ab
3	Wild white strain 1	14.11	4.97	abc
4	Traditional off-white strain 2	16.58	6.00	bc
5	Traditional off-white strain 3	17.07	3.95	bc
6	Commercial hybrid 2	17.08	4.25	bcd
7	Traditional off-white strain 4	18.54	4.06	cde
8	Traditional white strain 1	18.95	3.41	cde
9	Commercial hybrid 3	19.69	4.40	cde
10	Wild white strain 2	19.75	3.77	cdef
11	Commercial hybrid 4	20.84	3.24	cdefg
12	Wild white strain 3	21.28	3.77	defg
13	Traditional off-white strain 5	21.56	4.83	defg
14	Wild white strain 4	22.53	5.47	defg
15	Commercial hybrid 5	22.55	4.77	defg
16	Traditional white strain 2	22.69	4.46	defgh
17	Traditional white strain 3	23.12	5.00	efghi
18	Wild white strain 5	23.49	5.25	fghi
19	Traditional off-white strain 6	24.03	5.60	fghi
20	Traditional white strain 4	25.26	5.36	ghi
21	Wild white strain 6	25.88	6.57	hi
22	Traditional white strain 5	27.90	5.37	i
23	Wild white strain 7	28.23	4.85	j
24	Wild white strain 8	38.32	4.77	k

Table 3. Result of bruising-sensitivity of 22 brown *A. bisporus* strains after 60 min.
Least significant difference **(**LSD) shown at 0.05 level of significance.

Number	Strain	WI diff	Stdev	LSD
1	Wild brown strain 1	-0.10	7.42	a
2	Wild brown strain 2	0.29	5.80	a
3	Wild brown strain 3	1.04	8.43	a
4	Wild brown strain 4	1.06	5.43	a
5	Wild brown strain 5	3.03	4.54	ab
6	Wild brown strain 6	4.87	5.17	abc
7	Wild brown strain 7	5.07	6.20	abc
8	Wild brown strain 8	5.41	6.59	abc
9	Traditional brown strain 1	6.72	5.49	abcd
10	Wild brown strain 9	8.04	5.46	abcd
11	Wild brown strain 10	9.10	3.63	abcde
12	Wild brown strain 11	9.36	6.24	bcde
13	Wild brown strain 12	9.88	7.30	bcde
14	Traditional brown strain 2	10.68	6.08	bcde
15	Wild brown strain 13	11.60	4.11	cde
16	Wild brown strain 14	12.87	7.84	cdef
17	Traditional brown strain 3	13.60	5.07	cdef
18	Traditional brown strain 4	13.62	6.14	def
19	Traditional brown strain 5	17.96	3.54	ef
20	Wild brown strain 15	20.36	7.44	fg
21	Wild brown strain 16	20.61	5.02	fg
22	Wild brown strain 17	24.71	6.41	g

3 Results

3.1 Primary selection of strains

In order to select a representative set of mushrooms differing in bruising sensitivity a large number of old and present-day cultivars and wild collected strains from the ARP collection were grown. Mushrooms were picked at 35-50 mm diameter and bruised once. Both flush 1 and flush 2 mushrooms were used. As software had not been developed yet, pictures were classified into six classes (from no visible bruise until severe bruising). The more tolerant strains hardly showed any browning symptoms, indicating that repeated spatula treatments are probably necessary to differentiate within these strains. In total ten strains were selected (Table 1) to optimise the bruising method.

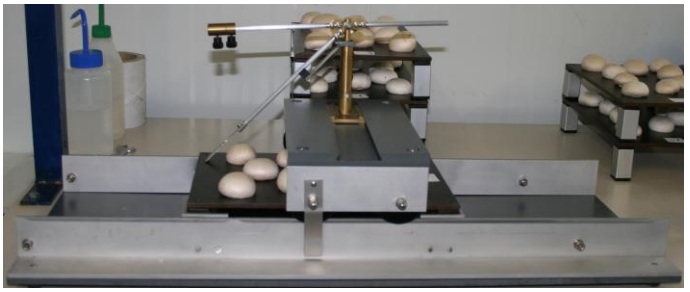


Figure 1. Bruising device. The black tray can hold ten mushrooms, with the stem placed through the hole. The spatula can be moved by hand over the tray. The weights on the horizontal bar can be adjusted.

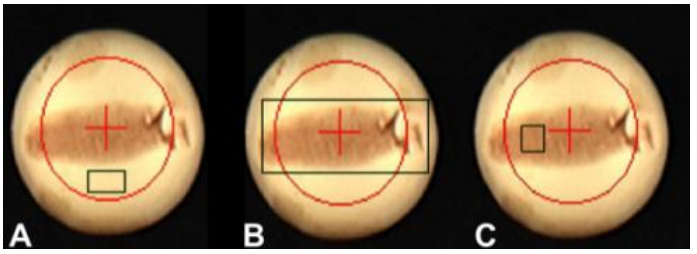


Figure 2. Region of interest and measurement area. Control area (A), Bruise boundary (B), Bruised area (C). The red circle is the measurement area (set at 50 % of detected surface).

3.2 Pictures taken at different time points after bruising

To optimize the bruise-related browning sensitivity method, several parameters were tested. First the time frame after bruising the mushrooms, in which pictures should be taken was analysed in order to determine the most differential time points. Ten mushrooms in a tray were bruised three times with the spatula. Time points beyond 120 min are not practical for large numbers of samples. Besides that it was shown with other experiments (data not included) that the division of bruised mushrooms into tolerant and sensitive does not change with increasing time points. There is an increase in discoloration in time, but this flattens out for longer time periods. This together with the practical point of view lead to the decision not to use time points after 120 min. The results are shown for four different *A. bisporus* strains (Figure 3 and 4).

The 0.01 time point was added for every strain with 0 as the starting WI difference before bruising of the mushrooms. The increase in WI difference was large in the first 5 minutes for all four strains, but increase was highest for Somycel X135 (as indicated by the slope in Figure 3). The slope was calculated with the curve fitted as [a×ln(x) + b], and a is used for the slope. After 60 min there was a clear difference between the bruising-sensitive strains, Bisp 051 and Somycel X135, and the bruising-tolerant strains, Horst U1 and Darlington 735 (Figure 4). Of the four strains tested, discoloration of Bisp 051 increased the fastest when the slope of the first 5 minutes was compared with that after 120 minutes. Discoloration speeds for Somycel X135 and Darlington 735 were almost identical after 5 and 120 min. The discoloration speed of Horst U1 was slightly increased after 120 min as compared to 5 min. The 60 min time point was chosen as suitable for the assessment of bruise-related browning and will be further used in this study. For more detailed analysis, for instance to differentiate between fast discoloration and the slower maintenance of discoloration also shorter time points might be used.

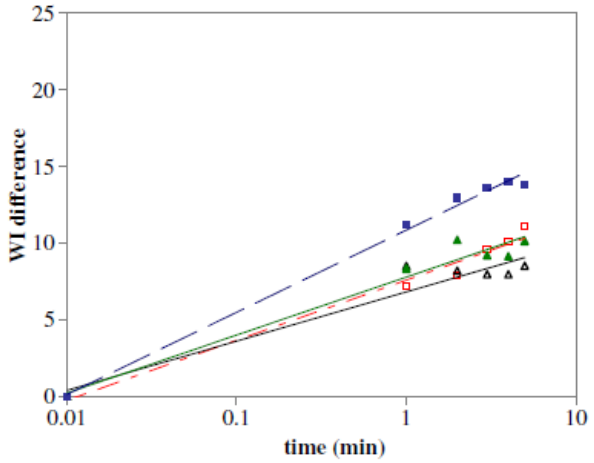


Figure 3. Discoloration followed in time. Mushrooms were bruised three times with the spatula. Mushroom strains used were Somycel X135 (■), Bisp 051 (□), Darlington 735 (▲) and Horst U1 (△).Trend line Somycel X135 (– –) y = 2.3241ln(x) + 10.878, R^2 = 0.9927. Trend line Bisp 051 (– · –) y = 1.6999ln(x) + 7.6199, R^2 = 0.9814. Trend line Darlington 735 (–) y = 1.6389ln(x) + 7.8171, R^2 = 0.9593. Trend line Horst U1 (--) y = 1.3916ln(x) + 6.8521, R^2 = 0.9254.

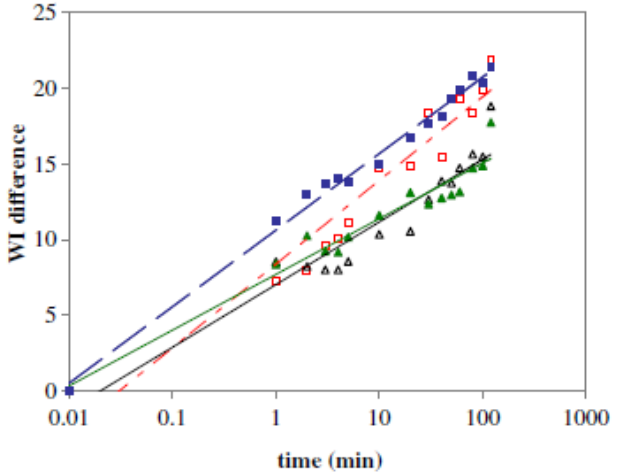


Figure 4. Discoloration followed in time. Mushrooms were bruised three times with the spatula. Mushroom strains used were Somycel X135 (■), Bisp 051 (□), Darlington 735 (▲) and Horst U1 (△). Trend line Somycel X135 (– –) y = 2.1945ln(x) + 10.632, R^2 = 0.9902. Trend line Bisp 051 (– · –) y = 2.401ln(x) + 8.3873, R^2 = 0.9332. Trend line Darlington 735 (–) y = 1.5978ln(x) + 7.7085, R^2 = 0.9428. Trend line Horst U1 (--) y = 1.7962ln(x) + 7.034, R^2 = 0.917.

3.3 Extent of bruising

The discoloration of the mushroom cap depended on the damage applied with the bruising device. The spatula could cause more bruising when more force was applied, but increased force introduced tissue damage and curled the upper epidermis tissue (pictures not shown). To prevent this, the number of times a similar shear and force stress was applied to the cap tissue of mushrooms was varied. To determine the optimal stress force, mushrooms were bruised 1, 3, 5 or 10 times with the spatula. A comparison between two strains and the number of spatula treatments after 60 min is presented in Figure 5. For the least sensitive strains, bruising only once was insufficient and only yielded a hardly visible brown discoloration. When bruising was repeated ten times, the damage to the cap tissue was too severe and skin tissue peeled off, because of repeatedly damaging the same area of the skin tissue. The difference between the WI difference of Le Lion X20 and Sinden A61 was highest when bruising is applied three times (Figure 5). In all future experiments mushrooms were bruised three times.

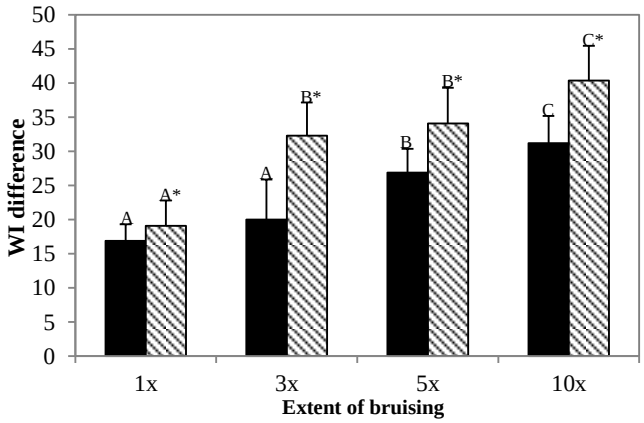


Figure 5. Bruising applied 1, 3, 5 or 10 times with the spatula. WI difference was determined for the 60 min pictures for two different strains. The black bar is Le Lion X20 and the striped bar is Sinden A61. Error bars are from standard deviation. Significance is indicated per strain, Le Lion X20 is normal letter, Sinden A61 with asterisk. Values from the same strain with the same letter are not significantly different at the 0.05 level.

3.4 Time between harvest and bruising

During large experiments, mushroom harvesting and bruising experiments cannot always be performed immediately consecutively. To analyse the influence of the time span between harvest and applying the bruise, mushrooms were harvested and stored in closed humidified boxes for 2, 4, 6 or 24 hours at 20 °C before they were bruised. Bruise-related browning sensitivity increased with increased length of the storage period after harvest of Le Lion X20 (Figure 6). Increased bruising-sensitivity was limited when comparing 2 and 4 hours of storage (no significant difference). Other strains analysed showed the same pattern, although the 6 hour time point was not always significantly different from the 2 and 4 hour storage periods. Bruising 24 hours after picking was always significantly different from the 2 and 4 hour time point. To obtain reproducible and standardized measurements, it was decided to analyse mushrooms within 4 hours after harvest.

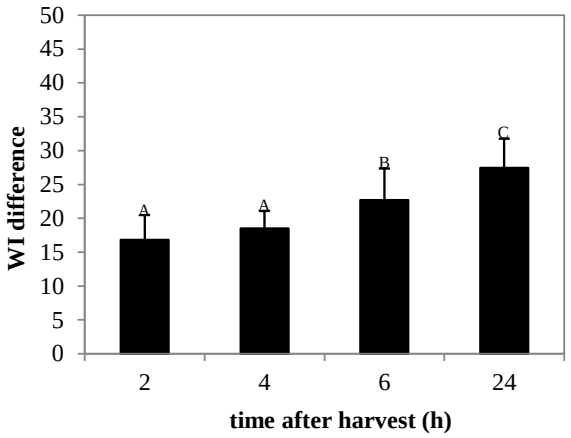


Figure 6. Influence of time after harvest on discoloration. Mushrooms were bruised three times with the spatula, 2, 4, 6 or 24 hours after harvest. WI difference was determined for the 60 min pictures of Le Lion X20. Error bars are from standard deviation. Values with the same letter are not significantly different at the 0.05 level.

3.5 Effect of developmental stage

Fruiting body formation can be very irregular and therefore it was analysed whether development stages of the mushroom influence its bruising-sensitivity. Mushrooms were divided into three groups based on size: small (25-35 mm), medium (35-55 mm) and large (55-70 mm). Within each size group a subdivision was made according to developmental stage: closed caps (cc), veiled caps (vc) or open caps (oc). These developmental stages were based on the Hammond and Nichols (1975 and 1976) classification system. It was not possible to obtain the different developmental stages for all strains. In Figure 7 the results are shown for strain Le Lion X20 which resulted in the most complete collection of developmental stages (no bar in the Figure means that there were no mushrooms available for the analysis). For Le Lion X20 developmental stages appeared to influence bruising sensitivity, large-oc, medium-cc and medium-vc gave the lowest WI difference, indicating a relative low bruising sensitivity (mutual not significantly different). Other developmental stages of Le Lion X20 gave higher WI differences, indicating a relative higher bruising-sensitivity for these developmental stages. This developmental stage dependent bruising-sensitivity was found for other strains as well although a strain like Sinden A61 did not show significant WI differences. To increase reproducibility and decrease variability between experiments, mushrooms from the same developmental stage should be used for quantitative comparisons. As medium sized mushrooms are by far the easiest obtained in a flush and most often used for commercial purposes, future experiments are limited to the medium sized, closed or veiled mushrooms.

3.6 Effect of flush number

It is known from previous research and commercial practice that flush number is of influence on discoloration of mushrooms. That is why the effect of flush for our tester strains was tested, using the developed method, in order to analyse

whether the bruising-tolerance level might vary due to flush number. In general, the later flushes produce fewer mushrooms and some strains did not produce enough mushrooms in the third flush to test bruising sensitivity. Nevertheless, the main trend observed was that mushrooms of the second flush were less sensitive to bruising than mushrooms of the first flush, which in turn were less sensitive than mushrooms from the third flush (data not shown). This is comparable to the results found by Burton and Noble (1993) and Bartley *et al.* (1991), who analysed browning prior to harvest and during storage with and without bruising, respectively. Both flush 1 and flush 2 material will be used in coming experiments as these flushes are also used in practice.

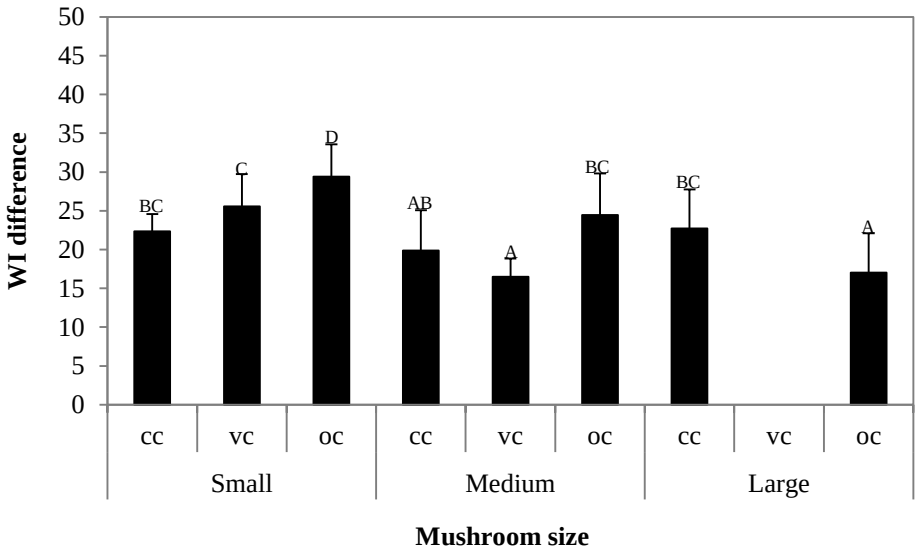


Figure 7. Influence of mushroom size on discoloration. Mushrooms were divided into three classes based on size: small (25-35 mm), medium (35-55 mm) or large (55-70 mm). Within each size group a subdivision was made according to developmental stage; closed caps (cc), veiled caps (vc) or open caps (oc). Mushrooms were bruised three times with the spatula. WI difference was determined for the 60 min pictures of Le Lion X20. No bar in the figure means that there were no mushrooms available for the analysis. Error bars are from standard deviation. Values with the same letter are not significantly different at the 0.05 level.

3.7 Overview of the developed bruising method

As described in the previous sections, medium sized closed or veiled mushrooms from the first and second flush are used to analyse bruising-sensitivity by applying three times a slip shear force with the spatula over the cap tissue (with the weight of the spatula adjusted to 40 g). Mushrooms are bruised within 4 hours after harvest. Pictures of the bruised mushrooms are taken 60 min after bruising and analysed with the computer image analysis system.

3.8 Bruising-sensitivity of 46 different A. bisporus strains

The method described above was now used to characterize the genetic variation of bruising sensitivity among a collection of 24 white and 22 brown *A. bisporus* strains (Tables 2 and 3). Comparison of the bruising-sensitivity of the tested strains indicated that a considerable variation in bruising-sensitivity exists among button mushrooms. At this stage of comparing bruising-sensitivity of white and brown capped mushrooms it is not clear if WI differences can be influenced by these different background colours. That is why both types of cap coloured mushrooms were analysed separately (Table 2 for white capped strains and Table 3 for brown capped strains). Among the white strains it was found that some of the commercial strains, such as commercial hybrid 1, 2 and 3, were among the least sensitive strains but that also some wild strains had low bruising sensitivity (Table 2). Among the white button mushroom strains also very sensitive strains were identified.

For the brown cap coloured strains only one old cultivar (traditional brown strain 1) was found among the most tolerant strains and was not significantly different in bruising-sensitivity in comparison to some of the wild strains tested. Data indicated that for the brown capped coloured strains the variation among the randomly selected strains was larger than for the white cap coloured strains (a WI diff of 10.8 to 38.32 for the white strains versus a WI diff of -0.1 to 24.71 for the

brown cap coloured strains). Within this small selection it was also found that thirteen brown strains have a lower WI difference than found for the most tolerant white strain. Five bruising sensitive white strains appeared more sensitive than the most sensitive brown strain, but the most sensitive white strain is only 3.8 times more sensitive than the most tolerant white strain. Several strains were included in more than one experiment and it was shown that the classification in to tolerant, moderate and sensitive was reproducible (data not shown).

4 Discussion

Discoloration of mushrooms due to bruising is a significant loss of quality caused by (mechanical) handling, conveyer belts or transportation. In order to unravel the mechanisms behind bruising-sensitivity, to compare pre- or postharvest treatments and to breed for bruising-tolerant strains, it will be necessary to have access to a reliable bruising quantification method. Here factors were studied that influence the reproducibility, internal sample variation and quantification of the bruise-related brown discoloration. This resulted in a standard protocol to determine bruising-sensitivity in a quantitative manner. The protocol takes into account the use of medium sized, closed or veiled mushrooms from flush 1 and 2. According to the protocol, mushrooms are bruised three times with the spatula within 4 hours after harvest and pictures are taken 60 min after bruising. Quantification of the discoloration is based on measuring the WI both of bruised and undamaged tissue of the same cap and subtracting the WI value of the undamaged tissue from the WI value of the bruised tissue. With the new bruising device it was possible to bruise a large number of samples in a short time period and in a reproducible way. This is the first research describing in detail the full method and the factors that have to be taken into account to quantify bruising-sensitivity. Research performed by Burton (2004) has shown the development of a device but has not shown the influences of above mentioned parameters and

therefore no robust protocol was available yet. Another improvement of our method compared to Burton is that the mushrooms are used as whole mushrooms and not dissected into three parts before bruising and quantification.

To increase reproducibility and decrease variability between experiments, mushrooms from the same developmental stage should be used for quantitative comparisons. As medium sized mushrooms are by far the easiest obtained in a flush and most often used for commercial purposes, future experiments are limited to the medium sized, closed or veiled mushrooms. Research on long time storage is also needed, because it is necessary to know whether a strain that is less sensitive to bruising can be stored for commercial use. In this research only storage between harvest and bruising at 20 °C was analysed. Mushrooms stored for 24 h showed a significant increase in bruising-sensitivity in comparison to 2, 4 and 6 hours of storage. This can be due to loss of water, membrane damage and enzyme activation, causing higher browning-discoloration sensitivity.

The bruising method is also useful for the comparison of a large collection of strains, of which a first result is shown in this article. Twenty-four white and 22 brown *A. bisporus* strains were tested. Although the selection was chosen randomly, it can be stated that there was a larger variation in sensitivity within the brown strains than in the white strains. A higher level of bruising-tolerance was found within the brown strains compared to the white strains tested.

Another use of the application can be found in breeding for bruising-tolerant mushrooms. Strains can be selected to generate new hybrid populations of which the bruising-discoloration will be compared to the parental lines. New mushroom breeding strategies, including marker assisted selection have been developed for *A. bisporus* (Sonnenberg *et al.,* 2006). Data for the development of quantitative trait loci markers should not only describe extremes, but also intermediate phenotypes. Besides accuracy of the trait, the size of the segregating populations and the density of the genetic map are important (Sonnenberg *et al.,* 2006). So, a high throughput

method as described in this article will be needed. The bruising-tolerant and bruising-sensitive strains as described here can be used to generate populations and study the segregation of the trait. By making use of genetic linkage maps like generated by Foulongne-Oriol *et al.* (2010) marker assisted selection can be used to find chromosomal loci that influence the bruising and discoloration trait.

The developed method can be used to quantitatively study the influence of agronomic and environmental factors on bruising-discoloration. Burton *et al.* (Burton, 2000; Burton and Rama, 2001; Burton, 2002; Burton *et al.*, 2003) have identified that the major factors influencing mushroom bruising sensitivity are related to water as casing water, humidity and, to a lesser extent, calcium chloride. In the present study these agronomic and environmental factors were kept constant for all strains so that differences were linked to a genetic origin rather than to other factors. The influence of agronomic and environmental factors will be studied when new hybrids become available, because then an optimal cultivation protocol for each new strain can be provided.

A last application of this new bruise-related browning sensitivity quantification method is in depth research to unravel the molecular and biochemical pathways behind the trait. These studies can be focused on substrate availability and enzyme activity, as reviewed in Jolivet *et al.* (1998). Different starting substrates lead to the formation of different types of melanin, of which the intermediate products have different colours. Dedicated transcriptomics, metabolomics and proteomics will be needed to unravel the molecular and biochemical pathways behind brown discoloration.

Acknowledgements

This project is supported by the governmental grant “TTI Green Genetics”, Dutch Board for Horticulture, Bromyc, Lutèce, Sylvan, Banken Mushrooms, the Greenery and WeBe Engineering.

References

Bartley, C.E., Beelman, R.B., Winnett, J.R. (1991) Factors affecting colour of cultivated mushrooms (*Agaricus bisporus)* prior to harvest and during postharvest storage. *Mushroom Science, XIII* , 689-694.

Burton, K.S. (2002) Bruising means lost mushroom sales. *Mushroom Journal, 624,* 23-24.

Burton, K.S. (2004) Cultural factors affecting mushroom quality - cause and control of bruising. *Mushroom Science, XVI*, 397-402.

Burton, K.S., Beecher, T.M. (2000) The effects of calcium chloride on mushroom whiteness. *Mushroom Journal, 601*, 21-22.

Burton, K.S., Malloy, S., Willoughby N. (2003) Water is the key to bruising. *Mushroom Journal, 641*, 26-28.

Burton, K.S., Noble, R. (1993) The influence of flush number, bruising and storage temperature on mushroom quality. *Postharvest Biology and Technology, 3*(1), 39-47.

Burton, K.S., Rama, T. (2001) Bruising a market effect. *Mushroom Journal, 617,* 20-22.

Fairman, H.S., Bril, M.H., Hemmendinger, H. (1997) How the CIE 1931 color-matching functions were derived from wright-guild data. *COLOR Research and Application, 22,* 11-23.

Foulongne-Oriol, M., Spataro, C., Cathalot, V., Monllor, S. Savoie, J.M. (2010) An expanded genetic linkage map of an intervarietal *Agaricus bisporus* var. *bisporus* x *A. bisporus* var. *burnettii* hybrid based on AFLP, SSR and CAPS markers sheds light on the recombination behaviour of the species. *Fungal Genetics and Biology, 47*(3), 226-236.

van Gils, J.J. (1988) Nutrition and compost. In: Laboratories Dm (Ed) *Nutrition and compost* (pp. 29-72) Rustington, United Kingdom.

Hammond, J.B.W., Nichols, R. (1975) Changes in respiration and soluble carbohydrates during the post-harvest storage of mushrooms (*Agaricus bisporus*). *Journal of the Science of Food and Agriculture, 26(6),* 835-842.

Hammond, J.B.W., Nichols, R. (1976) Carbohydrate Metabolism in *Agaricus bisporus* (Lange) Sing.: Changes in soluble carbohydrates during growth of mycelium and sporophore. *Journal of General Microbiology, 93(2),* 309-320.

Hunter L, a, b color space. Application note (2008) *9*(9), www.hunterlab.com accessed November 10th 2010.

Jolivet, S., Arpin, N., Wichers, H.J., Pellon, G. (1998) *Agaricus bisporus* browning: a review. *Mycological Research, 102*(12), 1459-1483.

Kerrigan, R.W. (1996) Characteristics of a large collection of edible wild mushroom germ plasm: the *Agaricus* Resource Program. In Samson, R.A. *et al.*, (Eds.) *Culture Collections to Improve the Quality of Life* (pp. 302-307) Centraalbureau voor Schimmelcultures, P.O. Box 273, 3470 AG Baarn, The Netherlands.

van Loon, P.C.C. (1996). Het bepalen van het ontwikkelingsstadium bij de champignon met computer beeldanalyse. *Champignoncultuur, 40*(9), 347-353.

van Loon, P.C.C., Sonnenberg, A.S.M., Swinkels, H.A.T.I., Van Griensven, L.J.L.D., van der Heijden, G.W.A.M. (1995). Objective measurement of developmental stage of white button mushrooms (*Agaricus bisporus*). *Mushroom Science*, *XIV*, 703-708.

Robertson, A.R. (1990) Historical development of CIE recommended colour difference equations. *Color Research and Application, 15*(3), 167-170.

Schanda, J. (2007) Colorimetry. *Understanding the CIE system.* Wiley-Interscience,. ISBN 9780470049044 (pp. 61-63).

Sonnenberg, A.S.M., Baars, J.J.P., Hendrickx, P.M., Kerrigan, R.W. (2006) Breeding mushrooms: state of the art. *Fifth international conference on mushroom biology and mushroom products, 12*, 163-173.

Chapter 3

Bruising-sensitivity of button mushrooms

A. Weijn[a,b], M.M.M. Tomassen[a], S. Bastiaan-Net[a], E.A.H.J. Hendrix[c], J.J.P. Baars[c], A.S.M. Sonnenberg[c], H.J. Wichers[a,b] and J.J. Mes[a]

a Food & Biobased Research, Wageningen University and Research Centre, Bornse Weilanden 9, 6708 WG Wageningen, The Netherlands.
b Laboratory of Food Chemistry, Wageningen University and Research Centre, Bomenweg 2, 6703 HD Wageningen, The Netherlands
c Plant Research International – Mushrooms, Wageningen University and Research Centre, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands

Proceedings of the 7th International Conference on Mushroom Biology and Mushroom Products (ICMBMP7) 2011
Section: Physiology and Development, pp 203-211

Abstract

To study sensitivity of mushroom to bruising, a reproducible method was developed to apply mechanical damage to mushroom caps and quantify the subsequent discoloration. The newly developed bruising device can apply damage to the cap tissue of intact button mushrooms by a slip-shear sliding process in a fast and reproducible way. A protocol has been developed to obtain the most reliable and reproducible method to compare bruising sensitivity of different *Agaricus bisporus* strains. The severity of the bruise is quantified with a computer image analysis system. Pictures of the bruised mushroom caps were taken under controlled lighting conditions and calibrated to a local reference. Image analysis software was developed to calculate the whiteness index (L-(3xb*), as defined by Hunter). This method of bruising and image based quantification was subsequently applied to a collection of wild, commercial and hybrid *Agaricus bisporus* strains. A significant difference was found between bruising sensitive mushrooms and bruising tolerant mushrooms. A correlation was found between discoloration by the bruising device and discoloration caused by transportation of mushroom on a conveyor belt. Less correlation was found between post-harvest discoloration of undamaged stored mushrooms and the bruising device. This indicates that discoloration caused by bruising or by storage of intact mushrooms might have different mechanisms.

1 Introduction

The quality of *Agaricus bisporus* button mushrooms is determined by colour, texture, cleanliness, maturity, flush number, and flavour. Of these, colour is the most important parameter because it is first perceived by consumers and discoloration decreases the commercial value (Burton, 2004). Due to picking, handling and storage, discoloration reactions are initiated. Contact-based discoloration, or bruising, is caused by a mechanical process known as 'slip-shear' (Burton, 2004), a downwards force and a sideways movement, which can occur during picking by hand or by robotic picking equipment. Mechanical harvesting is more cost-efficient than picking by hand, but cannot be applied yet to serve the fresh market, as commercial strains are too sensitive to bruising. It has been shown that enzymatic browning of mushrooms is caused by polyphenol oxidases (PPOs: tyrosinases and laccases) and peroxidases through an enzyme-catalysed oxidation of phenolic substrates into quinones (Jolivet *et al.*, 1998). These products undergo subsequent reactions leading to the formation of the dark pigment melanin.

This project aims to develop a high throughput tool to quantify bruising-sensitivity of mushrooms. This can support the selection of bruising-tolerant strains, suitable for mechanical harvesting of mushrooms for the fresh market. In order to breed for bruising-tolerant lines and to study the molecular and biochemical processes in depth it will be necessary to determine the bruise-related browning sensitivity in a reproducible way. Burton (2004) has developed a device to apply a slip-shear stress on mushroom slices. However, the damage applied was much stronger than in practise occurs and the method was too laborious to apply to a large number of samples.

Here we describe the use of a newly developed bruising device and image analysis system to quantify bruising-sensitivity (Weijn *et al.*, 2012). In order to develop a reliable and reproducible method, several parameters were studied in previous experiments. The parameters investigated were the influence of flush, the

effects of the developmental stage of the mushrooms, the time between harvest and applying the bruise, and the time between bruising and analysing discoloration. A collection of *A. bisporus* strains was screened for their bruising-sensitivity in order to analyse the phenotypic variation among strains. This method identified the genetic variation of bruising sensitivity among strains and now can support unravelling the molecular and biochemical basis for this trait. In this paper we describe the correlation between discoloration by the developed bruising method and discoloration caused by a conveyor belt and discoloration after seven days storage at 4 °C or 8 °C at 90 % RH of the same mushroom varieties.

2 Material and methods

2.1 Mushroom strains

Based on a previous collection screen of *A. bisporus* strains, several strains were chosen and cultivated. Eleven white mushroom strains were grown in seven replicates and four brown strains in two replicates (Table 1). In each experiment, the strains were randomly distributed over the growing room. Strains used in this research originated from the department of Plant Breeding at Wageningen UR and represent old and present day cultivars and wild collected varieties (ARP culture collection; (Kerrigan, 1996)). The mushrooms were grown as described elsewhere (Weijn *et al.*, 2012).

2.2 Bruising device

A new mushroom bruising device was constructed, which is able to apply damage to the cap tissue by a slip-shear sliding process using a spatula on a moving wheel (Figure 1) (Weijn *et al.*, 2012). Ten mushrooms were fixed on a tray and bruised in series. The force with which the spatula presses on the mushroom caps can be adjusted by the weights placed on the horizontal bar on top of the machine. After initial experiments the total weight of the spatula was set to 40 g. Mushrooms were kept at 20 °C at all time after harvest. After a recorded period of

time, the mushrooms were placed into an illuminated cupboard (four Philips Fluotone TLD on each side of the tray) equipped with a photo-camera (JVC KY-F30E colour video camera with a JVC TV UM lens) and pictures were taken.

Table 1. Names and abbreviations of the *A. bisporus* strains.

Strain	Abbreviation
Commercial hybrid 1	CH1
Commercial hybrid 2	CH2
Commercial hybrid 3	CH3
Traditional white strain 4	TW4
Traditional off-white strain 3	TO3
Traditional off-white strain 6	TO6
Traditional off-white strain 7	TO7
Traditional off-white strain 8	TO8
Wild white strain 1	WW1
Wild white strain 7	WW7
Wild white strain 9	WW9
Wild brown strain 2	WB2
Wild brown strain 4	WB4
Wild brown strain 16	WB16
Wild brown strain 17	WB17

2.3 Computer image analysis

The developed quantitative image analysis system can measure various parameters from colour images of mushrooms and gives a quantitative value of discoloration. All images of a single experiment were calibrated using the standard Gretag-Macbeth colour checker (MSCCC) in order to compensate for small, unintentional variations in colour and illumination during the course of an experiment. A sheet of white paper was used to calibrate for the brightness and to compensate for variations in illumination over the cupboard. The bruising-discoloration was analysed using specially developed software as described elsewhere (Weijn *et al.*, 2012).

The position of each of the ten mushrooms on the tray is identified and numbered automatically. The measurement area (Figure 2) is established such that 50 % of the cap area is selected to exclude shadow effects. The bruising parameters

used for the bruising applied by the bruising device are the whiteness index (WI) and the whiteness index difference (WI difference). WI is calculated as L-(3xb*), as defined by Hunter (2008). The WI difference is the difference between the average WI of a representative spot on the bruised area and the average WI of the control, not bruised tissue on the same mushroom (Figure 2). Tissue of the mushrooms after conveyer belt or after storage do not contain a control area. In this case the WI difference of the conveyor belt was calculated as the difference of the WI of the measurement area before and after the conveyer belt bruising at specific time points. The WI difference after storage was calculated as the difference between the WI of the measurement area before storage and the WI after seven days of storage.

2.4 Outlier test

After calculating the bruising parameters from the normalised pictures, the data were analysed with Matlab. This program calculated the most deviant values (outliers) for each ten mushrooms on a tray. This calculation is based on an analysis of variances of the trays. Mushrooms within a tray with a standardized residual of more than 2.3 were rejected and not used in further analysis.

2.5 Statistical analysis

Statistical analysis was performed using Genstat (Edition 13th). The linear mixed model (REML) was used to calculate significant differences between strains with a level of significance of 0.05, this was done with the least significant difference (LSD).

3 Results

3.1 Developed bruising method

In previous experiments, the optimal method to bruise mushroom with the bruising device was determined (Weijn *et al.*, 2012). Medium sized (35-55 mm) closed or veiled mushrooms from the first and second flush were used to analyse bruise-related browning sensitivity by applying three times a slip shear force with the spatula over the cap tissue (with the weight of the spatula adjusted to 40 g). Mushrooms were bruised within 4 hours after harvest. Pictures of the bruised mushrooms are taken 60 min after bruising and analysed with the computer image analysis system. The computer image analysis system can be used to determine the WI of a specific bruised area on the cap of the mushroom or of the whole cap surface, which is set at 50 % of the total cap area (circle in Figure 2).

Figure 1. Bruising device. The spatula can be moved by hand over the tray. The weights in the horizontal bar can be changed (set at 40 g in our experiments).

3.2 Bruise-related browning sensitivity of fifteen different A. bisporus strains

The method described above was used to characterize the genetic variation of bruising sensitivity among a small collection of eleven white and four brown *A. bisporus* strains (Figure 3 and 4). Comparison of the bruising-sensitivity of the tested strains indicated that a considerable variation in bruising-sensitivity exists among button mushrooms. At this stage of comparing bruising-sensitivity of white and brown capped mushrooms, we are not sure whether WI differences can be influenced by large differences in cap background colours. That is why both types of cap coloured mushrooms were analysed separately (Figure 3 for white capped strains and Figure 4 for brown capped strains). Among the white strains it was found that some of the commercial strains, such as commercial hybrid 1, 2 and 3, were among the less sensitive strains and also wild-type white strain 1 showed low bruising-sensitivity (Figure 3). Among the white button mushroom strains also very sensitive strains were identified, such as wild-type white strain 7. For the brown cap coloured strains only wild strains were tested, and these showed a significant difference in bruising sensitivity. Within this small selection it was found that the two tolerant brown strains have a lower WI difference than found for the most tolerant white strain. Several strains were included in more than one experiment and it was shown that the classification of discoloration after bruising into tolerant, moderate and sensitive was reproducible (data not shown).

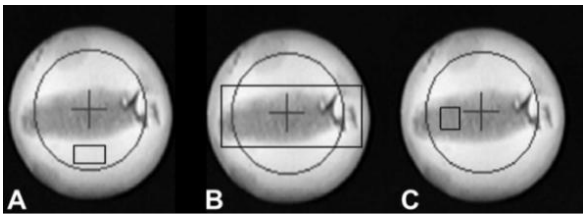


Figure 2. Computer image analysis areas. A control area. B bruise boundary. C bruised area. The circle on each mushroom is the measurement area (set at 50 % of the surface).

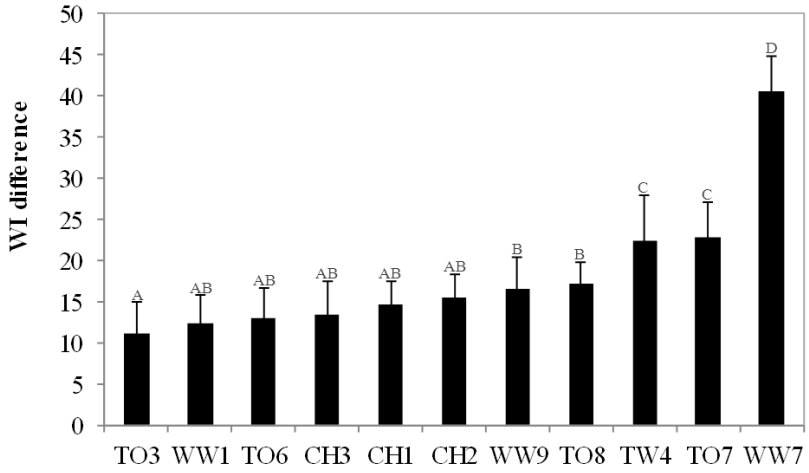


Figure 3. WI difference of eleven white button mushrooms of flush 1, 60 min after bruising. Error bars are from standard deviation. Values with the same letter are not significantly different at the 0.05 level. Abbreviations used correspond with Table 1.

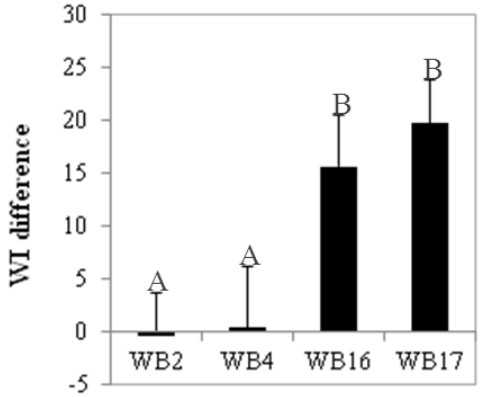


Figure 4. WI difference of four brown button mushrooms of flush 1, 60 min after bruising. Error bars are from standard deviation. Values with the same letter are not significantly different at the 0.05 level. Abbreviations used correspond with Table 1.

3.3 Bruising button mushrooms with a conveyor belt

Mushrooms of seven strains were bruised with a conveyor belt to compare the discoloration after bruising with the results of the in home developed bruising device. A mushroom grower was visited to check the times of bruising during mechanical harvesting and sorting. Approximately twenty falling motions occurred during the sorting process. Mushrooms were applied five times in a row on a conveyor belt of in total 5 m length (Figure 5). Mushrooms of the second flush were used and at least ten mushrooms per time point were used. The WI of the whole cap was measured and not of a specific bruised part. This was done by using the measurement area (the circle in Figure 2). A comparison was made between control (non-bruised) mushrooms at T = 0 min and bruised mushrooms at three time points, 5, 60, and 120 min after bruising.

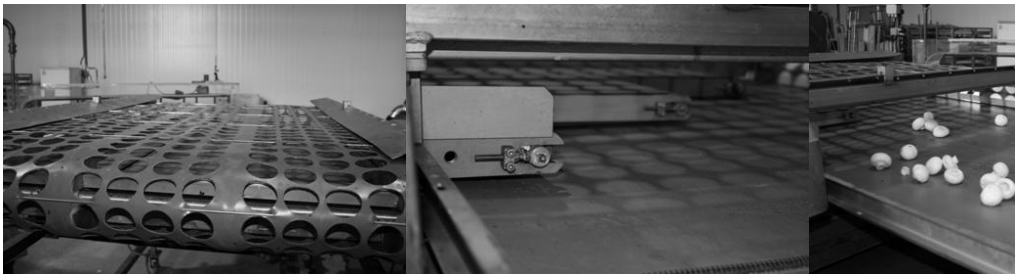


Figure 5. Conveyor belt used for the bruising experiment (from WeBe Engineering).

In Figure 6 the correlation is shown between the WI difference 60 min after bruising with the bruising device and the WI difference of the conveyor belt. The WI difference of the conveyor belt is calculated as the difference between WI control 0 min (C 0 min) and WI bruised 60 min (B 60 min) after bruising with the conveyor belt. A correlation of 0.84 (R^2) was found for the seven strains used. The in home developed bruising device gives a comparable bruising discoloration as industrial used machines. When the only brown strain used was left out, the correlation is higher ($R^2 = 0.96$).

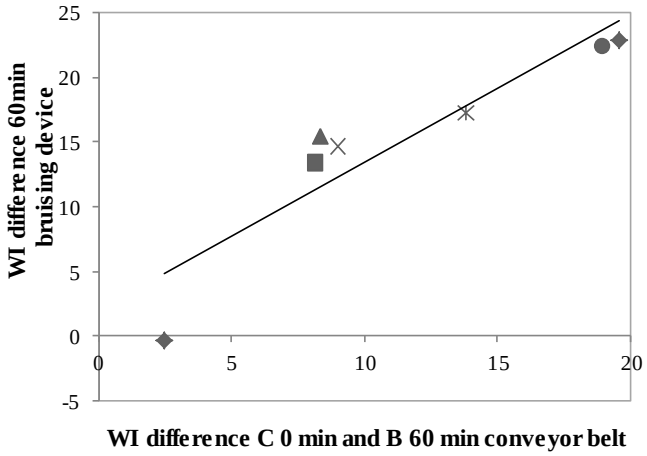


Figure 6. Correlation between conveyor belt bruising and discoloration after bruising with the bruising device. Y = 1.1388x + 2.0361, R^2 = 0.8444 (brown strain left out, gives Y = 0.749x + 7.9264, R^2 = 0.9612). C 0 min = control at T = 0 min, B 60 min = bruised T = 60 min. Abbreviations used correspond with Table 1.

◆ WB2 ■ CH2 ▲ CH3 x CH1 ✳ TO8 ● TW4 + TO7

3.4 Shelf life performance

Flush 2 mushrooms of eleven different strains were stored for seven days at 4 °C or 8 °C at 90 % humidity in boxes with a lid with small holes (as used in Dutch supermarkets). Pictures of the mushrooms were taken at the starting time point and at day 7. Discoloration upon storage was analysed and therefore the mushrooms were not bruised. The WI of the measurement area of the mushroom was analysed (Figure 2). The mushrooms were weighed at day 0 and day 7 to follow the change in weight upon storage, to achieve approximately the same weight in each box and that boxes were filled completely. In general both white and brown button mushrooms discolour more at 8 °C than at 4 °C, except for wild white strain 1 and traditional off-white strain 3 (Figure 7 for white capped strains and Figure 8 for brown capped strains). The difference between the discoloration upon storage at 4 °C and 8 °C is not the same for every strain. As shown before, the commercial hybrids 1, 2 and 3 show the least discoloration. For the other white and brown

strains that were analysed, there is less correlation between the discoloration after bruising compared to the discoloration after storage. This can be due to the fact that mushrooms strains showed a difference in development during this storage period; some strains were open after 7 days and sporulated, while some strains still contained closed or veiled mushrooms. Smith *et al.* (1993) followed mushroom development (cap opening) during storage at 18 °C for *A. bisporus* U3 and two *Agaricus bitorquis* strains and found a difference in the rate of maturation. One of the *Agaricus bitorquis* strains developed much slower, even after 5 days of storage stage 3 mushrooms were found in most cases (as based on Hammond and Nichols, 1976). The variation in discoloration after storage is less in the brown mushroom strains than found with the bruising device. The weight loss after seven days storage was always higher at 8 °C than 4 °C, except for traditional off-white strain 3 (Table 2).

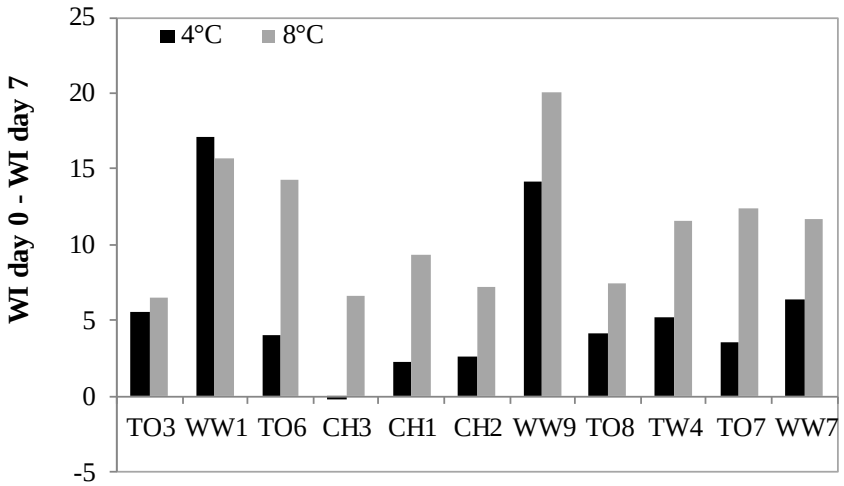


Figure 7. Difference between WI day 0 and WI day 7 after storage at 4 °C or 8 °C of eleven white button mushroom strains. Abbreviations used correspond with Table 1.

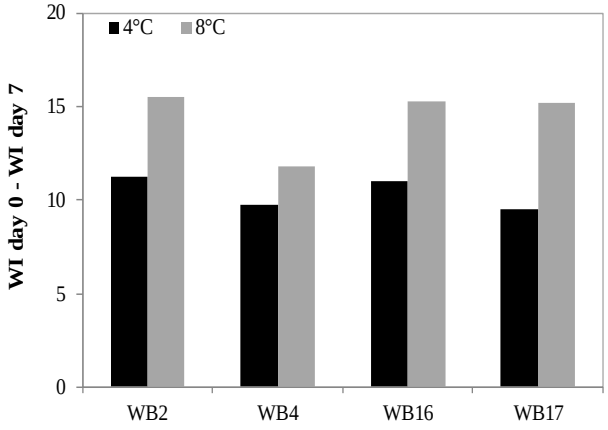


Figure 8. Difference between WI day 0 and WI day 7 after storage at 4 °C or 8 °C of 4 brown button mushroom strains. Abbreviations used correspond with Table 1.

Table 2. Weight loss in g per mushroom after storage for seven days.

Strain	at 4 °C	at 8 °C
Traditional off-white strain 3	1.93	1.08
Wild white strain 1	0.42	0.85
Traditional off-white strain 6	0.44	0.79
Commercial hybrid 3	0.36	2.09
Commercial hybrid 1	0.50	1.58
Commercial hybrid 2	0.54	1.60
Wild white strain 9	0.33	1.05
Traditional off-white strain 8	0.28	0.96
Traditional white strain 4	0.18	1.86
Traditional off-white strain 7	0.31	1.08
Wild white strain 7	0.35	1.11
Wild brown strain 2	0.36	0.69
Wild brown strain 4	0.05	1.51
Wild brown strain 16	0.33	2.53
Wild brown strain 17	0.59	1.92

4 Discussion

Bruising-related discoloration of mushrooms is an important loss of quality caused by picking, conveyor belts, or storage. In order to unravel the mechanisms behind bruising sensitivity, to compare pre- or post-harvest treatments and to breed for bruising tolerant strains, it will be necessary to have access to a reliable bruising quantification method. In previous experiments, factors were studied that influence the reproducibility, internal sample variation, and quantification of the bruise-related brown discoloration. This resulted in a standard protocol to determine bruising-sensitivity in a quantitative manner (Weijn *et al.*, 2012). Quantification of the discoloration is based on measuring the WI both of bruised and undamaged tissue of the same cap and subtracting the WI value of the undamaged tissue from the WI value of the bruised tissue.

Here, the bruising method is compared with bruising by a conveyor belt and by discoloration during cold storage using a collection of strains with different degrees of bruising sensitivity. Although the selection was chosen randomly, it can be stated that there was a larger variation in sensitivity within the brown strains than in the white strains upon bruising with the bruising device.

The results indicate that there is a correlation between the bruising device induced discoloration and conveyer belt damaging sensitivity. Including more strains and mushrooms from the same flush in this type of analysis will allow to further detail the correlation between different mushroom strains. Less correlation was found between the discoloration after cold storage of undamaged mushrooms and 60 minutes after bruising with the device. Possibly, different mechanisms are involved in causing storage or bruising-related discoloration, as the stored mushrooms were not bruised. In addition, the loss of water, membrane damage and enzyme activation might cause a different response and result in higher or lower browning sensitivity. The difference in shelf life performance of different strains is also an important factor to mention. For some strains, all mushrooms opened

during seven days of storage and for other strains the mushrooms were closed or veiled. Although not correlating to bruising-sensitivity, the possible genetic variation in shelf-life performance and discoloration of the strains tested can be of interest for the industry to increase shelf life performance by breeding.

The bruise-related browning sensitivity quantification method can be used for in depth research to unravel the molecular and biochemical pathways behind the trait. These studies can be focused on substrate availability and enzyme activity, as reviewed in Jolivet *et al.* (1998). Different starting substrates lead to the formation of different types of melanin, of which the intermediate products have different colours. Dedicated transcriptomics, metabolomics and proteomics can be used to unravel the molecular and biochemical pathways behind brown discoloration.

Acknowledgement

This project is supported by the governmental grant "TTI Green Genetics", Dutch Board for Horticulture, Bromyc, Lutèce, Sylvan, Banken Mushrooms, the Greenery and WeBe Engineering.

References

Burton, K.S. (2004). Cultural factors affecting mushroom quality - cause and control of bruising. *Proceedings of the XVIth International Congress of Science and Cultivation of Edible and Medicinal Fungi* (pp. 397-402). Maimi.

Hammond, J.B.W., Nichols, R. (1976). Carbohydrate Metabolism in *Agaricus bisporus* (Lange) Sing.: changes in soluble carbohydrates during growth of mycelium and sporophore. *Journal of Genetics and Microbioly*, *93*(2), 309-320.

Hunter L, a, b color space. Application note (2008) *9*(9), www.hunterlab.com accessed November 10th 2010.

Jolivet, S., Arpin, N., Wichers, H.J., Pellon, G. (1998). *Agaricus bisporus* browning : a review. *Mycological Research*, *102*(12), 1459-1483.

Kerrigan, R.W. (1996). Characteristics of a large collection of edible wild mushroom germ plasm: the *Agaricus* Resource Program. In Samson, R.A. *et al.*, (Eds.) *Culture Collections toImprove the Quality of Life*. (pp. 302-307) Centraalbureau voor Schimmelcultures P.O. Box 273, 3470 AG Baarn, The Netherlands.

Smith, J.F., Love, M.E., Burton, K.S. (1993). Comparative studies of the quality of fresh and stored mushrooms of *Agaricus bisporus* with two tropical *Agaricus bitorquis* strains. *Annual Applied Biology*, *122*, 593-603.

Weijn, A., Tomassen, M.M.M., Bastiaan-Net, S., Wigham, M.L.I., Boer, E.P.J., Hendrix, E.A.H.J., Baars, J.J.P., Sonnenberg, A.S.M., Wichers, H.J., Mes, J.J. (2012). A new method to apply and quantify bruising sensitivity of button mushrooms. *LWT - Food Science and Technolgy*, *47*(2), 308-314.

Chapter 4

Melanin biosynthesis pathway in *Agaricus bisporus* mushrooms

A. Weijn[a,b], S. Bastiaan-Net[a], H.J. Wichers[a,b] and J.J. Mes[a]

a Food & Biobased Research, Wageningen University and Research Centre, Bornse Weilanden 9, 6708 WG Wageningen, The Netherlands

b Laboratory of Food Chemistry, Wageningen University and Research Centre, Bomenweg 2, 6703 HD Wageningen, The Netherlands

Fungal Genetics and Biology, special issue *Agaricus bisporus* genome
Accepted October 2012, http://dx.doi.org/10.1016/j.fgb.2012.10.004

Abstract

With the full genome sequence of *Agaricus bisporus* available, it was possible to investigate the genes involved in the melanin biosynthesis pathway of button mushrooms. Based on different BLAST and alignments, genes were identified in the genome which are postulated to be involved in this pathway. Seven housekeeping genes were tested of which 18S rRNA was the only housekeeping gene that was stably expressed in various tissues of different developmental stages. Gene expression was determined for most gene homologs (26 genes) involved in the melanin pathway. Of the analysed genes, those encoding polyphenol oxidase (PPO), the PPO co-factor L-chain (unique for *Agaricus bisporus*), and a putative transcription factor (photoregulator B) were among the highest expressed in skin tissue. An in depth look was taken at the clustering of several PPO genes and the PPO co-factor gene on chromosome 5, which showed that almost 25 % of the protein encoding genes in this cluster have a conserved NACHT and WD40 domain or a P-loop nucleoside triphosphate hydrolase. This article will be the start for an in depth study of the melanin pathway and its role in quality losses of the button mushroom, an economically important product.

1 Introduction

Discoloration of mushrooms is a common phenomenon which decreases their commercial value. Due to picking, handling and storage, discoloration reactions are initiated, which are considered to be mediated by enzyme-catalysed oxidation of phenolic substrates into quinones, leading to the formation of brown coloured melanin (Burton, 2004; Jolivet *et al.*, 1998). Microbial infections can also cause brown discoloration and lead to decreased yield and economic losses of the crop. For instance, *Pseudomonas tolaasii* is the causal agent of brown blotch disease (Jolivet *et al.*, 1998), and *Lecanicillium fungicola* is a fungal pathogen that can cause dry bubble disease on button mushrooms (Foulongne-Oriol *et al.*, 2012). In both diseases, brown discoloration is part of the symptoms.

Two pathways for melanin synthesis are described in fungi (Eisenman and Casadevall, 2012). The pathway resulting in 1,8-dihydroxynaphthalene (DHN) melanin starts from the precursor molecule acetyl CoA or malonyl CoA. As far as we know this pathway is not found in *Agaricus bisporus* and therefore is not included in this study. The second melanin pathway is named the L-DOPA pathway after L-3,4-dihydroxyphenylalanine. The melanin forming pathway for *A. bisporus* starts from chorismate, derived from the shikimate pathway (Dewick, 1998). Enzymes convert chorismate into different compounds like γ-L-glutaminyl-4-hydroxybenzene (GHB), *p*-aminophenol (PAP) and L-DOPA. Subsequent steps convert these substrates in GHB-, PAP- and DOPA-melanin, respectively (Jolivet *et al.*, 1998) (Figure 1). Research indicated that GHB, tyrosine and their oxidative and/or hydrolysis products are the most abundant phenolic compounds in *A. bisporus* (Jolivet *et al.*, 1998). The pathway can also result in other compounds than melanin as phenylalanine can branch to the formation of compounds such as flavonoids and anthocyanins (Calla *et al.*, 2009). Another melanin pathway related to the L-DOPA pathway results in catechol melanin. The starting substrate is catechol (Figure 1), which has been identified in mushroom cap tissue (Paranjpe *et*

al., 1978). Little information on this pathway and the relevance for mushroom quality is known and therefore no other genes than the involved polyphenol oxidases were studied from this pathway.

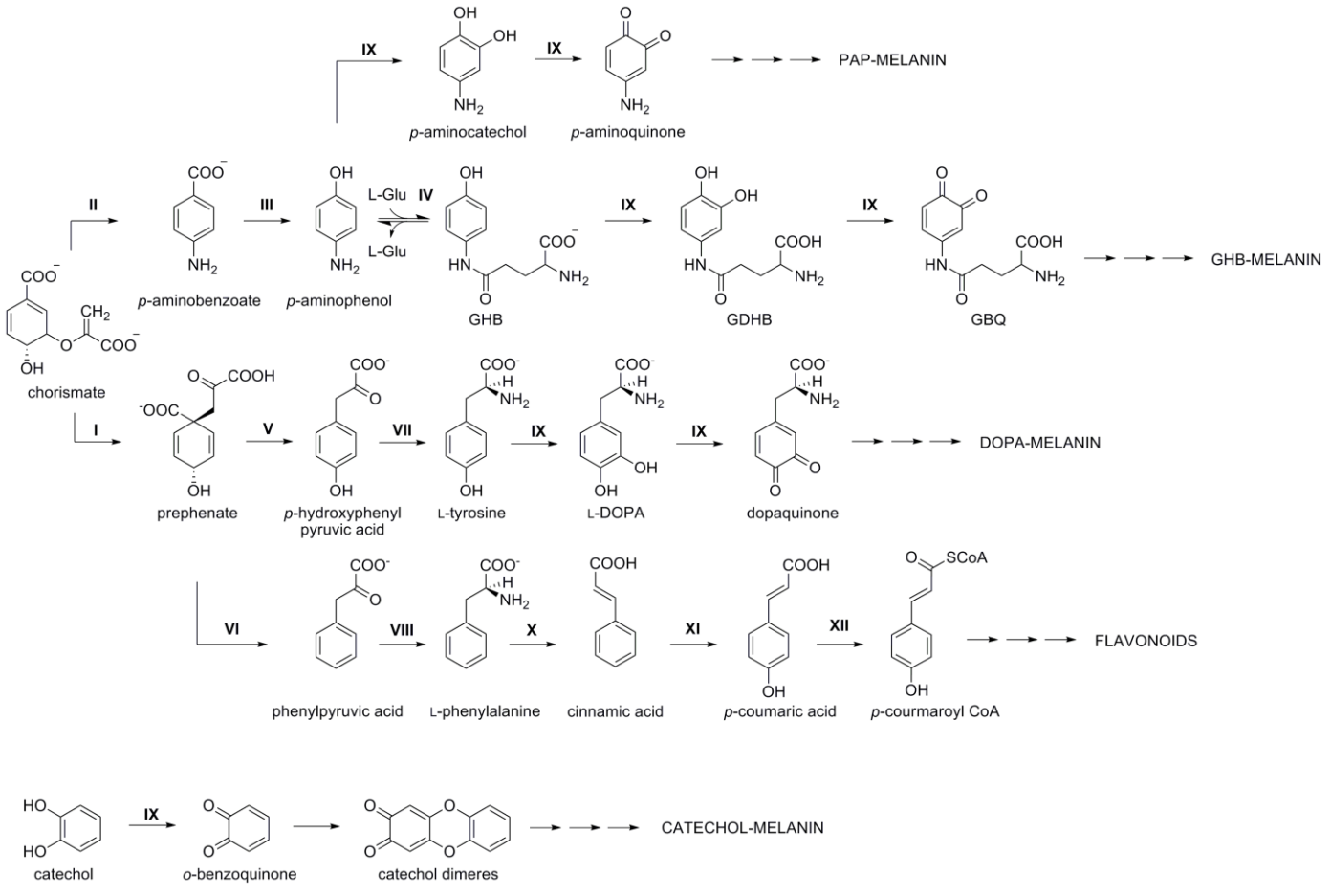
The best studied enzyme from the melanin pathway is polyphenol oxidase (PPO), an enzyme involved in the conversion of phenolic compounds (enzymatic step IX in Figure 1). Two types of PPOs, tyrosinase and laccase, have been described (Jolivet *et al.*, 1998). In this thesis, PPO is always referring to tyrosinase and not to laccase. Tyrosinase uses molecular oxygen to catalyse two different enzymatic reactions. The first one is the ortho-hydroxylation of monophenols to ortho-diphenols (monophenolase or cresolase activity) and the second is the oxidation of ortho-diphenols to ortho- quinones (diphenolase or catecholase activity) (Claus and Decker, 2006). The quinones undergo further, as far is known to date non-enzymatic, reactions leading to dark melanin pigments (Figure 1).

Another important class of enzymes probably involved in brown discoloration are peroxidases. Peroxidases are enzymes that use hydrogen peroxide to catalyse the oxidation of a variety of compounds (Conesa *et al.*, 2002, Hofrichter *et al.*, 2010). Known peroxidases of *A. bisporus* are manganese peroxidase (Lankinen *et al.*, 2005) and chloroperoxidase (GenBank ID: 9796009). Manganese peroxidase is an extracellular peroxidase involved in lignin degradation. Chloroperoxidase is a haloperoxidase that catalyses the oxidation of halides with peroxides such as hydrogen peroxide resulting in the halogenation of organic compounds (Hofrichter *et al.*, 2010).

The sequential enzymatic steps and conversions in the pathway illustrated in Figure 1 are still a working model as it has not been possible to analyse its genetics, enzymology and metabolism in detail yet. However, with the full genome sequence of *A. bisporus* available it is possible to investigate the genes involved in specific agronomic or economic traits of mushroom fruiting bodies more in-depth (http://genome.jgi-psf.org/Agabi_varbisH97_2/Agabi_varbisH97_2.home.html,

version 3.0). Here we try to list and characterize the most relevant genes in the genome of *A. bisporus* which are likely involved in the melanin biosynthesis pathway. Based on different BLAST and alignment approaches, genes were identified in the genome of *A. bisporus* that we postulate to be involved in the melanin biosynthesis pathway. Known gene sequences of *A. bisporus* or other fungi were used to identify new genes in the genome of *A. bisporus.* The automatic annotation made it possible to search directly in the KEGG and KOG databases that were based on the *A. bisporus* genome. Besides identification of genes involved in the melanin pathway, a putative co-factor of PPO (Ismaya *et al.*, 2011), a putative transcription factor (photoregulator B, PHRB) involved in this pathway and several peroxidases possibly involved in other oxidation processes were identified. All these gene products can have potential impact on the discoloration process (Table 1). For almost all individual genes, even when several homologs were identified in the genome, RT-qPCR analyses were performed to determine their expression, tissue specificity and to study gene expression in different developmental stages of the button mushroom. These analyses are a first attempt in unravelling the genetic background of the melanin biosynthesis pathway in mushrooms and, more over, should pave the way to support targeted breeding and selection strategies to develop mushroom strains that are less sensitive for discoloration.

Figure 1. Melanin synthesis pathway (on the next page). Genes presumed to be involved in the melanin biosynthesis pathway. Enzymes are indicated by Roman symbols, which are clarified in Table 1. GHB = γ-L-glutaminyl-4-hydroxybenzene. GDHB = γ-L-glutaminyl-3,4-dihydroxybenzene. GBQ = γ-L-glutaminyl-3,4-benzoquinone (Adapted from Jolivet *et al.*, 1998).


IX
IX
PAP-MELANIN
p-aminocatechol
p-aminoquinone
II
III
L-Glu
IV
L-Glu
IX
IX
GHB-MELANIN
chorismate
p-aminobenzoate
p-aminophenol
GHB
GDHB
GBQ
I
V
VII
IX
IX
DOPA-MELANIN
prephenate
p-hydroxyphenyl pyruvic acid
L-tyrosine
L-DOPA
dopaquinone
VI
VIII
X
XI
XII
FLAVONOIDS
phenylpyruvic acid
L-phenylalanine
cinnamic acid
p-coumaric acid
p-courmaroyl CoA
IX
CATECHOL-MELANIN
catechol
o-benzoquinone
catechol dimeres

2 Material and methods

2.1 Searching for homologous genes

The JGI website portal with the *Agaricus bisporus* var. *bisporus* H97 genome was used to identify new genes (http://genome.jgi-psf.org/Agabi_varbisH97_2/Agabi_varbisH97_2.home.html). The BLAST option was used with either protein or DNA sequences to search for homologous genes. Clone Manager 9 (Sci-Ed Software) was used to align either protein or DNA sequences with the multi way alignment. The standard linear scoring matrix was used for DNA sequences and the BLOSUM 62 scoring matrix was used for protein sequences. Complete sequences of known genes or conserved regions of these gene sequences were used to search for homologs in the H97 genome database of JGI. The sequences used to search for homologous genes in the H97 genome are depicted in Table 2. Enzyme commission numbers were used to search in the KEGG database of H97. Identified genes of the *A. bisporus* H97 genome are depicted in Table 1. The roman numbers correspond to the enzymatic steps in Figure 1. With the Protein ID number the genes can be found in the genome database.

2.2 Mushroom cultivation and sampling

Mushrooms (*A. bisporus* strain Horst U1) were grown as described in Weijn *et al.* (2012). Mycelium and mushrooms from stage 1 (pinhead) until stage 7 (flat open mushroom) (Hammond and Nichols, 1976) were collected. Mycelium, stage 1 and stage 2 samples were collected from the bed and directly frozen in liquid nitrogen. Stage 3 mushrooms were divided into cap (skin and flesh) and stem. From stage 4 until 7, the mushrooms were dissected in skin, flesh, gill and stem tissue before freezing in liquid nitrogen. For each stage a minimum of four mushrooms were used. Samples were stored at -80 °C.

Table 1. Identified genes of the *A. bisporus* H97 genome. Roman numbers in the column Number correspond with the enzymatic steps in Figure 1. Protein ID is the number with which the genes can be found in the genome of *A. bisporus* var. *bisporus*.

Number	Gene function	Gene abbreviation	Protein ID
I	Chorismate mutase	*CM*	193694
II	4-Aminobenzoate synthase	*4ABS_1*	189405
II	4-Aminobenzoate synthase	*4ABS_2*	192907
III	4-Aminobenzoate hydroxylase	*4ABH_1*	195454
III	4-Aminobenzoate hydroxylase	*4ABH_2*	239564
III	4-Aminobenzoate hydroxylase	*4ABH_3*	239563
III	4-Aminobenzoate hydroxylase	*4ABH_4*	195451
III	4-Aminobenzoate hydroxylase	*4ABH_5*	211127
IV	γ-Glutamyltransferase	*GGT_1*	239353
IV	γ-Glutamyltransferase	*GGT_2*	239362
V	Prephenate dehydratase	*PDHtase*	182401
VI	Prephenate dehydrogenase	*PDH*	226049
VII / VIII	(4-hydroxy)phenylpyruvate aminotransferase	*AT_1*	191838
VII / VIII	(4-hydroxy)phenylpyruvate aminotransferase	*AT_2*	201971
VII / VIII	(4-hydroxy)phenylpyruvate aminotransferase	*AT_3*	191531
IX	Polyphenoloxidase (tyrosinase)	*PPO_1*	194055
IX	Polyphenoloxidase (tyrosinase)	*PPO_2*	191532
IX	Polyphenoloxidase (tyrosinase)	*PPO_3*	239416
IX	Polyphenoloxidase (tyrosinase)	*PPO_4*	191507
IX	Polyphenoloxidase (tyrosinase)	*PPO_5*	239415
IX	Polyphenoloxidase (tyrosinase)	*PPO_6*	239414
IX	Polyphenoloxidase co-factor	*L-chain*	239342
X	Phenylalanine ammonialyase	*PAL_1*	192690
X	Phenylalanine ammonialyase	*PAL_2*	192776
XI	Trans-cinnamate-4-monooxygenase	*C4H_1*	239453
XI	Trans-cinnamate-4-monooxygenase	*C4H_2*	213259
XII	4-Coumarate CoA ligase	*4CL_1*	192760
XII	4-Coumarate CoA ligase	*4CL_2*	72962
XII	4-Coumarate CoA ligase	*4CL_3*	239433
XII	4-Coumarate CoA ligase	*4CL_4*	119772
XII	4-Coumarate CoA ligase	*4CL_5*	186749
XII	4-Coumarate CoA ligase	*4CL_6*	194515
XII	4-Coumarate CoA ligase	*4CL_7*	239428
XII	4-Coumarate CoA ligase	*4CL_8*	177762
XII	4-Coumarate CoA ligase	*4CL_9*	239435
XII	4-Coumarate CoA ligase	*4CL_10*	212169
	Catalase	*Cat_1*	115586
	Catalase	*Cat_2*	200291
	Catalase	*Cat_3*	239410
	Chloroperoxidase	*CPO*	239311
	Manganese peroxidase	*MNP*	221245
	Photoregulator B	*PHRB*	190401

Table 2. Protein sequences used for homology search in H97 genome. Gene function describes the function of the homolog in *A. bisporus.* GenBank ID is the number with which the genes can be found in the NCBI database. For the *A. bisporus* var. *burnettii* the sequence was used from the JGI genome database (http://genome.jgi-psf.org/Agabi_varbur_1/Agabi_varbur_1.home.html, version 1).

Gene function	Organism	GenBank ID
Chorismate mutase	*Cryptococcus neoformans*	58262028
Chorismate mutase	*Coprinopsis cinerea*	169858680
Chorismate mutase	*Saccharomyces cerevisiae*	295577
4-Aminobenzoate synthase	*S. cerevisiae*	6324361
4-Aminobenzoate hydroxylase	*A. bisporus*	1514422 (Tsuji *et al.*, 1996)
γ-Glutamyltransferase	*S. cerevisiae*	60389819
γ-Glutamyltransferase	*Schizosaccharomyces pombe*	34099622
γ-Glutamyltransferase	*Aspergillus flavus*	238500177
Prephenate dehydratase	*Laccaria bicolor*	164651390
Prephenate dehydratase	*C. cinerea*	299755662
Prephenate dehydrogenase	*S. cerevisiae*	6319643
Aminotransferase	*S. cerevisiae*	1723954
Polyphenoloxidase (tyrosinase)	*A. bisporus*	717171 (Wichers *et al.*, 2003)
Polyphenoloxidase (tyrosinase)	*A. bisporus*	895690 (Wichers *et al.*, 2003)
Polyphenoloxidase (tyrosinase)	*A. bisporus*	255687954 (Wu *et al.*, 2010)
Polyphenoloxidase (tyrosinase)	*A. bisporus*	255687956 (Wu *et al.*, 2010)
Phenylalanine ammonialyase	*C. cinerea*	299751908
Phenylalanine ammonialyase	*Ustilago maydis*	119364628
Phenylalanine ammonialyase	*Amanita muscaria*	30580473
Trans-cinnamate-4-monooxygenase	*A. bisporus* var. *burnettii*	104303 (JGI genome Protein ID)
Trans-cinnamate-4-monooxygenase	*Aspergillus clavatus*	121699404
4-Coumarate CoA ligase	*A. flavus*	238497886
4-Coumarate CoA ligase	*Coccidioides immitis*	119173778
4-Coumarate CoA ligase	*Yarrowia lipolytica*	49647360
Catalase	*C. neoformans*	58270756
Catalase	*L. bicolor*	170116610
Photoregulator B	*Lentinula edodes*	229365447 (Sano *et al.*, 2009)
Photoregulator B	*Neurospora crassa*	1835159
Photoregulator B	*Trichoderma atroviride*	51944888

2.3 RNA extraction and cDNA preparation

The mushroom samples were ground with mortar and pestle with liquid nitrogen. RNA was extracted from 40 mg mushroom powder using the RNeasy mini kit (Qiagen) according to the Qiagen Plants and Fungi protocol with two adjustments. The step in which the QIAshredder kit (Qiagen) is used to homogenize the sample was omitted. Instead, the mixture of mushroom powder

with RLT buffer was centrifuged for 10 min and the supernatant was transferred to a new tube. The RNA was eluted from the column membrane with 30 µl MilliQ water by incubation for 1 min at RT and centrifugation for 1 min. This elution was done twice and pooled. RNA quality and quantity were checked on a 1 % agarose gel and with a NanoDrop spectrophotometer (ND-1000 version 3.6.0). All samples were treated with DNase I to remove the genomic DNA, according to the protocol of Sigma (AMP-D1). 2 µg RNA, 2 µl 10 x Reaction buffer, 2 µl DNase I (1 unit/µl), and MilliQ water were used in a total volume of 20 µl. After DNase I treatment, quality was checked again with the NanoDrop. 200 ng DNase I treated RNA was used to make cDNA with the iScript cDNA synthesis kit (Biorad). For each sample the cDNA reaction was done four times. These reaction samples were pooled and diluted two and a half times with MilliQ water. RNA was stored at -80 °C, DNase I treated RNA and cDNA were stored at -20 °C.

2.4 Real Time-qPCR

In order to normalize gene expression data from RT-qPCR analysis it is important to have stable expressing housekeeping genes, which are expressed similarly in all tissues analysed. Seven housekeeping genes (β-tubulin, glyceraldehyde-3-phosphate dehydrogenase, translation elongation factor α, phosphoglycerate kinase, actin, protein kinase inhibitor α, and 18S rRNA) were tested for their expression in different tissues and stages of Horst U1 mushrooms (Table 3).

Primers were designed with Clone Manager 9 (Sci-Ed software, Table 3 and Table 4) and checked for alignment with homologous sequences. Primer efficiency, between 80 and 120 %, was analysed using dilution series (10, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) of cDNA from gill samples of stages 4, 5, 6 and 7 that were mixed before use. Amplified products were checked on a 2 % agarose gel.

20 ng cDNA was used in each RT-qPCR reaction with 10 µl iQ SYBR Green Supermix (Biorad) and forward and reversed primers (final concentration is indicated in Table 4) in a total volume of 20 µl. Each sample was measured in duplicate. The RT-qPCR was preheated at 95 °C for 1.5 min. This was followed by 40 cycles with a denaturing temperature of 95 °C for 10 s, annealing temperature of 58 °C for 10 s, and elongation temperature of 72 °C for 15 s, followed by a final annealing temperature of 72 °C for 2 min. For each sample a melting curve was determined to verify a single amplification product. The relative gene expression is determined as the relative expression to 18S rRNA, calculated as $\Delta C_T = 2^{\wedge}(Ct_{18S\ rRNA} - Ct_{sample})$. Relative gene expression data were log2 transformed. Squared Pearson correlation was used for similarity and UPGMA for clustering in GeneMaths XT (Applied Maths, version 2.12).

Table 3. Housekeeping genes of *Agaricus bisporus.* GenBank ID is the number with which the genes can be found in the NCBI database.

Gene function	Gene abbreviation	Genbank ID	Primer [µM]	Sequence (5' → 3')
β-Tubulin	*Tubulin*	AW324553	0.40	F - TCATGCGTGAAATCGTTCAT R - CAATACCGTGCTCGTCAGGAA
Glyceraldehyde-3-phosphate dehydrogenase	*GPD*	M81727	0.25	F - CGTTGGGAGCAACTATTCG R - ACCTCATCGCAGACTCTAC
Translation elongation factor α	*TefA*	X97204	0.25	F - CGCCTTCGAACGTCACTACT R - CGTTCTTGACGTTGAAACCA
Phosphoglycerate kinase	*PgkA*	X91105	0.40	F - AAATATGCCCTCGACAATGG R - GGCCACAGGTTTGAGTGAGT
Actin	*Actin*	AW444077	0.40	F - TACCCGATCGAACACGGTAT R - GCCACTCGCAATTCATTGTA
Protein kinase inhibitor α	*PkiA*	X97579	0.40	F - CGTGGTGAGGACGTAAGACA R - TTAGCGACACCTTGCTCATTT
18S rRNA	*18S*	AY787216	0.25	F - TGCTCTGCTGGGTCTTACCT R - CGGCGACTCTAGAAACCAAC

Table 4. Real Time-qPCR primer sequences. Roman numbers in the column Number correspond with the enzymatic steps in Figure 1. Protein ID is the number with which the genes can be found in the genome of *A. bisporus.*

Number	Gene	Protein ID	Primer [µM]	Sequence (5' → 3')
I	*CM*	193694	0.10	F - AATTCGAGGATGACGGTAAC R - ATGAACTTGGACTCGGAAAC
II	*4ABS_1*	189405	0.10	F - TCTGGGATGGGAGGTTCTTAG R - CCTCTTTCTCTGCCTTCGATAC
II	*4ABS_2*	192907	0.10	F - CGTTGCCCAAGGATACTC R - AGCACCCGTCATAGAACC
III	*4ABH_1*	195454	0.25	F - AAGTCACAGAAATCAATAGCATC R - CCTTGGCCTGATGAAGAGTG
III	*4ABH_3*	239563	0.25	F - TCATCACATTCATGCCCTAT R - ATACCGCTCATATCGCTGTG
III	*4ABH_2&4*	195454 & 239564	0.25	F - CCTGGACATAGAGCATTAACG R - TGCCACAACATTGATGAACT
III	*4ABH_2,4&5*	195454 & 239564 & 211127	0.25	F - GTGGAAGTAACACAAGAAGAA R - CACTTGGTGGGTTTCTTTATG
IV	*GGT_1*	239353	0.10	F - TGTATATCCGCAGGTATGCC R - ATACGACACGAGCATCATGG
IV	*GGT_2*	239362	0.10	F - TTGTATCCGCTGGTTCTG R - ACTGCCGCTATCCTATTG
V	*PDHtase*	182401	0.10	F - GCAATTTGCTCCAAAGTCTG R - ACGCTTTGATCTCGAACTAC
VI	*PDH*	226049	0.10	F - CTCAGTCACTGCCAATACCC R - CGACGTAAAGACCCTGTTCC
VII / VIII	*AT_1*	191838	0.10	F - ATTCGAGAGGCAAGGTGAAAC R - CATCAACAATGCTGCGACAAG
VII / VIII	*AT_2*	201971	0.25	F - GAAGGCTCGTCGTTCACTG R - GGCCTGTCTCCGGTAATTC
VII / VIII	*AT_3*	191531	0.25	F - CTTTCGGCTGGTATCAGG R - AGGGACGAGACTTGAAGG
IX	*PPO_1*	194055	0.10	F - ACGACTTGGCCTCATTTCAG R - GTTACCACCGACGAGGACAT
IX	*PPO_2*	191532	0.40	F - GAGCGACCTTCCATGAACCT R - TTTGCTCCAACACAGAAAGG
IX	*PPO_3*	239416	0.25	F - TTGCCGATGACCATAGCG R - GCAGTGAGACCGATGTTG
IX	*PPO_4*	191507	0.25	F - GCTTATGAACCCGTATTCTGG R - CCTTCGCTGACATAGACATC
IX	*PPO_5*	239415	0.25	F - GCGGATCACGAAGATGAC R - GAGACGTGAGTTCTCCTTCAG

Number	Gene	Protein ID	Primer [µM]	Sequence (5' → 3')
IX	*PPO_6*	239414	0.25	F - CAAGAAACACGAAGCGAAAG
				R - AGCTAGGAGGTAGGTTAAGG
IX	*L-chain*	239342	0.25	F - CGACGGACAGACTTGTAAATG
				R - GACTTGATGCAGGCTTGATAG
X	*PAL_1*	192690	0.10	F - GTCCCGTTTCTACTCACATTC
				R - CCACCAGCATTGACAAGAC
X	*PAL_2*	192776	0.10	F - GCTCGGTTATTTAGCGAATCC
				R - TTTGTCGGCCTGAGATCAAAG
XI	*C4H_1*	239453	0.25	F - ACAGTAAAGACCACTACGCTTG
				R - ACCTTGCTCGTCGGTCAATC
XI	*C4H_2*	213259	0.25	F - CACACCACTCTACTGCGCTC
				R - TTGCCATTCGCGTCCGTC
	PHRB	190401	0.25	F – AATGGCGGAAAGGTCCAG
				R - GCTTTGCCCAGCGTAAAC

3 Results

Starting from chorismate in the melanin biosynthesis pathway (Figure 1) and following the *p*-aminobenzoate branch leading to GHB- and PAP-melanin, two gene homologs were identified for 4-aminobenzoate synthase (4ABS, enzymatic step II in Figure 1). Next, 4-aminobenzoate hydroxylase (4ABH) is involved in the conversion of *p*-aminobenzoate into *p*-aminophenol (enzymatic step III in Figure 1). 4ABH is also involved in the biosynthesis of the 4-hydroxyaniline moiety of *N*-(γ-L-glutamyl)-4-hydroxyaniline (Tsuji *et al.*, 1996). *N*-(γ-L-glutamyl)-4-hydroxyaniline and agaritine (*N*-(γ-L-glutamyl)-4-(hydroxymethyl)phenyl-hydrazine) are aromatic compounds that are characteristic for *A. bisporus* (Walton *et al.*, 2001). Although not part of the melanin biosynthetic pathway, enzymes of the melanin pathway can be involved in the conversion of these compounds. We postulate 4ABH and γ-glutamyltransferase (GGT) for such roles. The five gene homologs identified for 4ABHs are all found on chromosome 12, clustered in tandem. For GGT (step IV in Figure 1) two genes were found.

Jolivet *et al.* (1998) described in the melanin biosynthesis pathway that there are two different aminotransferases, 4-hydroxy phenylpyruvate aminotransferase

(step VII in Figure 1) and phenylpyruvate aminotransferase (step VIII in Figure 1). Three genes for aminotransferases were identified. As it was not possible to assign a specific function to these three genes, they are therefore listed under the proposed gene function (4-hydroxy) phenylpyruvate aminotransferase (AT, Table 1).

The prephenate branch of Figure 1, starting from phenylpyruvic acid, does not lead to the formation of melanin, but to flavonoids. This branch can be important for the prevention of discoloration when the phenolic compound flux passes through this branch and so limits the available substrate for the branches leading to brown pigments. For the enzymes in this part two genes for phenylalanine ammonialyase (PAL, step X in Figure 1) and two for trans-cinnamate-4-monooxygenase (C4H, step XI in Figure 1) were identified. The enzyme for the next substrate conversion is 4-coumarate CoA ligase (4CL), for which ten putative gene homologs were identified. For these homologs no gene expression was determined, because no specific RT-qPCR primers were developed due to few differences in the gene sequences.

Peroxidases can contribute to or generate browning-like reactions (Zhang and Flurkey, 1997). Many peroxidase genes can be found in the genome sequence and no exhaustive analyses have been initiated to identify all. Manganese- and chloro-peroxidase genes were automatically annotated and therefore incorporated. Another interesting peroxidase is catalase (Cat), for which three gene homologs were identified. Catalase can catalyse the conversion of hydrogen peroxide to water and molecular oxygen (http://www.genome.jp/dbget-bin/www_bget?enzyme+1.11.1.6).

An overview of the physical localisation of the identified genes for the genome of H97 is shown in Figure 2. These genes can be found on different chromosomes and sometimes in clusters on a chromosome. Interesting is the cluster between *PPO_3* and *PPO_5* on chromosome 5 (shown and described in more detail in Figure 5 and section 3.2). *PPO*-homologs (described in more detail in section 3.1)

are found on two different chromosomes. *PPO_2*, *PPO_3*, *PPO_4* and *PPO_5* are located on chromosome 5, but *PPO_1* and *PPO_6* can be found on chromosome 8.

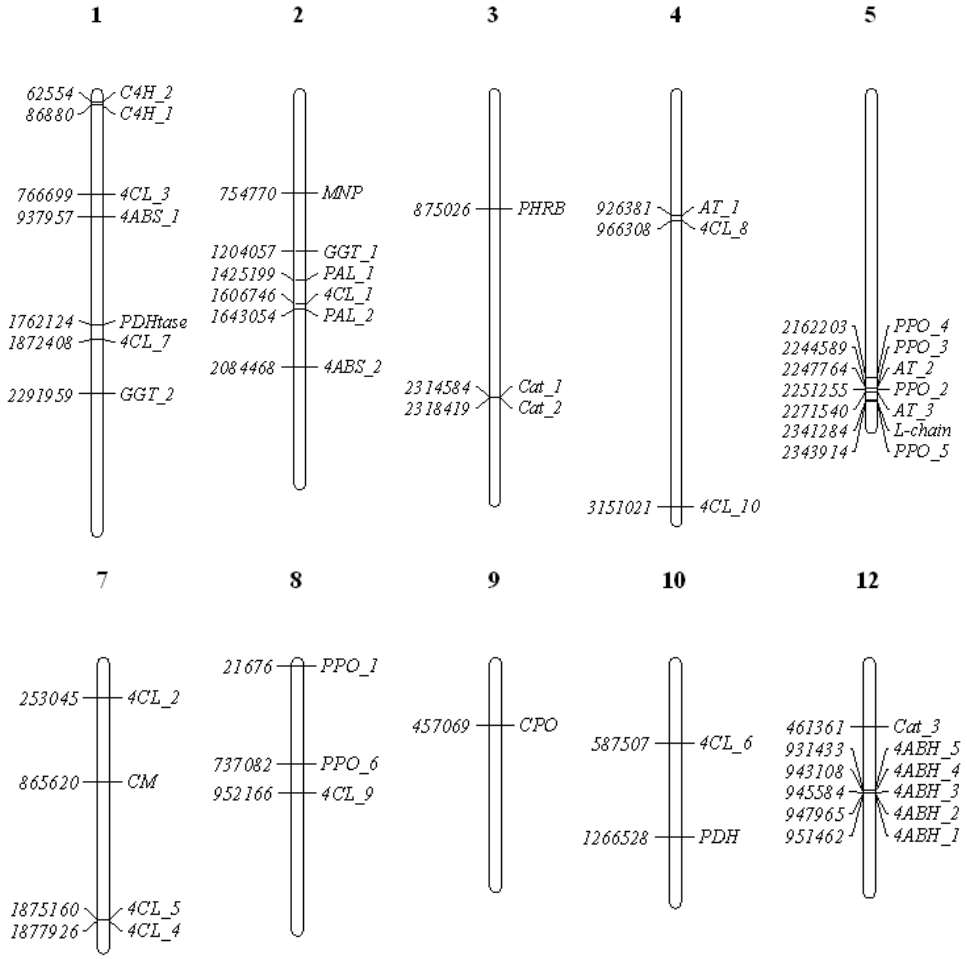


Figure 2. Chromosome map of H97. Each chromosome is indicated by the number at the top (chromosome numbers correspond with the linkage groups of Morin *et al.* (2012)). Genes are indicated on each chromosome by name (see Table 1) on the left side. The starting point of the gene is given in cM (Kosambi unit) to the right of the chromosome. Chromosomes 6, 11 and 13 are omitted from the figure, because there were no genes identified in this research on chromosome 6, 11 and 13. The figure is drawn using the software program MapChart 2.2 (Voorrips, 2002).

3.1 Polyphenol oxidase and L-chain

PPO is involved at six steps in the melanin biosynthesis pathway (Figure 1) performing two different types of reactions. Several genes coding for PPO were already identified. *PPO_1* and *PPO_2* were first described by Wichers *et al.* (2003). Wu *et al.* (2010) identified *PPO_3* and *PPO_4* and Li *et al.* (2011) also reported on these two new PPO genes in *A. bisporus.* Apparently, at the time of preparation of their paper, Li *et al.* (2011) were not aware of the publication of Wu *et al.* (2010) and their numbering of both genes is reverse to that given by Wu *et al.* We propose that denomination of the PPOs will be according to Wu *et al.* We have therefore used this numbering for annotating the sequences. Based on the full genome sequence of *A. bisporus,* we have identified two additional PPO-encoding sequences, which are named *PPO_5* and *PPO_6*. *PPO_6* has two starting codons and was initially annotated as two different PPOs. This appears to complete the *PPO*-gene family for *A. bisporus*. The six PPO protein sequences of H97 were aligned and compared for homology (Figure 3). Several highly conserved regions of PPO were identified and are indicated in Figure 3. The copper domains CuA and CuB, which are involved in the binding of copper via histidine (Jolivet *et al.*, 1998), are indicated in this alignment as well is the tyrosine region. The tyrosine region is involved in the overall structure of the protein, with Y/FxY as the conserved residues sequence (Marusek *et al.*, 2006). The dendrogram for the amino acid sequences in Figure 4 shows that PPOs 2, 3, 4 and 5 are closest related to each other. The products of PPO_1 and PPO_6, which are also found on a different chromosome (Figure 2), show lower similarity to the other PPOs and to each other. *PPO_3* and *PPO_5* have the highest homology based on sequence alignment similarity: 76 % for amino acid and 79 % for nucleotides. *PPO_2* and *PPO_4*, have a similarity of 57 % for amino acid and 61 % for nucleotides.

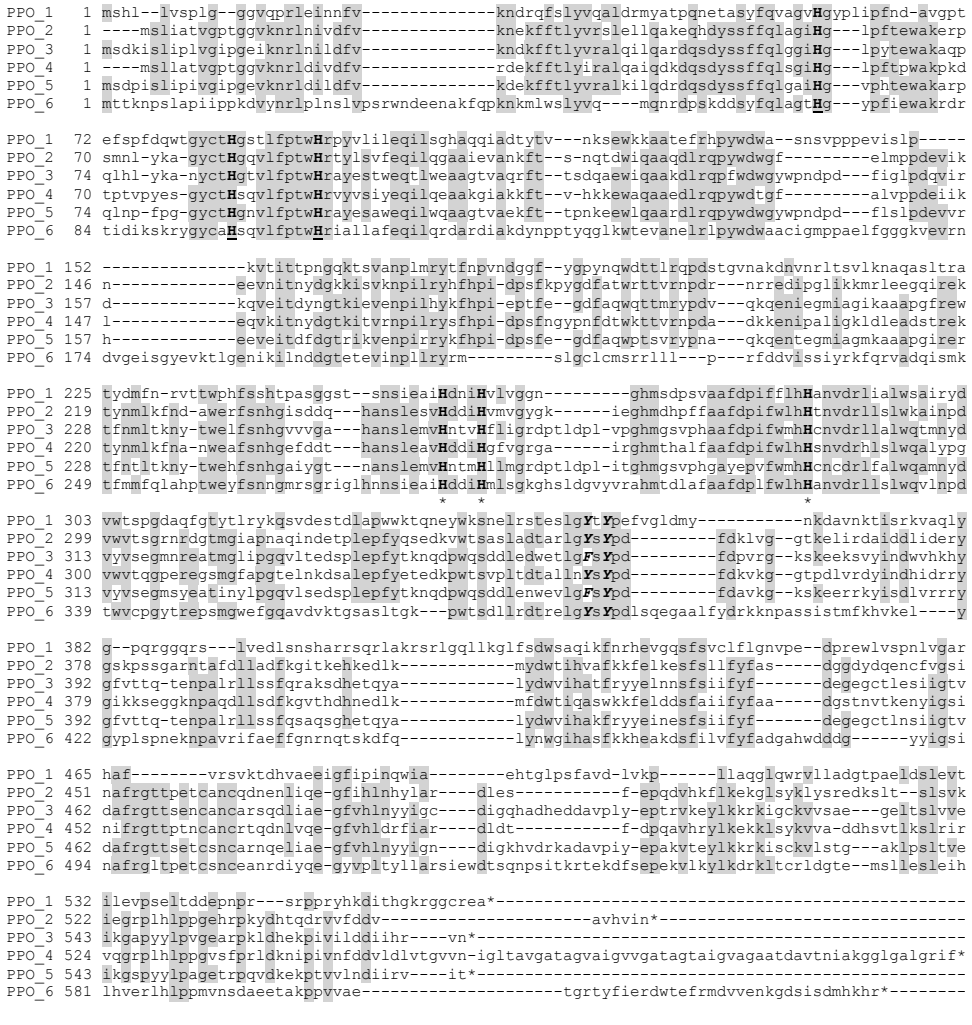


Figure 3. ***Agaricus bisporus*** **PPO amino acid alignment.** Highlighted areas have 60% or higher homology. First three underlined and bold histidines are of CuA. Second three * and bold histidines are of CuB. The tyrosine and phenylalanine residues in bold and italic form the tyrosine region (Flurkey and Inlow, 2008; Marusek *et al.*, 2006). Alignment was made with Clone Manager 9 (Sci-Ed Software), with a multi-way protein alignment with scoring matric BLOSUM 62.

Making use of known peptide sequences (Schurink *et al.*, 2007), crystal analysis data (Ismaya *et al.*, 2011) and the genome sequence we identified a protein with high affinity for PPO. This protein is known as the L-chain and characterized as the light chain (L-chain) involved in the H_2L_2 quaternary structure of PPO (Strothkamp *et al.*, 1976). The biological role of the L-chain is, as yet, not elucidated nor understood. With new BLAST methods, until to date no other proteins were identified that showed sequence homology with the L-chain (Schurink *et al.*, 2007 and Ismaya *et al.*, 2011). The L-chain is unique for *A. bisporus* var. *bisporus* and var. *burnettii*. Interesting is the fact that the *A. bisporus* genome only contains one *L-chain* sequence and six genes for possible PPOs with which the L-chain can form a stable and active tyrosinase protein structure. Remarkably, the *L-chain* clusters close to the PPO genes of *PPO_2, 3, 4* and *5* (Figure 2 and 5) which may suggest that this co-factor is not an artefact from biochemical purification of the PPO but has a real biological function in the PPO-involved pathways.

3.2 The cluster in-between PPO_3 and PPO_5

The clustering of PPO genes and the PPO co-factor L-chain gene triggered our curiosity to study this cluster further in an attempt to identify other genes that might be involved in the melanin biosynthesis pathway. We studied in depth the genes automatically annotated around *PPO_2*, *PPO_3* and *PPO_5* (Figure 5). Support for further studying this cluster for pathway related genes came from the observation that two out of three (4-hydroxy) phenylpyruvate aminotransferase genes are positioned in this cluster. A striking result was the observation that almost 25 % of the protein encoding genes in this cluster have a conserved NACHT and WD40 domain or a P-loop nucleoside triphosphate hydrolase (P-loop NTPase) (IPR001680 and IPR007111, http://www.ebi.ac.uk/interpro). These NACHT and WD40 domain-containing genes from *A. bisporus* have a high

similarity with NWD2 genes from *C. cinerea* okayama 7 (GenBank ID: 299755179, 299740726, 299748936, 169863252, and 299738356) and are therefore named NWD2 (Figure 5).

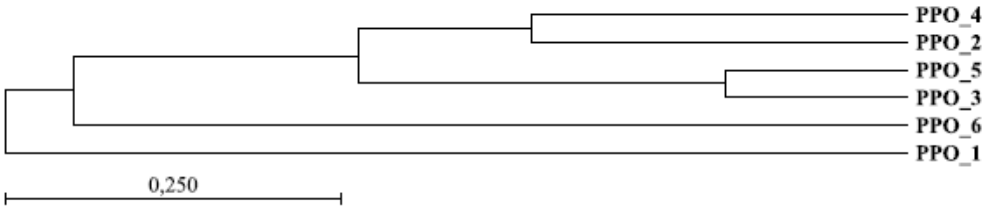


Figure 4. Alignment of PPO amino acid sequences. Alignment was made with CLC Genomics Workbench (CLC Bio, version 5.5.1). The dendrogram is constructed with a distance-based tree-building method using the UPGMA algorithm. The branch length is given in terms of expected numbers of substitutions per site.

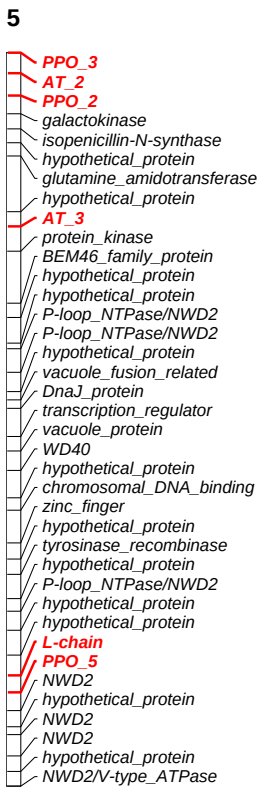


Figure 5. Chromosome map of the cluster on chromosome 5 between *PPO_3* and *PPO_5*. Genes indicated in red are from the melanin biosynthesis pathway (see Table 1). Genes in black are automatically annotated genes and are therefore putative genes of which some gave a high potential function and for some no possible function could be identified. The starting point of the genes is based on cM (Kosambi unit). The figure is drawn using the software program MapChart 2.2 (Voorrips, 2002).

Daskalov *et al.* (2012) also describe fungal genes containing a WD-repeat domain and a NACHT domain as NWD. WD40 repeats (IPR001680, http://www.ebi.ac.uk/interpro) are short 40 amino acids motifs. WD-repeat proteins are a large family found in all eukaryotes and are implicated in a variety of functions ranging from signal transduction and transcription regulation to cell cycle control and apoptosis. The NACHT domain is a 300 to 400 residue predicted NTPase domain, which is found in animal, fungal and bacterial proteins (IPR007111, http://www.ebi.ac.uk/interpro). The P-loop NTPase domain is characterized by a conserved nucleotide phosphate-binding motif, also referred to as the Walker A motif (GxxxxGK[S/T], where x is any residue), and the Walker B motif (hhhh[D/E], where h is a hydrophobic residue) (Marchler-Bauer *et al.*, 2009). P-loop NTPases are involved in diverse cellular functions and are comparable to the NACHT domain (Marchler-Bauer *et al.*, 2009).

Other interesting identified genes were vacuole-related genes; a gene which is possibly belonging to the V-type ATPase subfamily, and an isopenicillin N synthase like gene. These suggested gene functions could support the hypothesis that melanin formation is a consequence of release of monophenolic compounds from the vacuole. These compounds are then polymerized by enzymes in the cytoplasm or enzymes with membrane associated cellular location (Jolivet *et al.*, 1998) that make use of membrane transport in the biosynthetic pathways that leads to melanin formation. The isopenicillin N synthase is an oxidoreductase type of enzyme (like PPO) which catalyzes the formation of isopenicillin N from δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (LLD-ACV) (Roach *et al.*, 1997). This homolog might have a slightly different function as these substrates are yet not known to be present in *A. bisporus* and therefore might be involved in a similar process. The genes in this gene cluster will be very interesting to study further to elucidate their possible role in melanin formation and cellular events that will lead to the discoloration of mushrooms.

3.3 Selecting housekeeping genes

Good quantification of RT-qPCR based gene expression analysis is in need of normalization steps based on stable expressed housekeeping genes. In order to identify these genes for normalization seven genes were selected and analysed in a wide set of tissue samples (Table 3). GeNorm version 3.4 (Vandesompele *et al.*, 2002) was used to determine the most stably expressed housekeeping genes. 18S rRNA appeared the only housekeeping gene that is stably expressed in all tested stages and in the different analysed tissues (difference < 1 C_T). For the determination of the 18S rRNA gene expression the cDNA was diluted 500 times. Other housekeeping genes showed the same level of gene expression in the same tissues, but differed between different tissue types (results not shown). Skin tissue-specific housekeeping genes are protein kinase inhibitor α and actin. Housekeeping genes are preferably selected based on an expression in the same range as the genes of interest. So the disadvantage of using 18S rRNA as a housekeeping gene is its high level of expression, making the expression of the gene of interest appear relatively low.

3.4 Gene expression results

All genes identified were analysed for gene expression making use of material of the strain Horst U1. Gene expression was analysed in different tissue samples from different developmental stages. In Figure 6 the results are shown for all genes in all tissues analysed using a heat map and hierarchic cluster analysis. All genes showed expression in all tissues. Several genes are expressed at a low constitutive level in all tissues, like the chorismate mutase gene (*CM*), *AT_1*, and *AT_3*. Another first observation is that no or small differences between maturation stages 4, 5, 6 and 7 were found, although some variations are found between different tissues. From the expression data it is clear that the expression of some genes are correlating with maturation and some others are clearly tissue specific e.g. *4ABS_1*,

which has a low expression in mycelium and increases slightly during maturation, but not in the stem. The colour of the gills is light pink in stage 4 and dark brown in stage 7. This is natural discoloration associated with the formation of spores. This brown discoloration not necessarily results from the same mechanism as underlying bruising, storage or bacterial infections. If PPO expression is an indication of its involvement, then *PPO_4* is the most likely candidate as it is having the highest expression in gills. *4ABH_1* expression increased mainly in the stem. The prephenate dehydratase gene (*PDHtase*) is 100 times higher expressed in the gills from stage 4 onwards and also the prephenate dehydrogenase gene (*PDH*) is expressed relatively higher in gills than in flesh or stem tissue of the same developmental stage. The same could be concluded for *C4H_1* and *C4H_2* and both *GGT_1* and *GGT_2* genes that also are at least ten times higher expressed in gills than in other tissues of the same stage.

Homologous genes which probably have the same function do not always have the same expression pattern. As an example for this, *AT_3* has the highest expression of the three aminotransferase genes analysed. In stage 4 and beyond it can be seen that *AT_1* and *AT_2* have similar expression in gills, flesh and stem but in the skin tissue *AT_2* is higher expressed then *AT_1*.

Identical as for the ten *4CL* homologs, no specific primers could be designed for all five separate *4ABH* homologs. Therefore, it was decided to study *4ABH_1* and *4ABH_3* by specific RT-qPCR primers and study *4ABH_2*, *4ABH_ 4* and *4ABH_5* by two different primer sets that are expected to detect all three (labelled as the combined expression of 4ABH_2&4&5 in Figure 6 and Table 4) or expected to detect only the mRNA derived from *4ABH_2* and *4ABH_4* (referred to as 4ABH_2&4 in Figure 6 and Table 4). It was found that the expression of *4ABH_1* is more specific for stem and gills and increases in these tissues with increasing developmental stage. *4ABH_3* expression is highest in the skin and decreases during maturation.

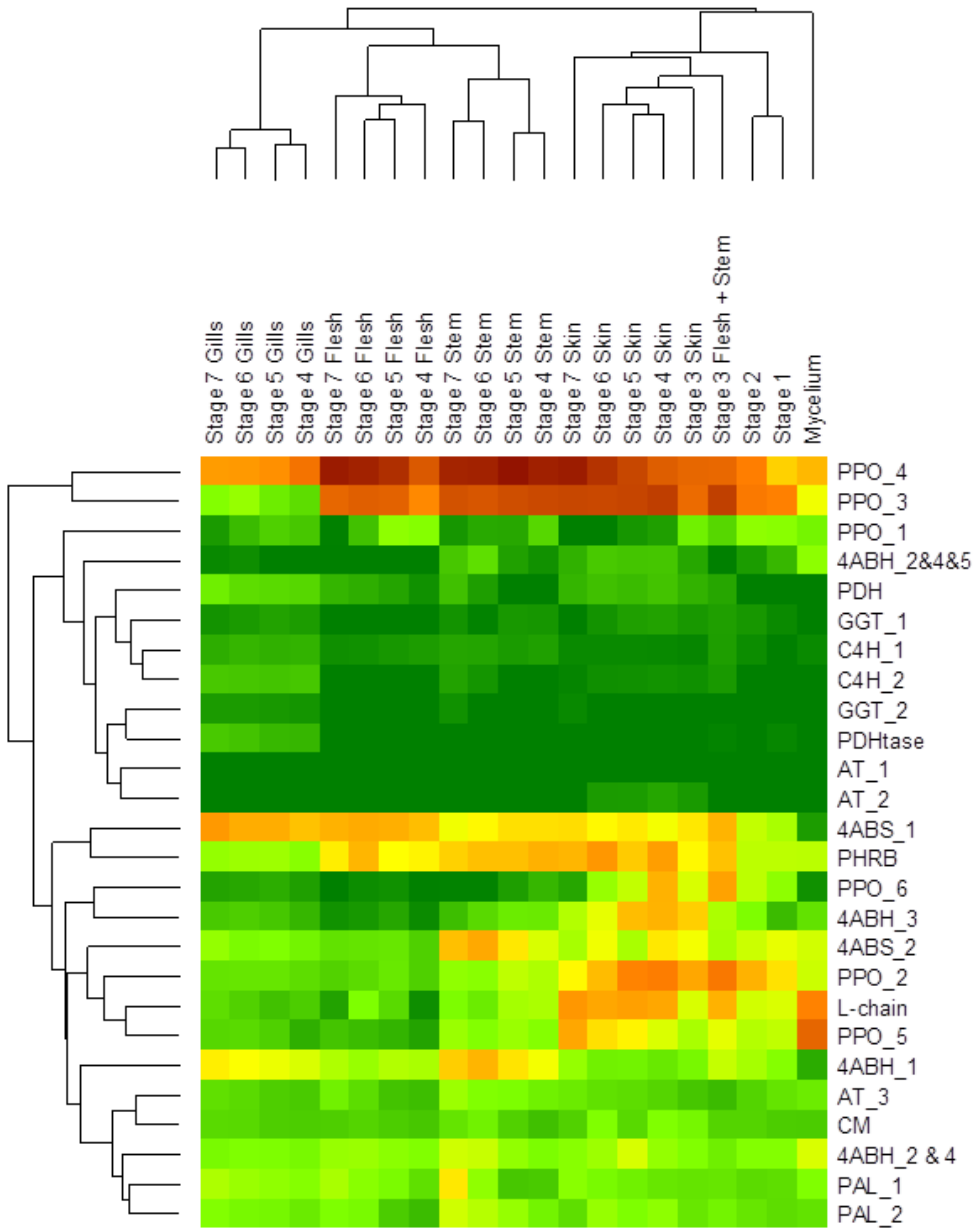


Figure 6. Heat map of the gene expression in Horst U1. The stages compared at the top are according to Hammond and Nichols (1976). The genes compared on the right side correspond to the enzymatic steps in Figure 1 and Table 1. The lowest expression is shown in dark green, the highest expression in dark red.

The primer 4ABH_2&4&5 detects fewer transcripts then 4ABH_2&4. Possibly, the 4ABH_2&4&5 primer is not specific enough for *4ABH*s and is bound by other cDNA, as a consequence of which the expression level is lower than expected.

The enzymatic steps in the biosynthesis pathway after chorismate are either PDHtase or PDH (step V or VI in Figure 1). Both enzymes are at the starting point of a branching point in the melanin biosynthesis pathway leading to the formation of different phenolic compounds. Different fluxes of phenolic compounds and enzymes (genes) in different tissues are therefore possible. The hierarchal clustering in Figure 6 shows that sequential genes in the pathway do not have a high correlation in expression levels and in tissue specificity.

From all genes tested, the family of PPO genes are relatively highest expressed. Of these six *PPOs*, *PPO_1* exhibits the lowest expression level, particularly in the skin tissue, in which the expression decreases slightly with increasing developmental stage. *PPO_2* expression is more specific for the early stages and the skin, in which expression of *PPO_2* decreased in stage 6 and 7. *PPO_3* and *PPO_4* have the highest expression of the PPO genes in all tissues. *PPO_4* is more specific for the gills than the other *PPOs*. *PPO_3* and *PPO_4* have a 100 fold higher expression in the flesh than other PPO genes. Interesting is the possible transcription factor PHRB which might regulate tyrosinase expression similar to what has been found for *Lentinula edodes* (Sano *et al.*, 2009). The expression of *PHRB* did not show a high similarity with one of the PPO genes and might be more involved with *4ABS_1* to which it is clustered in Figure 6. *PPO_4* and *PPO_5* have a 10 to 100 fold higher expression in the skin tissue of stages 4 until 7. *PPO_6* drops in expression in the skin from stage 4 until stage 7, which is opposite to the expression of *PPO_4* and *PPO_5*. The expression of the L-chain gene shows the highest correlation with expression of *PPO_5*, which might be explained by the fact that both genes are located next to each other in the genome (Figure 2 and 5), but in opposite direction, and probably make use of the same

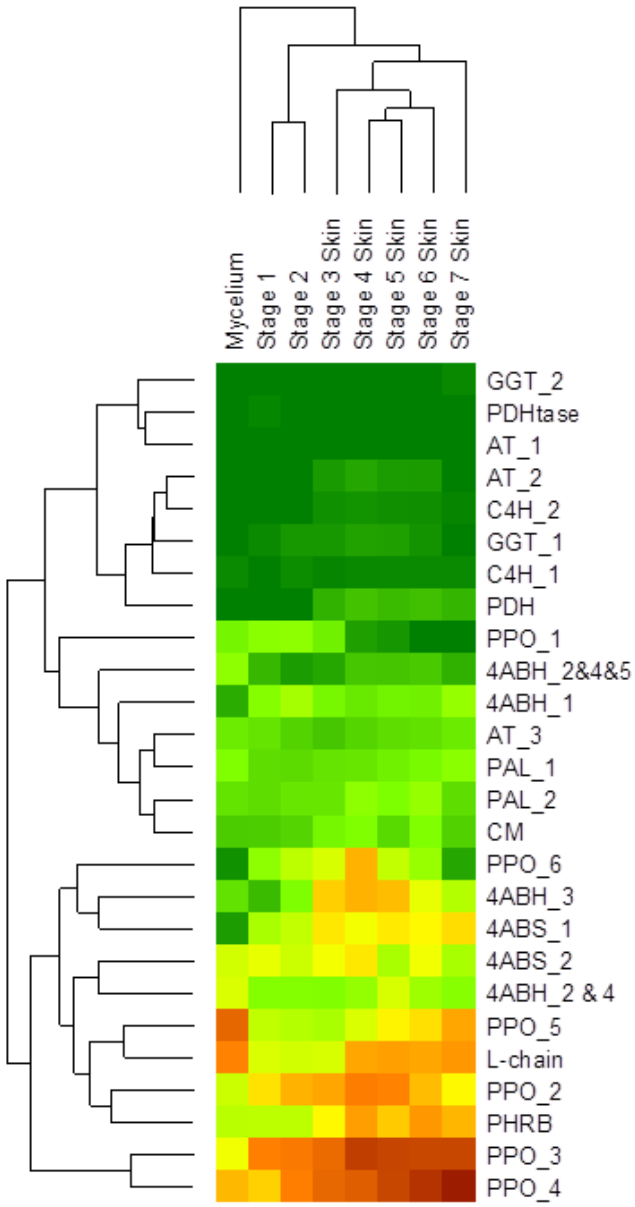


Figure 7. Heat map of the gene expression in Horst U1 skin tissue. The stages compared at the top are according to Hammond and Nichols (1976). The genes compared on the right side correspond to the enzymatic steps in Figure 1 and Table 1. The lowest expression is shown in dark green, the highest expression in dark red.

1.7 kb promoter fragment in between the two genes. As skin tissue determines most of the visual quality of mushrooms and as it is the tissue responding strongest, with discoloration, to damage and infection, an overview was prepared that illustrates the expression of all genes in the early stages and skin tissue of later developmental stages (Figure 7). It is clearly shown in Figure 7 that *PPO*, *L-chain* and *PHRB* are amongst the highest expressed genes in the skin.

4 Discussion

The completion of the genome sequence of the edible and commercially interesting mushroom *A. bisporus* facilitates the unravelling of the molecular background of agronomic important traits like fruiting body discoloration. For all proposed enzymes involved in the melanin biosynthesis pathway (Figure 1 and Table 1) one or more gene homologs were identified. Among these, only the PPO co-factor, named L-chain, is unique for *A. bisporus* var. *bisporus* and var. *burnettii* of which only one homolog was found in the genome of H97 while six homologs were found for PPO. Using BLAST analysis no proteins were identified that showed sequence homology with the L-chain. In several bacterial species of *Streptomyces* the tyrosinase gene (*melC2*) is preceded by an open reading frame (ORF) named *melC1* (Chen, 1992). Bernan *et al.* (1985) postulated several possible functions for MelC1; I) a regulatory protein for the induction of tyrosinase, II) an accessory protein for the secretion of tyrosinase or III) a copper-transfer protein for the activation of the tyrosinase. For *Streptomyces castaneoglobisporus* ORF378 was identified as MelC1 (Matoba *et al.*, 2006). These authors describe ORF378 (MelC1) as a caddie protein because it is postulated to mediate the transport of Cu(II) ions into the catalytic centre of tyrosinase. This is in agreement with the proposed function III of Bernan *et al.* (1985). When the sequences of MelC1 of different *Streptomyces* species and the mushroom L-chain are compared they show low homology (results not shown). For *S. castaneoglobisporus* the crystal structure

of MelC2 with ORF378 was determined (Matoba *et al.*, 2006). Recently, the crystal structure of *A. bisporus* as a heterodimer of PPO_3 and the L-chain was identified (Ismaya *et al.*, 2011). When these crystal structures were compared, the core structure of PPO_3 (that constitute the heavy chain) showed similarity to the tyrosinase crystal structure of *S. castaneoglobisporus*, but no homology was found between the L-chain and ORF378. The function of the L-chain is possibly the same as proposed for the caddie protein of *S. castaneoglobisporus*, although no sequence or structure homology could be identified (Ismaya *et al.*, 2011).

As already stated by Moquet *et al.* (1999) no relation was found between bacterial blotch susceptibility (caused by *P. tolaassii*) and tyrosinase, but it was stated that other enzymes of the melanin biosynthesis might still be involved in this trait. Now, knowing the genes in this pathway and being able to study different homologs separately new research could shed new light on this issue. Both Jolivet *et al.* (1998) and Moquet *et al.* (1999) showed that GGT, which is involved in the formation of the phenolic substrate GHB (Figure 1), was directly linked with the level of susceptibility for *P. tolaassii*. This study identified two possible candidate genes that are responsible for this GGT activity of which *GGT_1* might be the most logical candidate because of its ten fold higher expression in skin than *GGT_2* (Figure 7). However, pathogen recognition may result in a specific up regulation of one of these two genes, which will be interesting to test. The genetic linkage between natural cap colour and resistance to bacterial blotch suggests that mechanisms of melanin synthesis for natural or pathogenic browning are strongly linked (Moquet *et al.*, 1999). Follow-up research can combine the analysis of different *A. bisporus* strains for their browning after bruising (Weijn *et al.*, 2012) and the resistance for *P. tolaassii* making use of the enzymes identified in this research.

Foulongne-Oriol *et al.* (2012) tried to identify QTL's related to *L. fungicola*, which causes dry bubble disease, the most common and serious fungal disease of

A. bisporus (Largeteau *et al.*, 2010). One of their conclusions is that the browning reactions caused by *L. fungicola* and *P. tolaassii* may result from a shared mechanism of resistance based on the melanin biosynthesis pathway. They already used some molecular markers derived from tyrosinase and laccase but no relation with the specific trait was identified. Largeteau *et al.* (2010) analysed the expression of laccase, tyrosinase and heat-shock genes during fruiting body and bubble development, to identify differential expression associated with disruption of morphogenesis following infection by *L. fungicola*. Possibly, with the genes identified in this research, candidate genes underlying QTL's can be identified and better correlation studies between genes and important biological processes can be performed.

Most studies did not dissect the mushrooms in different tissues but studied the expression in whole fruiting bodies. For example, Largeteau *et al.* (2010) found that *PPO_1* was constitutively expressed from the mycelial aggregate to the sphorophore (stage 3) and *PPO_2* showed an up regulation at these stages. Li *et al.* (2011) found increasing expression of *PPO_3* and *PPO_4* during fruit body maturation (from stage 1 until stage 6). As we studied the dissected tissues separately it will be difficult to compare the results. In our study, *PPO_1* expression decreased in the skin tissue from stage 4 until 7 and *PPO_2* had a more constant level of expression. Our study is in line with Li *et al.* as we also observed up-regulation of *PPO_3* in flesh tissue during maturation from stage 4 to 7.

The group of Foster demonstrated that many genes in the antibiotic pleuromutilin gene cluster from *Clitopilus passeckerianus* are involved in the same biosynthetic pathway (Mattos-Shipley *et al.*, 2011). It would be interesting to see whether this will be the case for more biosynthetic pathways as genome analysis like performed here can become a major tool in identifying new interesting genes involved in complex pathways in order to change important traits or to reconstruct fluxes of compounds. As mentioned above, the melanin pathway has been related in the

literature with bacterial resistance and here we identified a gene related to antibiotic production in the gene cluster containing many PPOs, underlining the relevance of studying clustered genes in more detail. The genes in the gene cluster between *PPO_3* and *PPO_5* will be very interesting to study further to elucidate their possible role in melanin formation and cellular events that will lead to the discoloration of mushrooms.

The melanin biosynthesis pathway in button mushrooms is analysed molecularly for the first time with this research and this should facilitate targeted breeding and selection strategies to develop mushroom strains that are less sensitive for discoloration. However, still other parameters as for instance peroxidases, proteases as PPO-activators, other proteins/enzymes involved in brown discoloration, or enzyme inhibitors might play a role in the discoloration process. Follow up research can focus on the functionality of all homologs, substrate specificity of the enzymes, and the genetic variation. One possible approach includes expressed QTL analysis on a segregating population in order to identify the rate limiting factor in the melanin pathway. In the future this can lead to mushrooms that show less bacterial infections, less discoloration during storage and less discoloration by bruising.

Acknowledgements

This project is supported by the governmental grant "TTI Green Genetics", Dutch Board for Horticulture, Bromyc, Lutèce, Sylvan, Banken Mushrooms, the Greenery and WeBe Engineering.

References

Bernan, V., Filpula, D., Herberm W., Bibb, M., Katz, E. (1985). The nucleotide sequence of tyrosinase gene from *Streptomyces antibioticus* and characterization of the gene product. *Gene*, *37(1-3)*, 101-110.

Burton, K.S. (2004). Cultural factors affecting mushroom quality - cause and control of bruising. *Mushroom Science*, *16*, 397-402.

Calla, B., Vuong, T., Radwan, O., Hartman, G.L., Clough, S.J. (2009). Gene expresssion profiling soybean stem tissue early response to *Sclerotinia sclerotiorum* and in silico mapping in relation to resistance markers. *The Plant Genome Journal*, *2*(2), 149-166.

Chen, L.Y., Leu, W.M., Wang, K.T., Lee, Y.H.W. (1992). Copper transfer and activation of the *Streptomyces* apotyrosinase are mediated through a complex formation between apotyrosinase and its trans-activator MelC1. *The Journal of Biological Chemistry*, *267*(28), 20100-20107.

Claus, H., Decker, H. (2006). Bacterial tyrosinases. *Systematic and Applied Microbiology*, *29(1)*, 3-14.

Conesa, A., Punt, P.J., van den Hondel, C.A.M.J.J. (2002). Fungal peroxidases: molecular aspects and applications. *Journal of Biotechnology*, *93(2)*, 143-158.

Daskalov, A., Paoletti, M., Ness, F., Saupe, S.J. (2012). Genomic clustering and homology between HET-S and the NWD2 STAND protein in various fungal genomes. *PLoS ONE*, *7*(4), 1-16.

Dewick, P.M. (1998). The biosynthesis of shikimate metabolites. *Natural Products Report*, *15*, 17-58.

Eisenman, H.C., Casadevall, A. (2012). Synthesis and assembly of fungal melanin. *Applied Microbiology and Biotechnology*, *93*(3), 931-940.

Flurkey, W.H., Inlow, J.K. (2008). Proteolytic processing of polyphenol oxidase from plants and fungi. *Journal of Inorganic Biochemistry*, *102*(12), 2160-2170.

Foulongne-Oriol, M., Rodier, A., Savoie, J.M. (2012). Relationship between yield components and partial resistance to *Lecaniculium fungicola* in the button mushroom *Agaricus bisporus*, assessed by quantitative trait locus mapping. *Applied Environmental Microbiology*, *78*(7), 2435-2442.

Hammond, J.B.W., Nichols, R. (1976). Carbohydrate metabolism in *Agaricus bisporus* (Lange) Sing.: Changes in soluble carbohydrates during growth of mycelium and sporophore. *Journal of General Microbiology*, *93*(2), 309-320.

Hofrichter, M., Ullrich, R., Pecyna, M.J., Liers, C., Lundell, T. (2010). New and classic families of secreted fungal heme peroxidases. *Applied Microbiology and Biotechnology*, *87*(3), 971-897.

Ismaya, W., Rozeboom H.J., Weijn, A., Mes, J.J., Fusetti, F., Wichers, H.J., Dijkstra B.W. (2011). Crystal structure of *Agaricus bisporus* mushroom tyrosinase: identity of the tetramer subunits and interaction with tropolone. *Biochemistry*, *50*(24), 5477-5486.

Jolivet, S., Arpin, N., Wichers, H.J., Pellon, G. (1998). *Agaricus bisporus* browning: a review. *Mycological Research*, *102*(12), 1459-1483.

Lankinen, P., Hildén, K., Aro, N., Salkinoja-Salonen, M., Hatakka, A. (2005). Manganese peroxidase of *Agaricus bisporus*: grain bran-promoted production and gene characterization. *Applied Microbiology and Biotechnology*, *66*(4), 401-407.

Largeteau, M.L., Latapy, C., Minvielle, N., Regnault-Roger, C., Savoie, J.M. (2010). Expression of phenol oxidase and heat-shock genes during the development of *Agaricus bisporus* fruiting bodies, healthy and infected by *Lecanicillium fungicola*. *Applied Microbiology and Biotechnology*, *85*(5), 1499-1507.

Li, N.-Y., Cai, W.-M., Jin, Q.-L., Qin, Q.-P., Ran, F.-L. (2011). Molecular cloning and expression of polyphenoloxidase genes from the mushroom, *Agaricus bisporus*. *Agricultural Sciences in China*, *10*(2), 185-194.

Marchler-Bauer, A., Anderson, J.B., Chitsaz, F., Derbyshire, M.K., DeWeese-Scott, C., Fong, J.H., Geer, L.Y., Geer, R.C., Gonzales, N.R., Gwadz, M., He, S., Hurwitz, D.I., Jackson, J.D., Ke, Z., Lanczycki, C.J., Liebert, C.A., Liu, C., Lu, F., Lu, S., Marchler, G.H., Mullokandov, M., Song, J.S., Tasneem, A., Thanki, N., Yamashita, R.A., Zhang, D., Zhang, N., Bryant, S.H. (2009). CDD: specific functional annotation with the Conserved Domain Database. *Nucleic Acids Research*, *37*, D205-D210.

Marusek, C.M., Trobough, N.M., Flurkey, W.H., Inlow, J.K. (2006). Comparative analysis of polyphenol oxidase from plant and fungal species. *Journal of Inorganic Biochemistry*, *100*(1), 108-123.

Matoba, Y., Kumagai, T., Yamamoto, A., Yoshitsu, H., Sugiyama, M. (2006). Crystallographic evidence that the dinuclear copper center of tyrosinase is flexible during catalysis. *The Journal of Biological Chemistry*, *281*(13), 8981-8990.

Mattos-Shipley, K., de Hayes, P., Collins, C., Kilaru, S., Hartley, A., Foster, G.D., Bailey, A.M. (2011). Biobased antibiotics from basidios: a case study on the identification and manipulation of a gene cluster involved in pleuromutilin biosynthesis from *Clitopilus passeckerianus*. *Proceedings of the 7th International Conference on Mushroom Biology and Mushroom Products*, INRA Bordeaux (pp. 224-231).

Moquet, F., Desmerger, C., Mamoun, M., Ramos-Guedes-Lafargue, M., Olivier, J.M. (1999). A quantitative trait locus of *Agaricus bisporus* resistance to *Pseudomonas tolaasii* is closely linked to natural cap color. *Fungal Genetics and Biology*, *28*(1), 34-42.

Paranjpe, M.S., Chen, P.K., Jong, S.C. (1978). Phenolic and other organic compounds in morphologically different tissues of *Agaricus bisporus*. *Transactions of the Mycological Society of Japan*, *19*(2), 169-180.

Roach, P.L., Clifton, I.J., Hensgens, C.M., Shibata, N., Schofield, C.J., Hajdu, J., Baldwin, J.E. (1997). Structure of isopenicillin N synthase complexed with substrate and the mechanism of penicillin formation. *Nature*, *387*(6635), 827-830.

Sano, H., Kaneko, S., Sakamoto, Y., Sato, T., Shishido, K. (2009). The basidiomycetous mushroom *Lentinula edodes* white collar-2 homolog PHRB, a partner of putative blue-light photoreceptor PHRA, binds to a specific site on the promotor region of the *L. edodes* tyrosinase gene. *Fungal Genetics and Biology*, *46*(4), 333-341.

Schurink, M., van Berkel, W.J.H., Wichers, H.J., Boeriu, C.G. (2007). Novel peptides with tyrosinase inhibitory activity. *Peptides*, *28*(3), 485-495.

Strothkamp, K.G., Jolley, R.L., Mason, H.S. (1976). Quaternary structure of mushroom tyrosinase. *Biochemical and Biophysical Research Communications*, *70*(2), 519-524.

Tsuji, H., Oka, T., Kimoto, M., Hong, Y-M., Natori, Y., Ogawa, T. (1996). Cloning and sequencing of cDNA encoding 4-aminobenzoate hydroxylase from *Agaricus bisporus*. *Biochimica et Biophysica Acta*, *1309*(1-2), 31-36.

Vandesompele, J., de Preter, K., Pattyn, F., Poppe, B., van Roy, N., de Paepe, A., Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, *3*(7), 1-12.

Voorrips, R. E. (2002). MapChart: Software for the graphical presentation of likange maps and QTLs. *The Journal of Heredity*, *93*(1), 77-78.

Walton, K., Coombs, M.M., Walker, R., Ioannides, C. (2001). The metabolism and bioactivation of agaritine and of other mushroom hydrazines by whole mushroom homogenate and by mushroom tyrosinase. *Toxicology*, *161*(3), 165-177.

Weijn, A., Tomassen, M.M.M., Bastiaan-Net, S., Wigham, M.L.I., Boer, E.P.J., Hendrix, E.A.H.J., Baars, J.J.P., Sonnenberg, A.S.M., Wichers, H.J., Mes, J.J. (2012). A new method to apply and quantify bruising sensitivity of button mushrooms. *LWT - Food Science and Technology*, *47*(2), 308-314.

Wichers, H. J., Recourt, K., Hendriks, M., Ebbelaar, C.E.M., Biancone, G., Hoeberichts, F.A., Mooibroek, H., Soler-Rivas, C. (2003). Cloning, expression and characterisation of two tyrosinase cDNAs from *Agaricus bisporus*. *Applied Microbiology and Biotechnology*, *61*(4), 336-341.

Wu, J., Chen, H., Goa, J., Liu, X., Cheng, W., Ma, X. (2010). Cloning, characterization and expression of two new polyphenol oxidase cDNAs from *Agaricus bisporus*. *Biotechnology Letters*, *32*(10), 1439-1447.

Zhang, X., Flurkey, W.H. (1997). Phenoloxidases in *Portabella* mushrooms. *Journal of Food Science*, *62*(1), 97-100.

Chapter 5

Phenolic compounds of the melanin biosynthesis pathway in bruising-tolerant and bruising-sensitive *Agaricus bisporus* strains

A. Weijn[a,b], B.P.M. van den Berg-Somhorst[a], J.C. Slootweg[c], J.-P. Vincken[b], H. Gruppen[b], H.J. Wichers[a,b] and J.J. Mes[a]

a Food and Biobased Research, Wageningen University and Research Centre, Bornse Weilanden 9, 6708 WG Wageningen, The Netherlands
b Laboratory of Food Chemistry, Wageningen University, Bomenweg 2, 6703 HD Wageningen, The Netherlands
c Department of Medicinal Chemistry and Chemical Biology, University Utrecht, Universiteitsweg 99, 3584 CG Utrecht, The Netherlands

Abstract

Mushrooms are prone to develop brown discoloration due to bruising, which leads to reduced quality of the mushrooms. The skin tissue of a selection of white and brown *Agaricus bisporus* button mushroom strains was bruised using a slip-shear sliding process. The severity of the bruise was quantified with a computer imaging system and strains were classified as tolerant or sensitive to bruising. Phenolic compounds were extracted from the skin tissue with a sulphite containing solution and analysed with UHPLC-PDA-MS. In total 34 phenolic compounds were obtained. Small differences were found for the total phenolic content of tolerant and sensitive strains. A high correlation between concentrations of specific phenolics and the bruising sensitivity was observed. The γ-L-glutaminyl-4-hydroxybenzene (GHB) and γ-L-glutaminyl-3,4-dihydroxybenzene (GDHB) contents were higher in the non-bruised skin tissue of sensitive strains and also remained more abundant in bruised skin tissue. White capped bruising-sensitive strains contained 3 times more GDHB, and 2.5 times more GHB, in the non-bruised skin tissue compared to tolerant strains. In the same tissue of the brown strains, 10 times and 17 times higher concentrations were found in sensitive strains for GDHB and GHB, respectively. It can be assumed that GHB and GDHB lead to the formation of brown coloured GHB-melanin and therefore it is postulated that this pathway is the most relevant for bruising-discoloration of button mushroom.

1 Introduction

Due to picking, handling and storage of the button mushroom *Agaricus bisporus*, discoloration reactions are initiated that result from enzyme-catalysed oxidation (polyphenol oxidase, PPO) of phenolics into their respective quinones (Burton, 2004; Jolivet *et al.*, 1998). These quinones undergo further oxidative polymerization reactions leading to melanins, high molecular mass dark brown or black pigments (Fogarty and Tobin, 1996). This so-called enzymatic browning reaction decreases the commercial value of mushrooms. Based on a study by Jolivet *et al.* (1998) four pathways for melanin synthesis in *A. bisporus* were proposed (Weijn *et al.*, 2012a) (Figure 1). One hypothesised pathway starts from chorismate and branches into three different melanin pathways and one starts from catechol. Chorismate, which is derived from the shikimate pathway, is converted via several enzymatic steps into γ-L-glutaminyl-4-hydroxybenzene (GHB) which has been identified as the main phenolic in *A. bisporus* fruiting bodies (Choi and Sapers, 1994). γ-Glutamyltransferase (GGT, EC 2.3.2.2) can transfers the glutamyl part to *p*-aminophenol to yield GHB. In the presence of PPO (EC 1.10.3.1) and oxygen, GHB is readily oxidized into the corresponding diphenol (GDHB) and subsequently into *o*-quinone (GBQ), which polymerizes into melanins (Weaver *et al.*, 1971). Besides, amino-phenolics might react to different kinds of melanin following the same PPO-mediated enzymatic and autopolymerisation steps, i.e. PAP-melanin, formed from *p*-aminophenol (Jolivet *et al.*, 1998), and DOPA-melanin from L-3,4-dihydroxyphenylalanine (L-DOPA) (Bell and Wheeler, 1986). The fourth pathway leads to catechol melanins, which are formed by the oxidation of catechol by PPO or through free radicals or quinone-catechol adducts (Bell and Wheeler, 1986) (Figure 1). Catechol has been identified in mushroom cap tissue (Paranjpe *et al.*, 1978). In mushroom spores, the GHB-melanin pathway is believed to be, initially, most important for browning (Jolivet *et al.*, 1998). This is also true for the browning of mushroom skins (Jolivet *et al.*, 1995; Pierce and Rast, 1995).

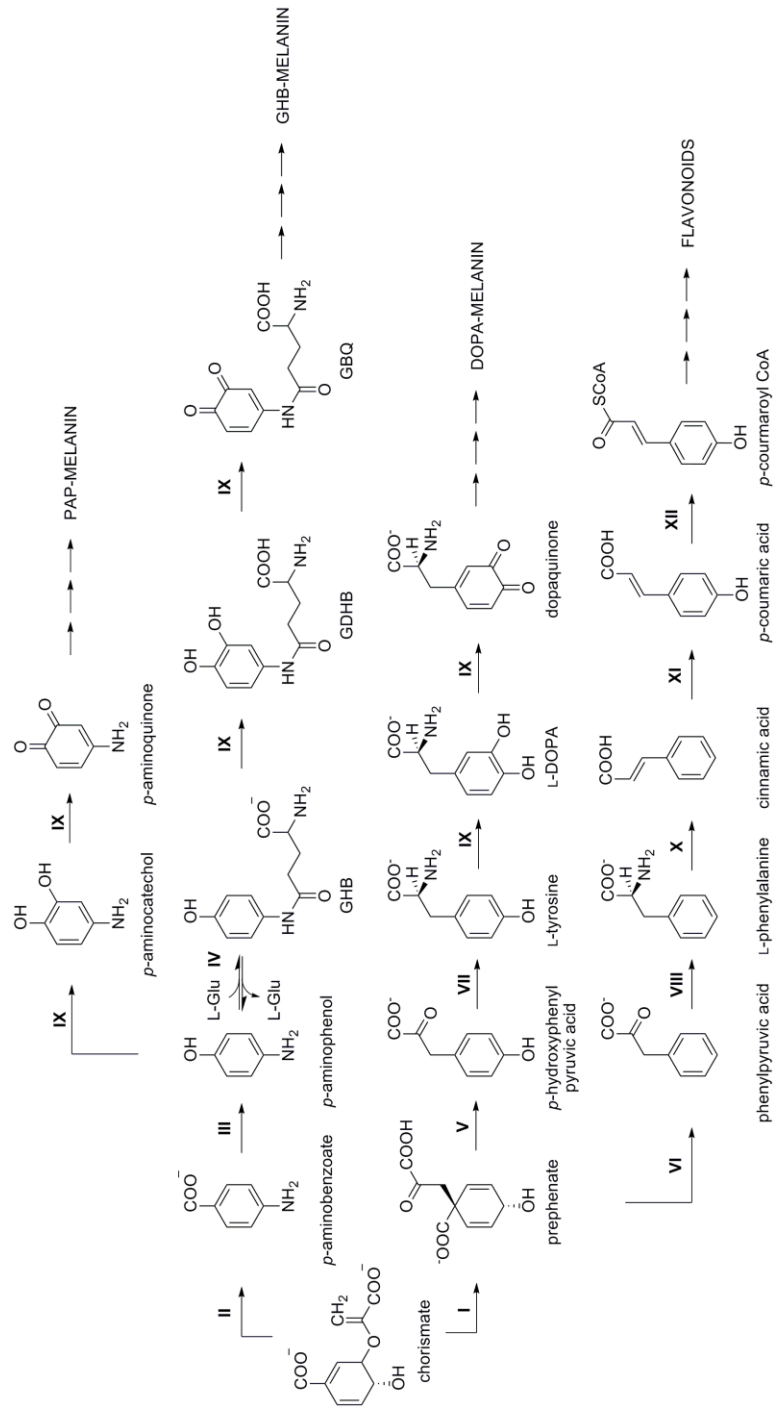
Due to the insolubility of melanins (Eisenman and Casadevall, 2012), no good methods are available to biochemically analyse and quantify them, and most studies on the discoloration reaction are dedicated to analysis their precursor phenolics. Most often HPLC analysis combined with UV measurements and/or mass spectrometry (MS) are used to identify and quantify individual phenolics that are involved in the browning reaction. Beaulieu *et al.* (1999) showed that a decrease in whiteness during 7 days of storage of *A. bisporus* var. *albidus* mushrooms resulted in an increase of chorismate, prephenate, and *p*-aminobenzoic acid. Unfortunately, Beaulieu *et al.* (1999) did not determine the concentration of GHB in the mushroom samples. Rast *et al.* (1979) measured the concentration of GHB in mushrooms of *A. bisporus* strain A-6 (ATCC 382581). The concentration of GHB increased in the whole fruiting body during maturation and the highest concentration was found in the lamellae (21.3 mg/g DW) of a 120 mm cap diameter mushroom. The contents of tyrosine, GHB and GDHB were nearly sevenfold higher in the cap skin of a browning-sensitive than in a less sensitive *A. bisporus* strain (Jolivet *et al.,* 1995). Mamoun *et al.* (1999) found that GHB contents from peel samples (epidermis and 2 mm cap tissue) were similar among wild strains which differed in natural colour. A significant difference was found between wild and commercial strains.

Tsai *et al.* (2007) analysed L-phenylalanine and L-tyrosine in *A. bisporus* mushrooms. The concentrations of both compounds were highest in stage 3 mushrooms (closed mushrooms with a cap diameter of 30-40 mm). Tseng and Mau (1999) also quantitatively analysed L-tyrosine and L-phenylalanine in button mushrooms and observed an increase for both compounds during storage. Sommer *et al.* (2010) studied these compounds in mushrooms with an intact veil and found a somewhat different concentration of tyrosine and phenylalanine than Tseng and Mau (1999) and Tsai *et al.* (2007). Besides the compounds discussed and shown in the pathway, some studies identified other phenolic compounds in *A. bisporus*

mushrooms (Kim *et al.*, 2008; Liu *et al.*, 2013; Palacios *et al.*, 2011), although some compounds were found only in one study and were not confirmed by others. These differences between studies may be related to the mushroom strain and developmental stage used and can be influenced by environmental factors, harvesting conditions, extraction solvents and detection methods (Rispail *et al.*, 2005). Most studies focussed on the analysis of a few phenolics from the same mushroom strain. There is a need for systematic studies which compare multiple mushroom strains and analyse the relation between phenolics and bruising-discoloration.

Here a UHPLC-PDA-MS method is described to analyse the phenolics of the melanin biosynthesis pathways resulting in a completer analysis of the individual biosynthetic intermediates in the skin tissue of *A. bisporus* button mushrooms than in previous studies. Besides, the results will give an impression of the most important fluxes through the major branches of the melanin pathway. Previously, *A. bisporus* strains with differential bruising sensitivity were identified (Weijn *et al.*, 2012b). Here, the phenolics of bruising-sensitive and bruising-tolerant button mushroom strains are analysed in order to correlate bruising-sensitivity to phenolic composition. Only the skin tissue of the cap was analysed because this tissue shows fastest and most pronounced discoloration after bruising. Our hypothesis is that these tools, together with gene expression analysis, will help in determining the key pathways and parameters for bruising-tolerance of *A. bisporus* strains. This will support targeted breeding and selection strategies to develop mushroom strains suitable for mechanical harvesting.

Figure 1. Putative melanin synthesis pathway of *A. bisporus*. Enzymes involved in the pathway are indicated by Roman symbols. I = chorismate mutase, II = 4-aminobenzoate synthase, III = 4-aminobenzoate hydroxylase, IV = γ-glutamyltransferase, V = prephenate dehydratase, VI = prephenate dehydrogenase, VII / VIII = (4-hydroxy) phenylpyruvate aminotransferase, IX = polyphenol oxidase, X = phenylalanine ammonia-lyase, XI = trans-cinnamate-4-monooxygenase, XII = 4-coumarate CoA ligase. GHB = γ-L-glutaminyl-4-hydroxybenzene. GDHB = γ-L-glutaminyl-3,4-dihydroxybenzene. GBQ = γ-L-glutaminyl-3,4-benzo-quinone (Adapted from Jolivet *et al.*, 1998).


PAP-MELANIN
p-aminocatechol
p-aminoquinone
GHB-MELANIN
GDHB
GBQ
L-Glu
L-Glu
p-aminobenzoate
p-aminophenol
GHB
DOPA-MELANIN
chorismate
prephenate
p-hydroxyphenyl pyruvic acid
L-tyrosine
L-DOPA
dopaquinone
FLAVONOIDS
phenylpyruvic acid
L-phenylalanine
cinnamic acid
p-coumaric acid
p-courmaroyl CoA
CATECHOL-MELANIN
catechol
o-benzoquinone
catechol dimeres

2 Material and methods

2.1 Materials used

The phenolic standards and other materials used were all HPLC grade compounds or solvents. *p*-Aminophenol, catechol, *p*-coumaric acid, L-DOPA, L-phenylalanine and L-tyrosine were purchased from Sigma-Aldrich (Steinheim, Germany). UHPLC-MS grade Milli-Q (MQ), acetonitrile (ACN), and formic acid (FA) were purchased from Biosolve BV (Valkenswaard, The Netherlands).

Chemicals used for the synthesis of GHB were obtained from commercial sources without further purification unless stated otherwise. The solvents used were peptide synthesis grade and stored on molecular sieves (4 Å).

2.2 Mushroom strains and cultivation

Mushrooms were grown as described elsewhere (Weijn *et al.*, 2012b). Nine *A. bisporus* strains (Table 2) were grown in seven replicates. The strains were randomly distributed over the growing room. Strains used originated either from the department of Plant Breeding at Wageningen University or from the ARP culture collection (Kerrigan, 1996). In order to minimise biological variation, all samples were collected during the same growing experiment under controlled pre- and post-harvest conditions.

2.3 Mushroom bruising and sampling

Mushroom bruising and discoloration analysis were performed as described elsewhere (Weijn *et al.*, 2012b). The bruised skin tissue of the cap was collected directly after taking the picture and immediately frozen in liquid nitrogen. As a control, samples of the skin tissue of non-bruised mushrooms were taken at the same time point. Samples were stored at -80 °C until use. The samples were ground with mortar and pestle with liquid nitrogen and for each experiment the

amount of mushroom powder needed was weighed. Weighed samples were handled with liquid nitrogen and stored at -80 °C.

2.4 SNP analysis of A. bisporus strains

A dendrogram was obtained for the *A. bisporus* strains based on single nucleotide polymorphism (SNP) analysis. With LTR-AFLP analysis it was determined previously that CH1, 2 and 3 are closely related to each other (personal communication with Patrick Hendrickx, Plant Research International). This information was used building the dendrogram, because SNP analysis was not performed for CH1 and 3. The similarity matrix was made with the simple matching method making use of the NTSYSpc2.1 program (Jamshidi and Jamshidi, 2011). For clustering SAHN was used, as the method UPGMA was used and, in the case of ties, Find was chosen.

2.5 GHB synthesis

Column chromatography was performed on Silicycle SiliFlash P60 silica gel (particle size 40-63 μm, Screening Devices b.v., Amersfoort, The Netherlands). Reaction progression was monitored using Thin Layer Chromatography (TLC) (Harwood and Moody, 1989). TLC was performed on Merck precoated silica gel 60F254 glass plates. Compound spots were evaluated by UV quenching of 254 nm black light and ninhydrin staining (Friedman, 2004). Analytical HPLC was performed on an automated HPLC system (Shimadzu) equipped with a UV/Vis detector operated at 220/254 nm using a Dr. Maisch ReproSil-Pur C18-AQ column (pore size: 120 Å, particle size: 5 μm, 250 × 4.6 mm, Screening Devices b.v.) at a flow rate of 1 ml/min (from 100 % buffer A (0.1 % TFA in CH_3CN/H_2O 95:5 v/v) to 100 % buffer B (0.1 % TFA in CH_3CN/H_2O 5:95 v/v) in 20 min. ESI-MS was performed on a Shimadzu LCMS-QP8000 electrospray ionization mass spectrometer operating in a positive ionization mode.

GHB (**3,** Supplementary Figure 1) was synthesized according to a literature procedure (Al-Obeidi *et al.*, 1990), because it is not commercially available. The synthesis started with coupling of Boc-Glu(OH)-OBn with *p*-aminophenol using BOP, which yielded N- and C-terminally protected GHB. Therefore, Boc-Glu(OH)-OBn (**1**) (1.01 g, 2.98 mmol) was dissolved in CH_2Cl_2 (30 ml) followed by addition of BOP (1.32 g, 2.98 mmol), *p*-aminophenol (390 mg, 3.58 mmol) and DiPEA (1.04 ml, 5.96 mmol). The dark coloured reaction mixture was stirred for 3 h at room temperature and concentrated in vacuum. The residue was taken up in EtOAc (30 ml) and the organic layer was subsequently washed with aq. 1 N $KHSO_4$ (3 × 20 ml), aq. 5% (v/v) $NaHCO_3$ (3 × 30 mL) and aq. sat. NaCl (3 × 20 ml). Then the EtOAc layer was dried (Na_2SO_4) and evaporated to dryness. The residue was purified by silica column chromatography and Boc-GHB-OBn (**2)** was obtained as an off-white solid in 50 % yield (683 mg).

In a two-step procedure the C-terminal benzyl-ester was deprotected using H_2 and Pd/C as a catalyst and finally the N-terminus was deprotected using 95% (v/v) TFA in H_2O. Therefore, Boc-GHB-OBn (**2)** (350 mg, 0.82 mmol) was dissolved in THF/H_2O (10 ml) and 10 % Pd/C (15 mg) was added to the solution. After flushing with H_2 (g), the reaction mixture was stirred for 5 h, pressurized with H_2, using a hydrogen-balloon. Then, the catalyst was filtrated off using Celite and the solution was evaporated to dryness. Subsequently, the residue was dissolved in 95 % (v/v) TFA in H_2O and stirred for 30 min at room temperature. The product was precipitated as its TFA salt, using MTBE/Hexane (30 ml, 1:1) and obtained as an off-white solid. After centrifugation (5 min, 3500 rpm (HERMLE rotor) at room temperature) the supernatant was decanted. The pellet was suspended a second time in MTBE/Hexane (30 ml, 1:1), centrifuged (5 min, 3500 rpm (HERMLE rotor) at room temperature) and the supernatant was decanted. After drying the pellet using a N_2 flow, the product was dissolved in *t*-BuOH/H_2O (30 ml, 1:9) and lyophilized yielding the TFA salt of GHB as a white powder in 73 % yield (200

mg). The product purity was confirmed by HPLC with a single peak at 6.55 min (Supplementary Figure 2). The calculated mass of GHB ($C_{11}H_{14}N_2O_4$) is 238.10, with ESI-MS m/z $[M+H]^+$ 239.00 was found (Supplementary Figure 3).

2.6 Sample preparation for UHPLC-PDA-MS analysis

For quantitative extraction of native phenolic compounds from the mushroom skin tissue, samples were extracted with a sulphite-containing solution. Kuijpers *et al.* (2012) found that sodium bisulphite ($NaHSO_3$) has a dual inhibitory effect in PPO-catalysed browning. On the one hand, it inhibits the enzymatic activity directly, and on the other hand it forms colourless sulfo-adducts, which cannot react further into melanin. Ten mg mushroom powder was weighed in a 1.5 ml dark Eppendorf tube using liquid nitrogen to cool the samples. To the sample 500 µl of a sulphite solution (0.5 % (w/v) sodium metabisulphite ($Na_2S_2O_5$) in 1 % (v/v) acetic acid solution) was added (based on Jolivet *et al.*, 1995; Soler-Rivas *et al.*, 1998). Each sample was vortexed for 1 min and put in an ultrasonic water bath for 10 min followed by centrifugation (5 min, 14462 g, 4 °C). The supernatants obtained were transferred to a new dark 1.5 ml Eppendorf tube. A second extraction was performed with the sulphite solution and the supernatants were combined. The samples were filtered with a 0.2 µm filter (Spartan 13/0.2 RC, Whatman) and stored at -20 °C until use.

2.7 UHPLC-PDA-MS

Samples were injected in an Accela UHPLC system (Thermo Scientific, San Jose, CA) equipped with a pump, autosampler and photo-diode array detector (PDA) using a Hypersil gold aQ column (150 mm x 2.1 mm i.d.; particle size 1.9 µm, Thermo Scientific) at 20 °C. The eluents used were 0.1 % FA in MQ (eluent A) and 0.1 % FA in ACN (eluent B). The elution program was 0-6 min 100 % A, 6-14 min 100 % - 90 % A with 0 - 10 % B, 14-15 min 10 % - 100 % B, 15-20 min

100 % B, 20-21 min 0-100 % A, 21-30 min 100 % A. The flow rate was 300 µl/min and the injection volume was 5 µl. The PDA detector was set to measure between 200 and 600 nm. MS^n analysis was performed on a Thermo Scientific LTQ-XL using electrospray ionisation (ESI) and detection in the positive and negative mode, with a source voltage of 4.7 kV and an ion transfer tube temperature of 260 °C. The instrument was tuned to optimise the ionisation process and sensitivity using L-tyrosine for the positive mode and catechol for the negative mode. A full-scan mass spectrum over a range of m/z values of 100-500 was recorded. From each branch of the melanin biosynthesis pathway (Figure 1) two standard compounds were chosen, except for the catechol-melanin branch for which only catechol was used. The other standard compounds were *p*-aminophenol and GHB, L-DOPA and L-tyrosine, L-phenylalanine and *p*-coumaric acid. Standard compounds were dissolved in 0.5 % (w/v) sodium metabisulphite ($Na_2S_2O_5$) in 1 % (v/v) acetic acid. Calibration curves were run at 254 nm (Table 1). Aromatic compounds often absorb light in the near-UV (200-400 nm), for analysis 254 nm was chosen due to L-phenylalanine, which has a smaller region of molar absorbance than the other standard compounds used (Schmid, 2001).

2.8 UHPLC-PDA-MS data analysis

Data analysis was performed with Xcalibur (Thermo Scientific, version 2.1.0). A background subtraction of a MQ sample was used for each sample. Peak integration was done at 254 nm with the ICIS algorithm. Peak parameters were set as follows; baseline window at 500, area noise factor at 10, and peak noise factor at 20. For all other settings the defaults were used. The automatic peak integration was checked to verify if the baseline was followed correctly and if peaks were integrated correctly (otherwise peak integration was performed manually). Peaks were considered for analysis when the peak area exceeded 0.5 % of the total peak area of that particular sample. In this way, 34 peaks were quantified with a high

reliability and reproducibility. Results are represented as the average of four determinations; the data were combined from two independent extracts which both were analysed in duplicate. Peak area was calculated per g fresh weight (g FW). Heat map analysis was done with Squared Pearson correlation for similarity and UPGMA for clustering in GeneMaths XT (Applied Maths, version 2.12). Identification of phenolic compounds was performed based on the standard compounds and the molecular mass of the parent ion, MS^n and UV/Vis spectra data. After comparison with the standards, unidentified peaks were compared with other phenolic compounds from the melanin pathway and compared to literature data. Besides, metabolite databases were used for confirmation of peak identity (http://metlin.scripps.edu/metabo_search_alt2.php and http://www.massbank.jp/). For conversion of peak area to concentration the corresponding standard compound was used. When this was not possible, a standard compound from the same branch in the melanin pathway was used.

Table 1. Standard compounds used for UHPLC-PDA-MS analysis. Linear range was determined at 254 nm.

Standard compound	Formula	MW (g/mol)	Rt time (min)	Linear range (µg)	Equation	Correlation R^2
p-aminophenol	C_6H_7NO	109.13	1.44-1.52	0.003-0.185	$y = 2 \cdot 10^7 x + 12673$	0.9988
L-DOPA	$C_9H_{11}NO_4$	197.19	2.78-2.97	0.004-0.198	$y = 9 \cdot 10^6 x - 12555$	0.9982
L-Tyrosine	$C_9H_{11}NO_3$	181.19	4.16-4.52	0.003-0.160	$y = 8 \cdot 10^6 x - 2890.4$	0.9966
GHB	$C_{11}H_{14}N_2O_4$	238.10	5.15-5.46	0.003-0.142	$y = 1 \cdot 10^8 x + 441882$	0.9961
L-Phenylalanine	$C_9H_{11}NO_2$	165.19	7.63-8.06	0.003-0.162	$y = 3 \cdot 10^6 x + 8065.5$	0.9949
Catechol	$C_6H_6O_2$	110.10	10.32-10.52	0.004-0.212	$y = 2 \cdot 10^7 x - 20849$	0.9985
p-coumaric acid	$C_9H_8O_3$	164.15	16.40-16.44	0.003-0.154	$y = 5 \cdot 10^7 x + 247625$	0.9911

3 Results

3.1 Browning-related bruising

A selection of white, off-white and brown mushroom strains, with different levels of bruising-sensitivity, were grown, picked, and bruised. Discoloration of each mushroom strain was quantified by comparing the colour of the non-bruised control area with that of the bruised area. The resulting Whiteness Index difference is shown in Table 2 (WI diff). Mushrooms with a white/off-white cap were statistically analysed separately from brown cap mushrooms, because the difference in background colour could not be entirely compensated for with computer image analysis. The results supported our previous findings (Weijn *et al.*, 2012b), as the strains showed the expected bruising-sensitivity and tolerance. In particular WB2 and WB18 showed very high levels of bruising-tolerance, with a WI difference around zero (Table 2) and the bruised skin area was hardly visible by the eye. The differences in WI diff between tolerant and sensitive strains is larger in the brown strains (WI diff -0.37 to 20.31) than for the white strains (WI diff 13.51 to 22.95).

Table 2. Bruise-related browning of nine *A. bisporus* mushroom strains. Strain names are according to Weijn *et al.* (2012b). LSD was calculated for white and off-white strains separately from the brown strains, which is indicated by #.

Strain	Abbreviation	Classification	WI diff	St dev	LSD
Commercial hybrid 3	CH3	Tolerant	13.51	4.43	a
Commercial hybrid 1	CH1	Tolerant	14.66	2.88	a
Commercial hybrid 2	CH2	Tolerant	15.45	3.30	a
Traditional white strain 4	TW4	Sensitive	22.45	5.37	b
Traditional off-white strain 7	TO7	Sensitive	22.95	4.39	b
Wild brown strain 2	WB2	Tolerant	-0.37	4.06	a#
Wild brown strain 18	WB18	Tolerant	0.13	6.56	a#
Wild brown strain 17	WB17	Sensitive	15.67	4.86	b#
Wild brown strain 16	WB16	Sensitive	20.31	4.81	b#

3.2 Genetic relationship of the selected strains

As differences in bruising-tolerance and the presence of phenolic compounds might be related to the genetic origin of the strain, a similarity matrix analyses was performed based on single nucleotide polymorphism (SNP) analysis (Figure 2). The results might indicate that the genetic origin of the tolerance trait of the three commercial strains originated from the same genetic background. The sensitive white strains TW4 and TO7 are genetically related and have a similar WI difference. The same is found for the sensitive brown strains, WB16 and WB17. The tolerant brown strains WB2 and WB18 cluster differently, although the WI difference is similar. Therefore it is postulated that different genes might underlay the bruising-tolerance trait, and possibly hinting at different mechanisms involved in the bruising-tolerance.

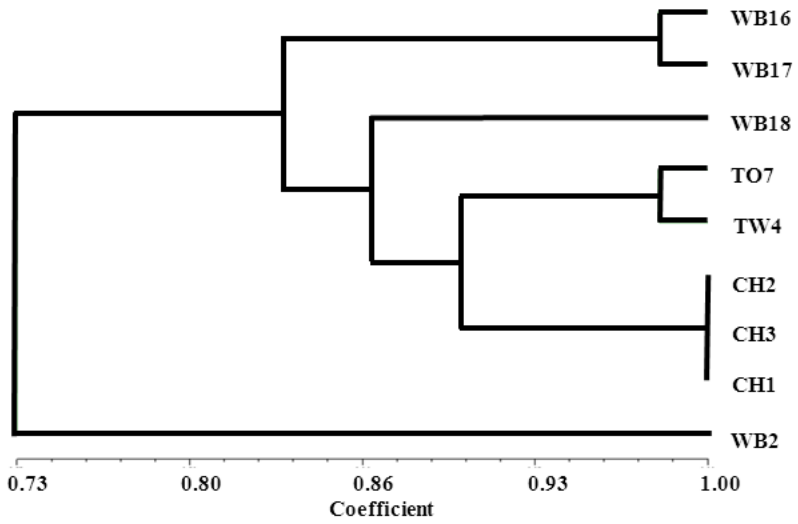


Figure 2. Dendrogram of the *A. bisporus* strains analysed, based on SNP analysis.

3.3 Identification of phenolic compounds from Agaricus bisporus skin tissue

The non-bruised and bruised skin tissue were peeled from the cap of the nine *A. bisporus* strains, the phenolic compounds were extracted and analysed with

UHPLC-PDA-MS. Two representative elution patterns are shown in Figure 3. The UV/Vis-spectra and MS data obtained for the standard compounds were used to identify these compounds in the mushroom skin tissue (Table 3) and all seven were identified in the mushroom skin tissue. In order to identify peaks additional to the standard compounds the UV/Vis and MS data obtained were compared with other phenolics from the melanin pathway, and subsequently with phenolic metabolites in general. This resulted in the identification of GDHB, *p*-hydroxybenzaldehyde, tryptophan, agaritine, agaritane and agaritinic acid. Quinones are highly reactive species and, therefore, not found with this analysis. In total, 13 of the 34 peaks in the chromatograms were identified. This covered almost all compounds as hypothesized in the pathways (*p*-aminophenol, GHB, GDHB, L-tyrosine, L-DOPA, L-phenylalanine, *p*-coumaric acid and catechol) except for the precursors at the start of the melanin pathways and the quinones (for the latter explained earlier).

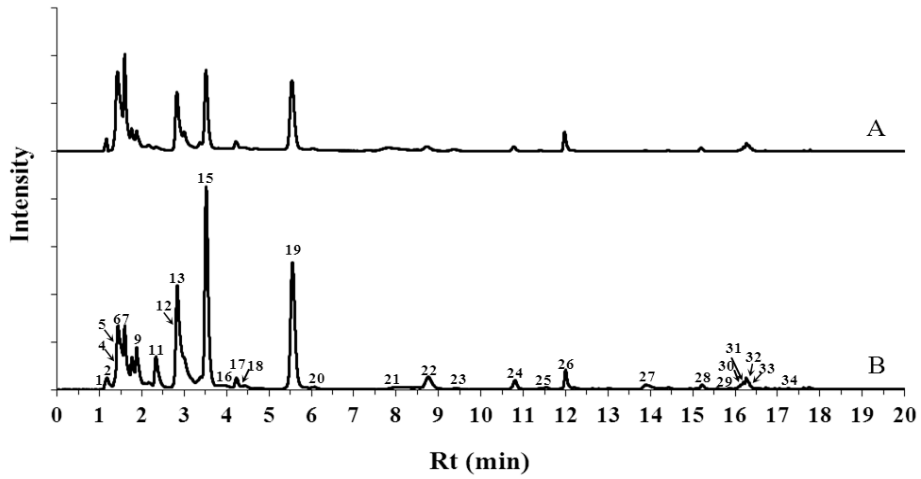


Figure 3. Elution profile of the skin tissue phenolics of two representative, non-bruised white *A. bisporus* strains. Tolerant CH3 (A) and sensitive TO7 (B).

Table 3. Identification of eluted peaks based on MS $[M+H]^+$ and UV/Vis data. For MS^2 are the ions written in order of intensity, the first one is the base peak. N/D = not determined.

Peak #	R_t(min)	MS (m/z)	MS^2 (m/z)	UV-vis λ_{max}	Tentative identification
1	1.05	123	82	204, 215	
2	1.14	179	161, 123	276, 229	
3	1.22	123	81, 79, 96, 93	276, 232	*p*-hydroxybenzaldehyde
4	1.32	205	76, 130, 84	216, 255	
5	1.39	205	76, 130, 84	262, 217	
6	1.43	110	-	271, 235	*p*-aminophenol
7	1.57	230	186, 127	258, 229	
8	1.73	118	72, 59, 88	243	
9	1.85	335	129, 205, 235	249, 297	
10	2.15	256	130, 206, 300, 289, 278, 98	262, 217	
11	2.24	241	210, 130, 84	246, 217	
12	2.74	268	232, 121	240	agaritine
13	2.93	198	138, 135, 162	279, 236	L-DOPA
14	3.19	132	86	245, 218	
15	3.38	255	126, 130, 192, 238, 108, 84	249, 285	GDHB
16	3.77	150	88, 70	217, 245, 282	
17	4.07	150	88, 70	260, 230	
18	4.20	182	146, 136, 123, 119	274, 231	L-tyrosine
19	5.30	239	110, 129, 193, 176, 222, 84	246	GHB
20	5.74	285	N/D	235, 306	
21	7.64	332	N/D	262	
22	8.32	166	120, 130, 135, 93	257, 220	L-phenylalanine
23	9.38	193	N/D	217, 226, 255	
24	10.41	(109)	- (negative mode)	274, 233	catechol
25	11.22	252	N/D	247	agaritane
26	11.71	280	130, 249, 240, 216	248, 298	agaritinic acid
27	13.69	259	232, 121	245, 216	
28	14.92	205, 188	146, 134, 92, 76	279, 229	tryptophan
29	15.57	157	106, 116, 125, 97	204, 254	
30	16.21	145	114, 60, 99	216, 238, 269	
31	16.26	215	187, 154, 86	269, 235	
32	16.30	145	60, 115, 95	276, 236	
33	16.39	165	119, 147, 123, 165	293, 241	*p*-coumaric acid
34	17.10	150	88, 70	216, 262	

3.4 Quantification of phenolic compounds

After peak identification, the total phenolic content of both the non-bruised and bruised skin tissue of the nine strains was compared. A reasonable correlation between bruising-sensitivity and total phenolics in the non-bruised tissue was found for both the white and brown strains (R^2 = 0.84 for the white strains and R^2 = 0.75 for the brown strains). The correlation between total phenolics in bruised skin tissue and the bruising-sensitivity was lower for both brown and white strains (R^2 = 0.68 for the white strains and R^2 = 0.32 for the brown strains). This might be explained when part of the phenolics are auto-polymerised into melanins, which are not detected with the used methods. The total content of phenolics was higher in the bruised than non-bruised tissue, except for strain WB16 (Figure 4). Next to that, the samples were compared based on peak area per gram FW for each peak individually. This was done to identify compounds that are different between non-bruised and bruised samples, and different between tolerant and sensitive strains. None of the phenolics was specifically present in non-bruised or bruised tissue, or in tolerant or sensitive strains, or in brown or white strains. However, a correlation between the content of specific phenolic compounds and bruising sensitivity was observed, as is clarified below.

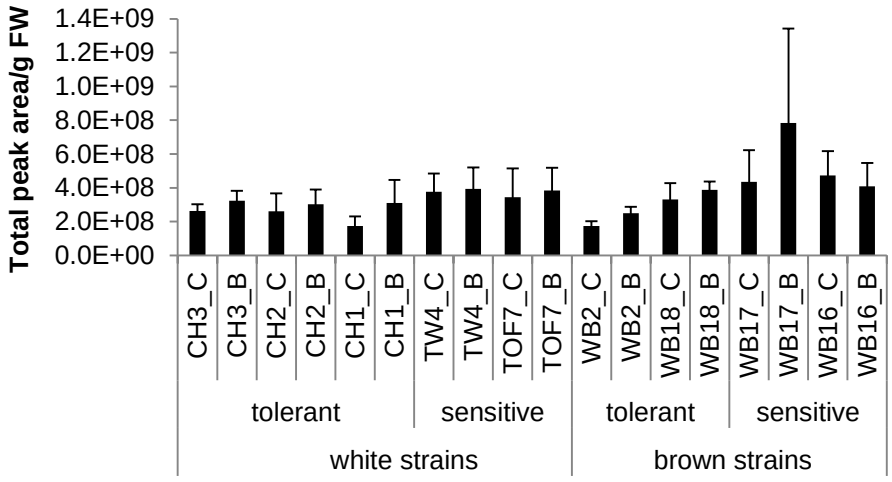


Figure 4. Total phenolics content in the *A. bisporus* strains analysed (previous page). The total phenolic content is based on total peak area per gram fresh weight (FW). C = non-bruised and B = bruised skin tissue.

3.5 Changes in phenolic compounds as a result of bruising

The changes in phenolic compounds upon bruising were different for each strain. The compounds that increased upon bruising in all nine strains were the unidentified peaks 10, 17, 29, and 30 (Figure 5). The percentage of the peak area of these four peaks together was approximately between 2.5 and 10 % of the total peak area which indicated that these peaks were not the most abundant phenolics (Figure 5). Tyrosine (peak 18) increases upon bruising in the white strains, except for strain CH2. For the brown strains, WB17 also showed an increase in tyrosine upon bruising, whereas for WB16 and WB18 a decrease was observed.

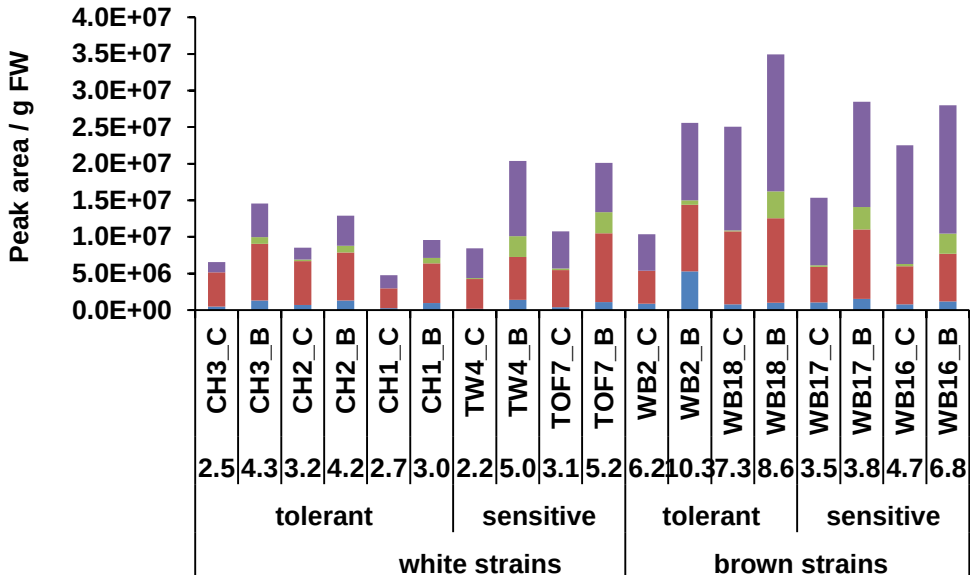


Figure 5. Peak area of four selected unidentified peaks that showed a consistent increase in concentration in cap tissue after bruising of nine *A. bisporus* strains.. Peak 10 in blue, peak 17 in red, peak 29 in green, and peak 30 in purple. The number below the bar indicates the percentage of these four peaks together of the total peak area of that strain.

Tryptophan (peak 28) slightly increased in all strains upon bruising, except for WB18 in which the concentration is the same in non-bruised and bruised skin tissue. Supplementary Figure 4 summarizes the difference in the content of phenolics between the bruised and non-bruised skin tissue samples of each strain. Considering the modest increase in phenolics content upon bruising, it might be that upon bruising of the mushroom skin phenolics are converted into more downstream phenolic compounds of the melanin pathways, whereas at the same time these phenolic compounds are replenished from chorismate, although it can not be predicted which specific phenolics are involved. The phenolic compounds formed were not the same for all strains.

3.6 Differences between bruising-sensitive and tolerant strains before bruising

When the non-bruised skin tissue of bruising-sensitive and tolerant strains were compared, the total content of phenolics and bruising-sensitivity did not seem to be correlated. When the elution profile of the extracts from the non-bruised skin tissue of a white tolerant and sensitive strain were compared (Figure 3), a clear difference in the intensity of the individual phenolics was observed; the same was found for the brown strains. Results of all strains, before bruising, were summarized in a heat map-based comparison using an unsupervised clustering of the samples and all peaks detected are shown in order of elution (Figure 6, shown as peak area per g FW). Clustering of the *A. bisporus* strains showed a division in sensitive and tolerant strains. It is striking that GDHB and GHB were more abundant in the sensitive strains, whereas its direct precursor in the GHB-melanin biosynthesis pathway, *p*-aminophenol showed similar abundance in sensitive and tolerant strains. Furthermore, the GHB and GDHB contents seemed to vary among the tolerant strains. Next to that, it was observed that compound 9 was also higher in sensitive than in tolerant strains. On average, compound 9 is 2.5 higher in the sensitive white strains than in the tolerant white strains, whereas GDHB and GHB

are 3 times and 2.5 times higher, respectively. For the brown strains, a more pronounced difference was found between sensitive and tolerant strains for compound 9, GHB and GDHB: 4 times for compound 9, 10 times for GDHB and 17 times for GHB. For the non-bruised white strains it was found that compounds 25, 27 and 30 were 4, 3 and 2.5 times higher in the sensitive strains compared to the tolerant strains, respectively. This was not observed for the brown strains. The difference between bruising-sensitive and tolerant strains could, therefore, be related to the presence of phenolic compounds in the skin before bruising, in particular compound 9, GDHB, and GHB. These compounds comprise between 36 - 52 % of the total peak area for the white and brown sensitive strains and, therefore, are likely to have a major impact on melanin formation. The very tolerant strain WB2 had an extremely low content (less than 10%) of these three compounds which might be the key to obtain new tolerant strains.

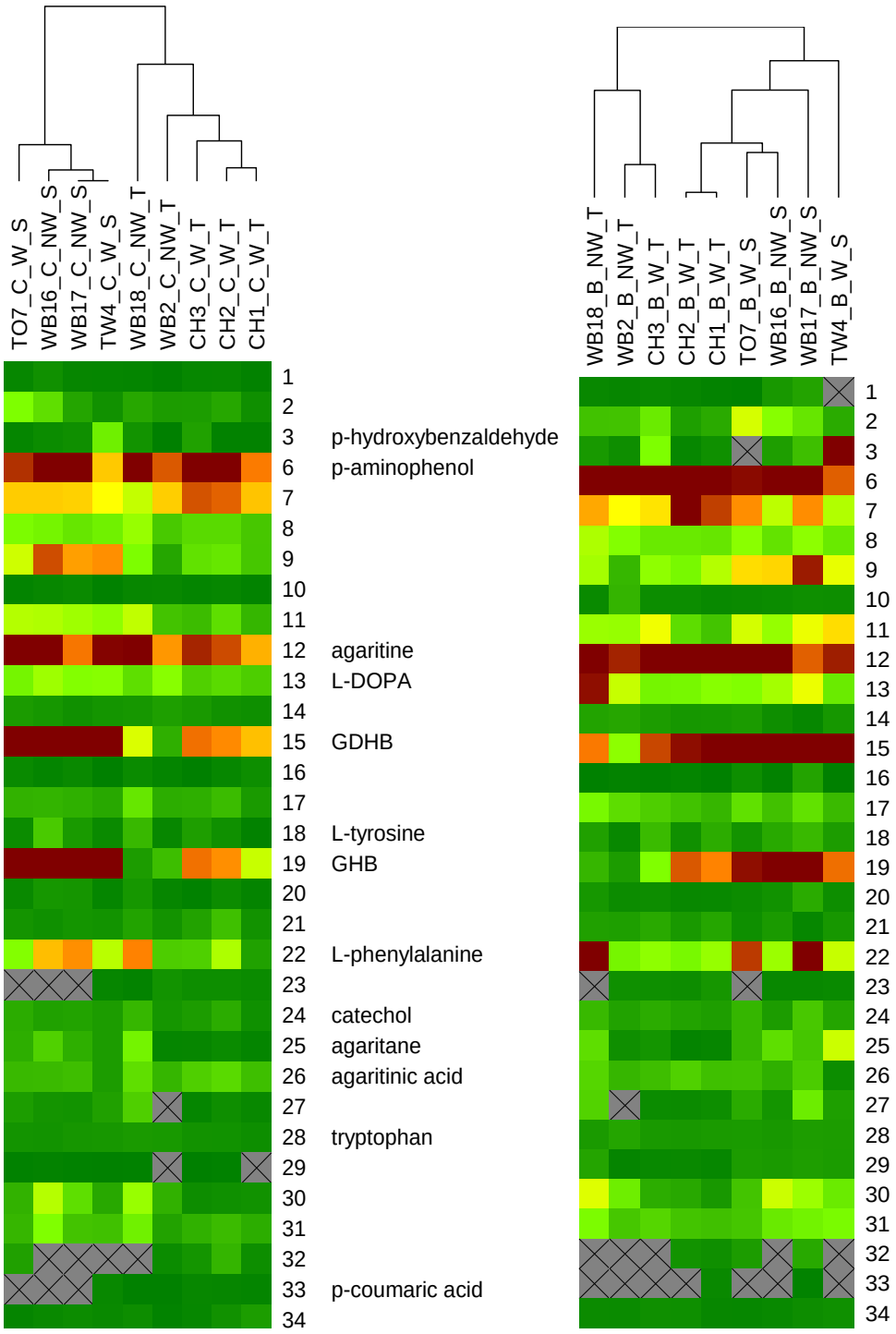
3.7 Difference between bruising-sensitive and tolerant strains after bruising

Differences in bruising sensitivity between the strains might also originate from the presence of specific enzymes that are able to convert the phenolic compounds into melanins or intermediate compounds within the melanin pathway. Therefore, also the bruised skin tissues, 60 min after applying the bruise, of the nine strains were analysed (Figure 7). Data revealed that upon bruising some of the phenolic compounds increased in one strain, but decreased in other strains. Unsupervised clustering of the bruised samples resulted in two main clusters, but these were not based on the bruising-sensitivity as was found for the non-bruised skin tissue. The tolerant strains commercial hybrid 1 and 2 are closer related to the sensitive strains than to the other tolerant strains. After bruising, the same phenolic compounds as found in the non-bruised tissue (compound 9, GDHB and GHB) were still higher in the sensitive strains. However, a different ratio was found comparing the bruised skin tissue of the bruising-sensitive and bruising-tolerant strains than found for the

non-bruised tissue. Compound 9 was 1.5 times higher in the bruised sensitive white strains and 3 times in the bruised sensitive brown strains compared to the tolerant strains with the same cap colour. These ratios were slightly lower than in the corresponding non-bruised skin samples. The sensitive white strains contained on average 2 and 1.5 times more GDHB and GHB, respectively, than the tolerant white strains. The brown strains contained 7 and 29 times higher concentration of GDHB and GHB, respectively, in the sensitive than the tolerant strains. Probably induced by bruising, GHB is formed and GDHB is converted into melanin in the brown bruising-sensitive strains. In the bruised tissue of the white strains it was found that both compounds 27 and 29 were 3 times higher in the sensitive strains compared to the tolerant strains. This was not found for the brown strains. In the brown strains compound 1 was two times higher in the bruised tissue of the sensitive strains than that in the tolerant strains. Again it is striking that strain WB2 still has very low concentrations of GHB, GDHB, and compound 9. Besides, WB2 also does not have the clear presence of DOPA as found in the other brown tolerant strain WB18. WB2 will therefore be a perfect strain for detailed analyses of bruising tolerance and for commercial applications.

Figure 6. Heat map analysis of non-bruised skin tissue samples based on peak area. The lowest concentration is shown in dark green, the highest concentration in dark red. A grey square with a cross indicates peaks that could not be integrated. C = non-bruised and B = bruised skin tissue. W = white and NW = non-white strains. T = tolerant and S = sensitive.

Figure 7. Heat map analysis of bruised skin tissue samples based on peak area. The lowest concentration is shown in dark green, the highest concentration in dark red. A grey square with a cross indicates peaks that could not be integrated. C = non-bruised and B = bruised skin tissue. W = white and NW = non-white strains. T = tolerant and S = sensitive.


TO7_C_W_S
WB16_C_NW_S
WB17_C_NW_S
TW4_C_W_S
WB18_C_NW_T
WB2_C_NW_T
CH3_C_W_T
CH2_C_W_T
CH1_C_W_T
WB18_B_NW_T
WB2_B_NW_T
CH3_B_W_T
CH2_B_W_T
CH1_B_W_T
TO7_B_W_S
WB16_B_NW_S
WB17_B_NW_S
TW4_B_W_S
1
2
3
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
p-hydroxybenzaldehyde
p-aminophenol
agaritine
L-DOPA
GDHB
L-tyrosine
GHB
L-phenylalanine
catechol
agaritane
agaritinic acid
tryptophan
p-coumaric acid

4 Discussion

In total 34 compounds were used to analyse the bruising-discoloration. The main phenolics involved in bruising-discoloration were identified to be GHB, GDHB and unidentified compound 9. It can therefore be concluded that the main melanin formed upon bruising in the sensitive strains is GHB-melanin. The different accumulation levels of these compounds is most pronounced for the brown strains, where these phenolics comprised around 10 % of the total content of phenolics in the tolerant strains, and around 50 % in the sensitive strains (Table 4).

Table 4. Percentage of compound 9, GHB and GDHB together of the total peak area of each strain.

Strain	Bruising-sensitivity	Non-bruised	Bruised
CH3	Tolerant	26.17	17.36
CH2	Tolerant	23.51	26.33
CH1	Tolerant	26.54	29.57
TW4	Sensitive	51.30	31.80
TO7	Sensitive	36.94	31.66
WB2	Tolerant	8.86	8.65
WB18	Tolerant	9.65	12.10
WB17	Sensitive	50.77	55.16
WB16	Sensitive	45.29	41.35

The precursor of GHB, *p*-aminophenol, was found in high quantities in all strains, suggesting that the conversion into GHB is rate-limiting in WB2 and WB18. This might be caused by genetic mutations resulting in changes in the expression of genes involved in this conversion step or mutations leading to changed enzyme activity. The very tolerant strains WB2 and WB18 contained less GHB and GDHB than the tolerant commercial white strains CH1, 2, and 3. At the same time they also differ in the content of phenylalanine and DOPA in non-bruised and bruised samples, respectively. This might indicate that both WB2 and WB18 have a different mechanism of modifying phenolics, and that the current tolerance level in the white commercial strains might be improved by introducing the genes

responsible for the tolerance in the brown strains, like those present in WB2 and WB18.

The phenolic content (unidentified compound 9, GHB and GDHB) was higher in the non-bruised and bruised skin tissue of bruising-sensitive strains of both white and brown strains. Jolivet *et al.* (1995) found a nearly sevenfold higher phenolic content in the skin (combined quantity of tyrosine, GHB and GDHB) of a browning-sensitive strain than in a less sensitive strain. In our study tyrosine (peak 18) did not show a consistent correlation with bruising sensitivity level. It can be stated that the concentrations of compound 9, GHB and GDHB in a mushroom are a good indication for the bruising-sensitivity; the higher the relative concentrations, the more sensitive. A correlation of R^2 of 0.78, 0.81, and 0.73 was found in the non-bruised skin tissue of white strains between bruising sensitivity and compound 9, GDHB and GHB, respectively. An even higher correlation was found for the non-bruised tissue of brown strains: R^2 of 0.94, 0.98 and 0.85 for compound 9, GDHB and GHB, respectively. Based on this correlation, GDHB-concentration currently provides the best indication for bruising-sensitivity and GDHB might be used as a marker to predict bruising-sensitivity of a strain.

The concentration of agaritine (0.25-0.74 mg/kg FW, as equivalent of GHB) reported here is lower than previously found by Sharman *et al.* (1990) for two other button mushroom strains (between 80-250 mg/kg FW, based on an agaritine standard). The concentrations of L-tyrosine and L-phenylalanine found by Tsai *et al.* (2007), Tseng and Mau (1999) and Sommer *et al.* (2010) were also higher than found in this research. This is probably due to the difference in use of fresh or dried samples and the fact that only skin tissue was analysed and not the whole mushroom. Choi and Sapers (1994) found that the contents of GHB plus tyrosine and GDHB plus L-DOPA (it was impossible to isolate and quantify the compounds separately, due to the overlapping of GHB and tyrosine peaks and of GDHB and L-DOPA peaks) was highest in the gills of dry-packed mushrooms, followed by skin,

stipe and cap. With the new developed method described in this report it was possible to analyse these four phenolic compounds separately.

Not all peaks were identified based on the UV/Vis and MS data obtained. First UHPLC results were compared with the standard compounds which confirmed the presence of these compounds in all samples. After that, the unidentified peaks were compared with the phenolic compounds from the melanin biosynthesis pathway. This did not lead to the identification of the unknown compounds except for GDHB. Next, the literature was studied for phenolic compounds identified in *A. bisporus.* MW of these compounds was calculated and compared with the obtained m/z $[M-H]^+$ values. This led to the identification of *p*-hydroxybenzaldehyde, tryptophan, and agaritine and related compounds agaritinate and agaritinic acid, but still most peaks were not identified. This might be due to the fact that in the research presented here only cap skin tissue is analysed, which probably comprises different phenolic compounds than mycelium or the flesh and stem of the fruiting body. A full library of phenolic compounds will be needed to identify all peaks. The main compounds related to bruising-sensitivity (GHB and GDHB) were confirmed; leaving only a limited number of intriguing compounds unidentified (e.g. compounds 9, 25, 27, 29 and 30). These will retain our attention until they are identified and their possible role in the melanin biosynthesis pathway is hypothesized.

Based on this study it is tempting to speculate what exactly happens in the cells upon bruising. A remarkable observation is that the overall phenolic content in the sample increases while it was expected that, upon bruising, phenolic components present in cells (perhaps inside vacuoles) would be transported to the cytosol were they polymerised into melanins mediated by enzymes present in the cytoplasm (Marusek *et al.*, 2006; Mayer, 2006; Rast *et al.*, 2003). This might still be the case but then it should be concluded that phenolic compounds used for these melanins are directly replenished with new compounds, indicating a dynamic flux of

components from a reservoir of precursors. The increase of GHB and GDHB would then originate from a reservoir of phenolic compounds like *p*-aminophenol, *p*-aminobenzoate and, even further upstream, chorismate. However *p*-aminobenzoate and chorismate were not identified among the peaks and moreover none of the unidentified compounds showed a large inverse correlation with the increase in GHB. This could either indicate that there are alternative pathways for the synthesis of GHB or a very fast release of precursors combined with enzymatic conversion into compounds like GHB.

More in-depth analysis of the melanin pathway, in which gene sequences, gene expression, enzyme activity and phenolic compounds are analysed, will be required to identify the full mechanism for bruising-tolerant strains. In particular, the conversion of *p*-aminophenol in GHB and GDHB by enzymes such as γ-glutamyl transferase and polyphenol oxidases will be of major interest.

Acknowledgements

This project is supported by the governmental grant "TTI Green Genetics", Dutch Board for Horticulture, Bromyc, Lutèce, Sylvan, Banken Mushrooms, the Greenery and WeBe Engineering. J.C. Slootweg performed the synthesis of GHB and is funded by the Netherlands Organization of Scientific Research (NWO) ECHO grant 700.57.014. Mark Sanders (Laboratory of Food Chemistry, Wageningen University) is thanked for his help with the UHPLC-PDA-MS setup. The similarity matrix was made by Patrick Hendrickx from Plant Research International, Wageningen University.

References

Al-Obeidi, F., Mulcahy, M., Pitt, V.S., Begay, V., Hadley, M.E., Hruby, V.J. (1990). Synthesis and actions of a melanotropin conjugate, Ac-[Nle^4, Glu(gamma-4′-hydroxyanilide)^5, D-Phe^7]α-MSH_{4-10}-NH_2, on melanocytes and melanoma cells in vitro. *Journal of Pharmaceutical Sciences, 79(6)*, 500-504.

Beaulieu, M., Beliveau, M., D'Aprano, G., Lacroix, M. (1999). Dose rate effect of gamma irradiation on phenolic compounds, polyphenol oxidase, and browning of mushrooms (*Agaricus bisporus*). *Journal of Agricultural and Food Chemistry*, *47*(7), 2537-2543.

Bell, A.A., Wheeler, M.H. (1986). Biosynthesis and functions of fungal melanins. *Annual Reviews Phytopathology*, *24*, 411-451.

Burton, K.S. (2004). Cultural factors affecting mushroom quality - cause and control of bruising. *Mushroom Science*, *16*, 397-402.

Choi, S.W., Sapers, G.M. (1994). Effects of washing on polyphenols and polyphenol oxidase in commercial mushrooms (*Agaricus bisporus*). *Journal of Agricultural and Food Chemistry*, *42*(10), 2286-2290.

Eisenman, H.C., Casadevall, A. (2012). Synthesis and assembly of fungal melanin. *Applied Microbiology and Biotechnology*, *93*(3), 931-940.

Fogarty, R.V., Tobin, J.M. (1996). Fungal melanins and their interactions with metals. *Enzyme and Microbial Technology*, *19*(4), 311-317.

Friedman, M. (2004). Applications of the ninhydrin reaction for analysis of amino acids, peptides, and proteins to agricultural and biomedical sciences. *Journal of Agricultural and Food Chemistry*, *52*(3), 385-406.

Harwood, L.M., Moody, C.J. (1989). Thin Layer Chromatography. *Experimental Organic Chemistry: Principles and Practice* (pp. 159-173). John Wiley and Sons.

Jamshidi, S., Jamshidi S., (2011). NTSYSpc 2.02e Implementation in molecular data analysis (clustering, screening and individual selection). *International Conference on Environmental and Computer Science IPCBEE volume 19*, 165-169.

Jolivet, S., Arpin, N., Wichers, H.J., Pellon, G. (1998). *Agaricus bisporus* browning : a review. *Mycological Research*, *102*(12), 1459-1483.

Jolivet, S., Voiland, A., Pellon, G., Arpin, N. (1995). Main factors involved in the browning of *Agaricus bisporus*. *Mushroom Science*, *14*, 695-702.

Kerrigan, R.W. (1996). Characteristics of a large collection of edible wild mushroom germ plasm: the *Agaricus* resource program. In R.A. Samson, *et al.* (Eds.),*Culture collections to improve the quality of life* (pp. 302-307). Centraalbureau voor Schimmelcultures P.O. Box 273, 3470 AG Baarn, The Netherlands.

Kim, M.-Y., Seguin, P., Ahn, J.-K., Kim, J.-J., Chun, S.-C., Kim, E.-H., Seo, S.-H., Kang, E.-Y., Kim, S.-L., Park, Y.-J., Ro, H.-M., Chung, I.M. (2008). Phenolic compound concentration and antioxidant activities of edible and medicinal mushrooms from Korea. *Journal of Agricultural and Food Chemistry*, *56*(16), 7265-7270.

Kuijpers, T.F.M., Narváez-Cuenca, C.-E., Vincken, J.-P., Verloop, A.J.W., van Berkel, W.J.H., Gruppen, H. (2012). Inhibition of enzymatic browning of chlorogenic acid by sulfur-containing compounds. *Journal of Agricultural and Food Chemistry*, *60*(13), 3507-3514.

Liu, J., Jia, L., Kan, J., Jin, C.-H. (2013). In vitro and in vivo antioxidant activity of ethanolic extract of white button mushroom (*Agaricus bisporus*). *Food and Chemical Toxicology*, *51*, 310-316.

Mamoun, M., Moquet, F., Savoie, J.-M., Devesse, C., Ramos-Guedes-Lafargue, M., Olivier, J.M., Arpin, N. (1999). *Agaricus bisporus* susceptibility to bacterial blotch in relation to environment: biochemical studies. *FEMS Microbiology Letters*, *181*(1), 131-136.

Marusek, C.M., Trobaugh, N.M., Flurkey, W.H., Inlow, J.K. (2006). Comparative analysis of polyphenol oxidase from plant and fungal species. *Journal of Inorganic Biochemistry*, *100*(1), 108-23.

Mayer, A.M. (2006). Polyphenol oxidases in plants and fungi: going places? A review. *Phytochemistry*, *67*(21), 2318-2331.

Palacois, I., Lozano, M., Moro, C., Rostagno, M.A., Martínez, J.A., García-Lafuente, A., Guillamón, E., Villares, A. (2011). Antioxidant properties of phenolic compounds occurring in edible mushrooms. *Food Chemistry*, *128*(3), 674-678.

Paranjpe, M.S., Chen, P.K., Jong, S.C. (1978). Phenolic and other organic compounds in morphologically different tissues of *Agaricus bisporus*. *Transactions of the Mycological Society of Japan*, *19*, 169-180.

Pierce, J.A., Rast, D.M. (1995). A comparison of native and synthetic mushroom melanins by Fourier transform infrared spectroscopy. *Phytochemistry*, *39*(1), 49-55.

Rast, D.M., Baumgartner, D., Mayer, C., Hollenstein, G.O. (2003) Cell wall-associated enzymes in fungi. *Phytochemistry*, *64*(2), 339-366.

Rast, D., Stussi, H., Zobrist, P. (1979). Self-inhibition of the *Agaricus bisporus* spore by CO_2 and / or y-glutaminyl-4-hydroxybenzene and y-glutaminyl-3,4-benzoquinone: a biochemical analysis. *Physiologia Planetarum*, *46*(3), 227-234.

Rispail, N., Morris, P., Webb, K.J. (2005). Phenolic compounds: extraction and analysis. In A.J. Marquez (Ed.), *Lotus japonicus handbook* (pp. 349-355).

Sharman, M., Patey, A.L., Gilbert, J. A survey of the occurrence of agaritine in U.K. cultivated mushrooms and processed mushroom products. *Food Addit. Contam.* **1990**, *7*, 649-656.

Schmid, F.-X. (2001). Biological macromolecules: UV-visible spectrophotometry. *Encyclopedia of Life Sciences* (pp. 1-4). Macmillan Publishers Ltd.

Soler-Rivas, C., Jolivet, S., Yuksel, D., Arpin, N., Oliver, J.M., Wichers, H.J. (1998). Analysis of *Agaricus bisporus* tyrosinase activation and phenolics utilization during *Pseudomonas tolaasii* or tolaasin-induced discolouration. *Mycological Research*, *102*(12), 1497-1502.

Sommer, I., Schwartz, H., Solar, S., Sontag, G. (2010). Effect of gamma-irradiation on flavour 5′-nucleotides, tyrosine, and phenylalanine in mushrooms (*Agaricus bisporus*). *Food Chemistry*, *123*(1), 171-174.

Tsai, S.-Y., Wu, T.-P., Huang, S.-J., Mau, J.-L. (2007). Nonvolatile taste components of *Agaricus bisporus* harvested at different stages of maturity. *Food Chemistry*, *103*(4), 1457-1464.

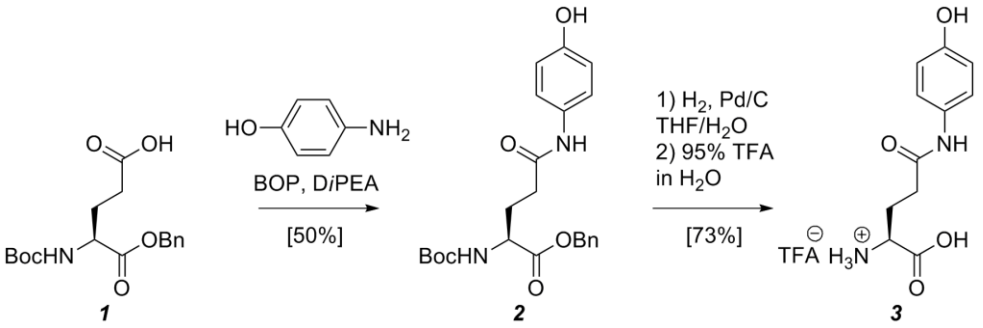
Tseng, Y.-H., Mau, J.-L. (1999). Contents of sugars, free amino acids and free 5'-nucleotides in mushrooms, *Agaricus bisporus*, during post-harvest storage. *Journal of the Science of Food and Agriculture*, *79*(11), 1519-1523.

Weaver, R.F., Rajagopalan, K.V., Handler, P., Byrne, W.L. (1971). y-L-Glutaminyl-3,4-benzoquinone structural studies and enzymatic synthesis. *The Journal of Biological Chemistry*, *246*(7), 2015-2020.

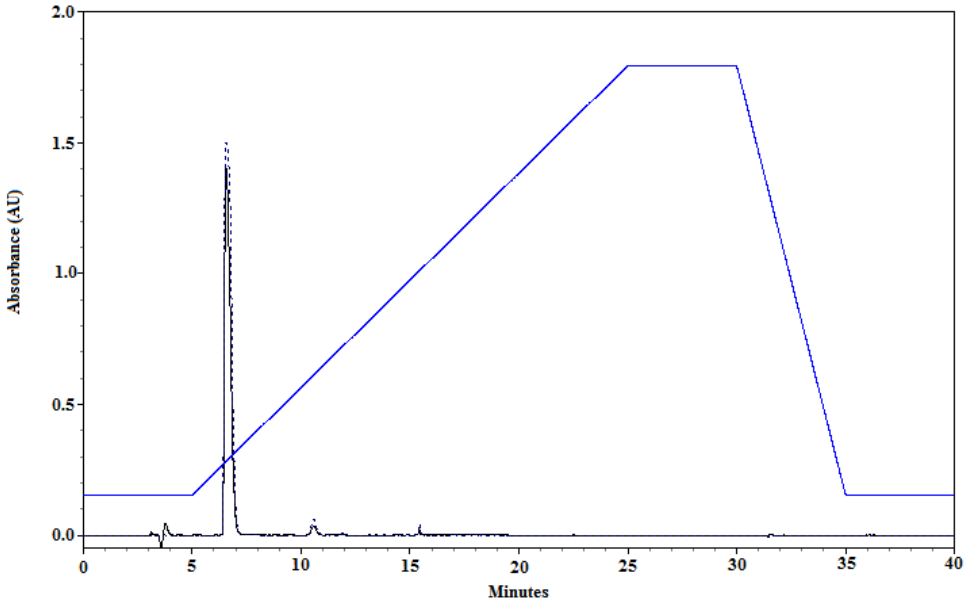
Weijn, A., Bastiaan-Net, S., Wichers, H.J., Mes, J.J. (2012a). Melanin biosynthesis pathway in *Agaricus bisporus* mushrooms. *Fungal Genetics and Biology*. http://dx.doi.org/10.1016/j.fgb.2012.10.004

Weijn, A., Tomassen, M.M.M., Bastiaan-Net, S., Wigham, M.L.I., Boer, E.P.J., Hendrix, E.A.H.J., Baars, J.J.P., Sonnenberg, A.S.M., Wichers, H.J., Mes, J.J. (2012b). A new method to apply and quantify bruising sensitivity of button mushrooms. *LWT - Food Science and Technology*, *47*(2), 308-314.

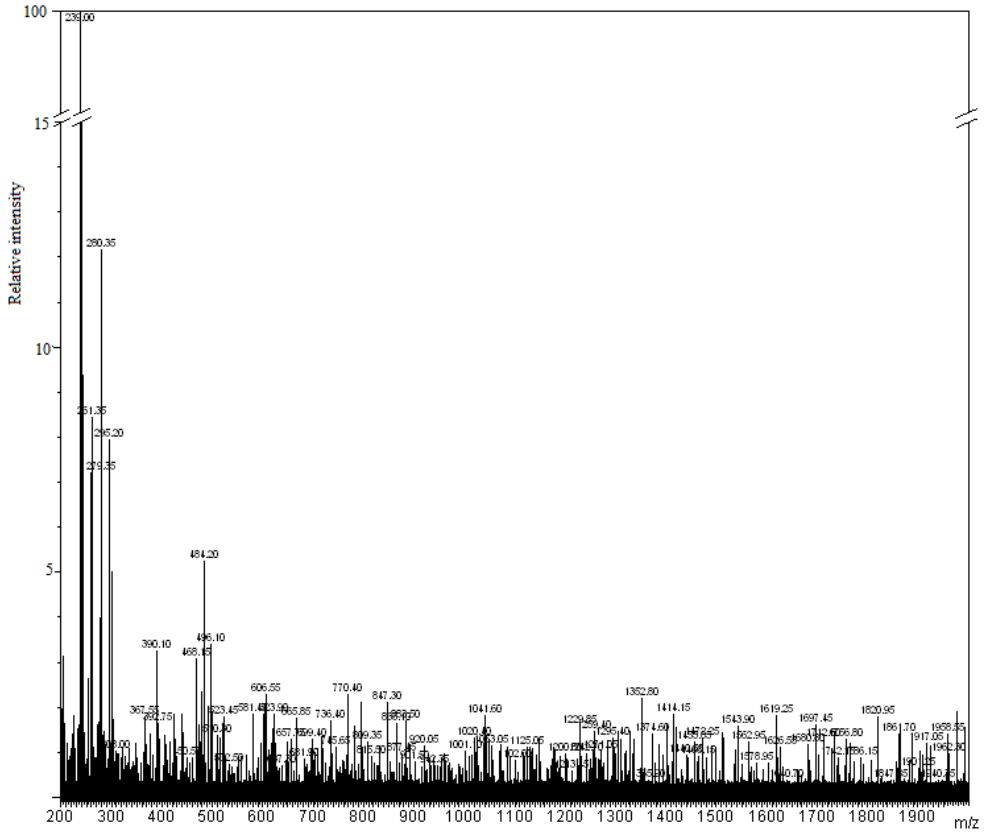
Supplementary Figure 1. Reaction scheme of GHB synthesis.

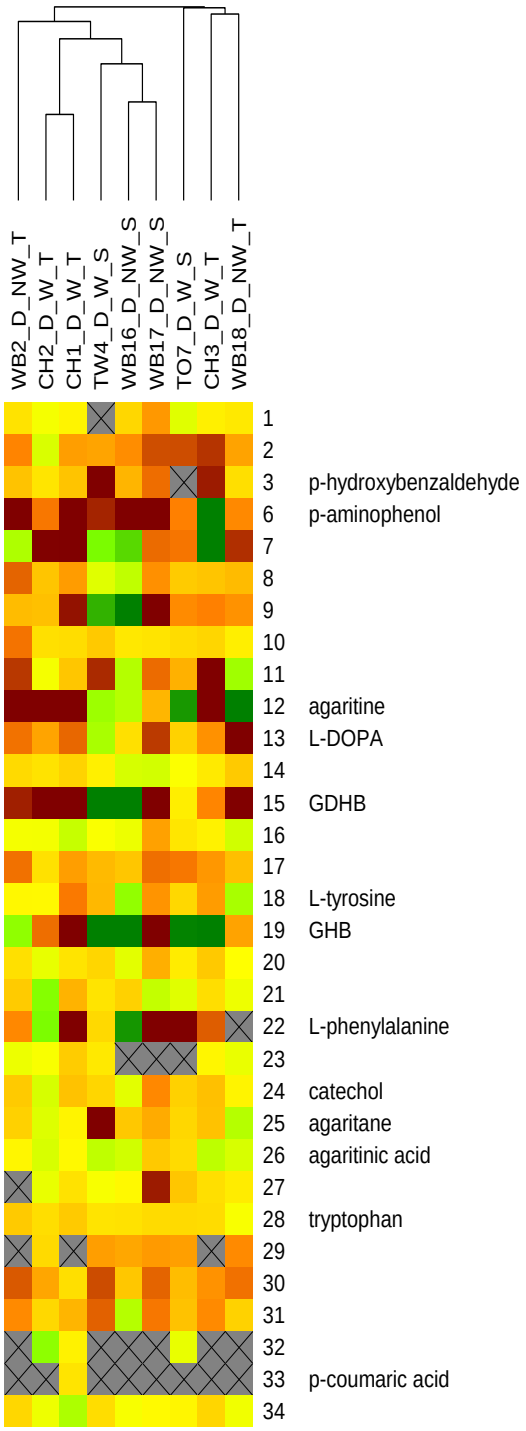


Supplementary Figure 2. HPLC of synthesized GHB.



Supplementary Figure 3. ESI-MS of synthesized GHB.





Supplementary Figure 4. Heat map analysis of the difference of phenolic compounds between the bruised and non-bruised skin tissue (based on peak area). The highest decrease is shown in dark green, the highest increase in dark red. A grey square with a cross indicates peaks that could not be integrated in either bruised or non-bruised skin tissue and therefore the difference could not be calculated. D = difference between bruised and non-bruised skin tissue. W = white and NW = non-white strains. T = tolerant and S = sensitive.

Chapter 6

A molecular approach to identify key components involved in bruising-tolerance of the button mushroom *Agaricus bisporus*

A. Weijn[a,b], D.P. Sintiski[a], M.M.M. Tomassen[a], H.J. Wichers[a,b] and J.J. Mes[a]

a Food & Biobased Research, Wageningen University and Research Centre, Bornse Weilanden 9, 6708 WG Wageningen, The Netherlands
b Laboratory of Food Chemistry, Wageningen University and Research Centre, Bomenweg 2, 6703 HD Wageningen, The Netherlands

Abstract

Button mushrooms are prone to develop brown discoloration upon bruising. Nine *Agaricus bisporus* strains were selected with significant different bruising-sensitivity, among them are two strains that hardly show bruising-discoloration. Genes related to the melanin biosynthesis pathway were studied for expression in the cap skin tissue in order to identify differential expression between bruising-sensitive and tolerant strains. Results indicated that all 30 genes analysed showed very small differences in expression between the strains and did not show clear indications for rate-limiting genes involved in the bruising-tolerance. A limited set of genes showed to respond to bruising, among them phenylalanine ammonia-lyase 2 and some of the polyphenol oxidases but also these genes did not show consistent differences in expression between bruising-tolerant and bruising-sensitive strains. The genomes of several strains with different bruising-sensitivity were fully sequenced and the coding sequence of the 30 genes involved in the melanin pathway were aligned. This resulted in the identification of at least four genes for which the sequence showed alteration in a wild brown bruising-tolerant strain. The mutations found are at the start in γ-glutamyltransferase 1, an elongation of 15 nucleotides at the C-terminus of aminotransferase 3 and a nucleotide mutation resulting in a premature stop codon in both aminotransferase 1 and polyphenol oxidase 3 leading to truncated proteins. Mutations were confirmed with RT-qPCR and were specific for the sequenced wild brown bruising-tolerant strain. These genomic mutations might be the key to bruising-sensitivity and can possibly be used in breeding and selection of bruising-tolerant mushrooms.

1 Introduction

Discoloration of fruit and vegetables has a major impact on their quality. The compounds most commonly involved in discoloration are phenolics, which are relevant for the appearance, taste and flavour of food products (Tomas-Barberan and Espin, 2001). Button mushrooms are prone to discoloration, which decreases their commercial value and can be caused by picking, handling and storage of the mushrooms. Discoloration reactions are mediated by enzyme-catalysed oxidation of phenolic compounds into quinones, leading to the formation of brown coloured melanin (Burton, 2004; Jolivet *et al*., 1998). Phenolic compounds are secondary metabolites that are biosynthesized through the shikimic acid pathway followed by the phenylpropanoid biosynthesis in plants (Dixon and Paiva, 1995). For a hypothesized scheme of the conversions of the melanin pathway in button mushrooms see Figure 1 of Chapter 4.

The best studied enzymes from the melanin biosynthesis pathway are phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO). PAL is the first enzyme in the phenylpropanoid metabolism and therefore possibly related to discoloration processes. In plants, an increase in PAL provokes an increase in the concentration of phenolic compounds, which can be substrates for oxidase enzymes such as PPO and peroxidase. PAL is encoded by a multi-gene family in several species; in potato plants approximately 40-50 homologs were identified of which at least ten genes were active and responded to wounding of the leaves and fungal pathogen infection (Joos and Hahlbrock, 1992). In tomato 26 PAL genes were identified in the genome of which only one gene was expressed and responded to pathogen infection or wounding (Chang *et al.*, 2008).

PPO enzymes can oxidize many different mono- and di-phenolic compounds leading to reactive quinones that can auto-polymerize in their subsequent melanins. In the genome of many species PPO genes are commonly present as a gene family with complex expression profiles. In tomato, PPOs show a spatial and temporal

regulation in vegetative and reproductive organs and one of the PPOs responded to wounding or pathogen infection (Thipyapong *et al.,* 1997). Similar results have been found for potato and apple (Thipyapong *et al.*, 1995; Boss *et al.*, 1995; Kim *et al.*, 2001). In mushrooms, Largeteau *et al.* (2010) analysed the expression of *PPO_1* and *PPO_2* in a commercial *A. bisporus* strain (2100 Amycel). Analysis showed a comparable expression for *PPO_1* in mycelial aggregates, pin stage, primordium and sporophore. *PPO_2* was up-regulated at fructification (primordium compared with mycelial aggregates) and down-regulated in expanding sporophore. They also stated that *PPO_2* increased with tissue discoloration in bubbles, which are caused by the fungus *Lecanicillium fungicola*.

Antisense down-regulation of constitutive and induced PPO expression was achieved in tomato (Thipyapong *et al.*, 2004). This transformation resulted in a hypersensitivity to pathogen infections, suggesting a critical role for PPO-mediated phenolic oxidation in plant defence. Bachem *et al.* (1994) showed that the antisense-based reduction of PPO expression in potato resulted in a significantly lower level of browning. Also studies based on the wild potato species *Solanum hjertingii* with low levels of PPO isoenzymes indicated the possible key role of PPO in obtaining species with reduced discoloration (Sim *et al.*, 1997).

Recently a full family of six PPOs was identified in *A. bisporus* var. *bisporus* together with other genes from or related to the melanin pathway and discoloration, including two PAL genes (Weijn *et al.*, 2012a). Besides that, strains with a different response to bruising were selected (Weijn *et al*., 2012b) and the phenolic compounds in these lines were studied (Chapter 5). Here we present the results of a study dedicated to identify the difference between bruising-sensitive and bruising-tolerant strains on a molecular level using genome sequences and transcriptional analysis.

2 Materials and methods

2.1 Mushroom strains and cultivation

Mushrooms were grown as described in Weijn *et al.* (2012b). Nine *A. bisporus* strains (Table 1) were grown in seven replicates. The strains were randomly distributed over the growing room. Strains used in this research originated either from the department of Plant Breeding at Wageningen UR or from the *Agaricus* resource program culture collection (Kerrigan, 1996). In order to minimise biological variation, all samples were collected from mushrooms at the same developmental stage (35-50 mm, closed or veiled) taken in the middle of the first flush under controlled pre- and post-harvest conditions.

Table 1. Result of bruise-related discoloration of nine *Agaricus bisporus* mushroom strains. Strain names are according to Weijn *et al.* (2012b). WI diff = WI difference. Least sifnificant difference (LSD) was calculated for white and off-white strains separately from the brown strains, which is indicated by #. Data is reproduced from Chapter 5.

Strain	Abbreviation	Classification	WI diff	St dev	LSD
Commercial hybrid 3	CH3	Tolerant	13.51	4.43	a
Commercial hybrid 1	CH1	Tolerant	14.66	2.88	a
Commercial hybrid 2	CH2	Tolerant	15.45	3.30	a
Traditional white strain 4	TW4	Sensitive	22.45	5.37	b
Traditional off-white strain 7	TO7	Sensitive	22.95	4.39	b
Wild brown strain 2	WB2	Tolerant	-0.37	4.06	a#
Wild brown strain 18	WB18	Tolerant	0.13	6.56	a#
Wild brown strain 17	WB17	Sensitive	15.67	4.86	b#
Wild brown strain 16	WB16	Sensitive	20.31	4.81	b#

2.2 Mushroom bruising and sampling

Mushroom bruising and discoloration analyses are performed as described in Weijn *et al.* (2012b). The bruised skin tissue was collected directly after taking the picture as described in Chapter 5.

2.3 RNA extraction, cDNA preparation and Real Time-qPCR

RNA extraction, cDNA preparation and RT-qPCR were performed according to Weijn *et al.* (2012a). Primers were designed with Clone Manager 9 (Sci-Ed software, Table 2 from this Chapter; Tables 3 and 4 from Weijn *et al.*, 2012a) and checked for alignment with homologous sequences. Primer efficiency was analysed using dilution series (10, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) of cDNA from gill samples of stages 4, 5, 6 and 7 of Horst U1 that were mixed before use. Primers and conditions were used when primer efficiency was between 80 and 120 % and amplified products were checked on a 2 % agarose gel.

In order to normalize gene expression data from RT-qPCR analysis it is important to have stably expressing housekeeping genes, which are expressed similarly in the skin tissues analysed. Seven housekeeping genes, β-tubulin, glyceraldehyde-3-phosphate dehydrogenase, translation elongation factor α, phosphoglycerate kinase, actin, protein kinase inhibitor α (PkiA), and 18S rRNA were tested for their expression in the skin tissue of different *A. bisporus* strains (primers are the same as used in Chapter 4, Table 3). GeNorm version 3.4 (Vandesompele *et al.*, 2002) was used to determine the most stably expressed housekeeping genes. Actin and PkiA were the most stably expressed housekeeping genes in the skin tissue of the nine strains analysed (results not shown) and chosen for normalisation of all expression analysis. The relative gene expression is determined as the relative expression to actin and PkiA, calculated as $\Delta C_T = 2^{\wedge}(Ct_{geomean(actin:PkiA)} - Ct_{sample})$. Relative gene expression data were log2 transformed. Squared Pearson correlation was used for similarity and UPGMA for clustering analysis in GeneMaths XT (Applied Maths, version 2.12). Table 2 list the RT-qPCR primers sequences that were used adjacent to the primers already specified in Chapter 4 (Table 4).

Table 2. Real Time-qPCR primer sequences. Protein ID is the number with which the genes can be found in the genome of *A. bisporus* var. *bisporus*.

Gene function (abbreviation)	Protein ID	Primer [µM]	Sequence (5' → 3')
MNP	239311	0.33	F - GGCAACTCTTCGCTACTG R - GATCGTTCGCGGAATGAC
CPO	221245	0.33	F - TTCATCGCATGTTTGGAAG R - ATTGAATGCCGCGAATGTC
Cat_1	115586	0.10	F - GACGTTGGTAAAGTCACTACC R - GGCCCGAATGTGAAAGTT
Cat_2	200291	0.10	F - AATTGTCTGTTTGGGCTGCT R - GCAGCAGAGATGCACTTGAG
Cat_3	239410	0.10	F - CCGACGTCTCTAAGCCTGAA R - GGATCAAGGCGGATGACA

2.4 Recovery of parental lines

Homokaryotic parental lines were obtained by protoplasting the vegetative mycelium and were identified using PCR (Gao *et al.*, 2011). Line Z8 is a homokaryotic parental line derived from strain wild white 7 (WW7) and line WB2A was recovered from WB2 (WB2A is identical to MES09143 from Gao *et al.*, 2011, the terminology WB2A is used for convenience in this and next chapters).

2.5 Sequenced genomes

Both parental lines (H97 and H39) of tolerant strain Horst U1 were sequenced previously. H97 was sequenced with Sanger sequencing with a coverage of 8.29x (Morin *et al.*, 2012). The H97 sequence is available via the JGI website (http://genome.jgi-psf.org/Agabi_varbisH97_2). The sequence of H39 was acquired via the Illumnia platform (Service XS, Leiden). The genome of *A. bisporus* var. *burnettii* strain JB137-S8 (Bur is used as abbreviation for this strain), a tetrasporic line, was sequenced using 454 pyrosequencing and Illumina HiSeq (Morin *et al.*, 2012). Because of the availability of this genome it was used although bruising-sensitivity of the strain is not known. This genome is also available via the JGI website (http://genome.jgi-psf.org/Agabi_varbur_1). WW7 is

a wild white strain very sensitive to bruising as determined previously (Weijn *et al.*, 2012b). Both constituent nuclear types of this line were recovered as homokaryons via protoplasting and are designated as parental lines Z8 and Z6 (Gao *et al.*, 2011). Crossings with other *A. bisporus* haploid lines indicated that both Z8 and Z6 introduced high bruising-sensitivity (Gao *et al.* 2011) and therefore the whole genome of one of these parental lines (Z8) was sequenced using the Illumina platform (Service XS, Leiden). As shown before (Weijn *et al*., 2012b) and confirmed in Chapter 5, strain WB2 is one of the most bruising-tolerant strains. Gao *et al.* (2011) also succeeded in recovering one of the parental lines of this strain and showed that this line (WB2A) can pass on this tolerance when combined with other parental lines indicating its dominant inheritance pattern for bruising tolerance. The genomic sequences of H39, Z8 and WB2A were obtained in collaboration with Anton Sonnenberg (PRI, Wageningen UR) using the Illumnia platform (Service XS, Leiden).

2.6 Alignment studies

Clone Manager 9 (Sci-Ed Software) was used to align either DNA or protein sequences with the multi way alignment. For the alignments standard program settings were used. The standard linear scoring matrix was used for DNA sequences and the BLOSUM 62 scoring matrix was used for protein sequences. Differences in DNA and protein sequences are based on 60 % similarity between the five parental lines analysed. An x in the sequence of H39 means unknown amino acid because of not correctly sequenced DNA. Consensus similarity was analysed with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Consensus similarity is indicated in the alignments (in which x as amino acid was ignored) with a * for identical amino acids, a : for a conserved substitution, and a . for a semi-conserved substitution (Clustal W2).

2.7 Real Time-qPCR of identified mutations

Primers were designed with Clone Manager 9 (Sci-Ed software, Table 3) to analyse identified mutations. For each mutation a primer set was based on the H97 sequence (wild type) and the WB2A sequence (mutation). H97 and WB2A DNA were a kind gift from Wei Gao (PRI, Wageningen UR). DNA was isolated from the nine *A. bisporus* strains (Table 1) according to the standard protocol of the DNeasy Plant Mini kit (Qiagen). RT-qPCR was performed with 5 µl (1 ng/µl) DNA with 10 µl iQ SYBR Green Supermix (Biorad) and a final concentration of 0.25 µM forward and reversed primers in a total volume of 20 µl. Each sample was measured in duplicate. The RT-qPCR was preheated at 95 °C for 1.5 min. This was followed by 40 cycles with a denaturing temperature of 95 °C for 10 s, annealing temperature of 70 °C (60 °C was used for GGT_1) for 10 s, and elongation temperature of 72 °C for 15 s. For each sample a melting curve was obtained to verify a single amplification product.

Table 3. Real Time-qPCR primers for gene mutations in WB2A. Gene mutations are underlined and bold in the primers sequences.

Gene mutation	Primer name	Sequence (5' → 3')
15 nucleotides extra at the C-terminus	AT_1_H97_F	CTTCTATGGTGAAGATCGGCTGTG**A**
	AT_1_H97_R	ACGTAGGACTGGGTACAGGGTTATT
	AT_1_WB2A_F	TATGGTGAAGATCGGCTGTG**C**
	AT_1_WB2A_R	ACATTTACGTAGGACTGGGTACAGG
Premature stop codon at amino acid # 17	AT_3_H97_F	CCTTCAAGAAACTTATGATGC**GC**
	AT_3_H97_R	GGTATCCGTTAACGTACTAGGACTT
	AT_3_WB2A_F	CTGCCTTCAAGAAACTTATGATGC**AT**
	AT_3_WB2A_R	GGTATCCGTTAACGTACTAGGACTT
Mutation at the start of the gene	GGT_1_H97_F	TTACTAACCGTCTCTC**C**CAT**G**
	GGT_1_H97_R	CAAGTGCCACCAGTAGAGCA
	GGT_1_WB2A_F	TTACTAACCGTCTCTC**A**CAT**C**
	GGT_1_WB2A_R	CAAGTGCCACCAGTAGAGCA
Premature stop codon at amino acid # 48	PPO_3_H97_F	AGAG**A**TCCTTCAAGCTCGCGA**CC**
	PPO_3_H97_R	AGTAATTGGCCTTGTAGAGGTGGAG
	PPO_3_WB2A_F	AG**G**TCCTTCAAGCTCGCGA**TT**
	PPO_3_WB2A_R	AGTAATTGGCCTTGTAGAGGTGGAG

3 Results

3.1 Bruising tolerance of the selected strains

Discoloration of the mushrooms was quantified by comparing the control with the bruised area. The resulting Whiteness Index difference is shown in Table 1 (WI diff) and is reproduced from Chapter 5. The white and brown mushrooms were divided into two groups because is it not known if the background colour subtraction is influencing the bruising-sensitivity quantification. A clear distinction was observed between bruising-tolerant and sensitive strains based on this computer image analysis but also by eye. Especially the bruising zone of strains WB2 and WB18 are difficult to distinguish, indicating their very high level of bruising-tolerance. Commercial hybrids 1, 2, and 3 have a similar bruising-sensitivity level which can be explained by their close genetic background as indicated by single nucleotide polymorphism analysis (see Chapter 5). Between other strains less genetic homology has been found and therefore possible different mechanisms of bruising-sensitivity or tolerance can be hypothesized.

3.2 Gene expression analysis in non-bruised skin tissue of bruising-sensitive and tolerant strains

The nine strains were subjected to gene expression analysis using genes previously identified to be involved in the melanin biosynthesis pathway (Weijn *et al.*, 2012a). Discoloration can be found within minutes after bruising of the skin tissue therefore it was hypothesized that accumulated gene products, expressed in the mushrooms in the days before picking and at the time the bruise was applied, lead to differences in bruising-sensitivity. These differences might be attenuated at the time of harvest when samples are taken. So, first gene expression was studied using dissected cap tissue of non-bruised mushrooms in order to identify natural variation in gene expression between bruising-sensitive and tolerant strains. The five white strains (Figure 1, left panel) show very similar expression profiles

although hierarchic clustering showed a difference between the three tolerant commercial hybrids in one group and the two bruising sensitive lines in a separate group. The main gene responsible for this difference between tolerant and sensitive strains is *GGT_1*, which shows to be less expressed in the sensitive strains. Also the genes that cluster together with *GGT_1* like *PHRB*, *Cat_2*, *4ABH_3*, and *PPO_2* show a little different expression in TW4 and TO7 compared with the tolerant white strains.

Similar analysis was performed for the four brown cap colour strains, also using non-bruised skin tissue (Figure 1, right panel). Both bruising sensitive strains WB16 and WB17 gave a similar expression profile and somewhat different from the two bruising-tolerant strains, which is reflected by the hierarchic cluster at the top of the figure. The main differences between the tolerant and sensitive brown strains are found in the cluster that reaches from PPO_1 to PPO_6. In this cluster *PPO_1*, *PPO_5* and *L-chain* show a somewhat lower expression in the tolerant strains compared to the sensitive strains while for the genes *PAL_2*, *AT_3*, *PAL_1* and *PPO_6* this is the other way around. However, when the bruising mechanism is different between the two brown tolerant strains it can be speculated that also other genes, that show a more specific profile for one of these strains, might be involved in the trait. As an example for this *GGT_1*, *C4H_2, PPO_2* and *Cat_3* can be mentioned.

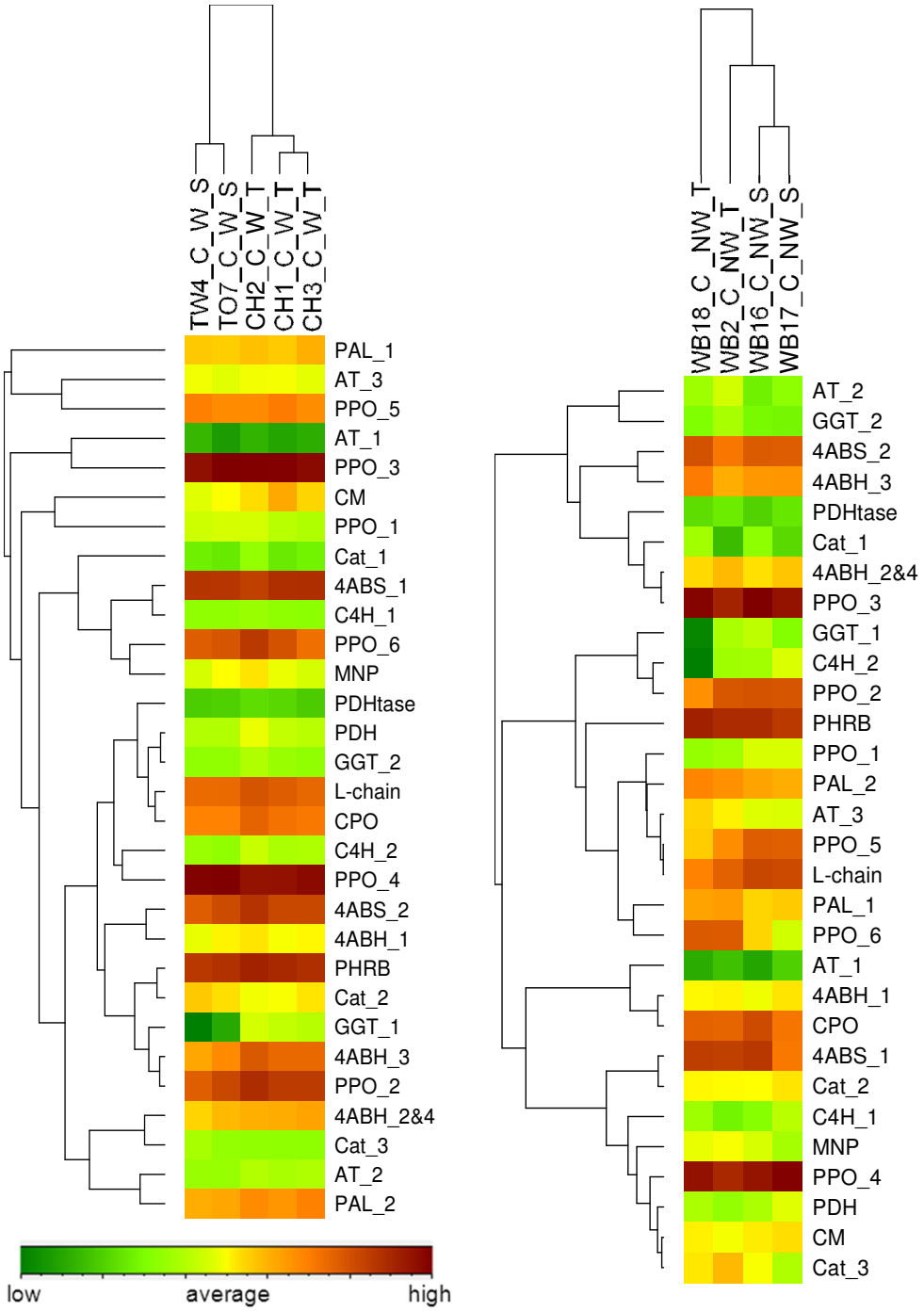
When combining the gene expression data from both the white and brown mushrooms and their bruising-sensitivity level no clear hypothesis can be suggested for the key genes involved in the bruising trait. No correlation was found between gene expression and natural cap colour, which might have a background in these same genes. It should be kept in mind that expression was analysed at the time of harvest and expression does not reflect the abundance of the corresponding enzymes. As RT-qPCR primers were designed on the sequence of H97 and the

genomic sequences from most of the analysed strains are unknown it is possible that some primer mismatches resulted in variation in RT-qPCR values.

3.3 Comparison of non-bruised and bruised skin tissue

Non-bruised and bruised skin tissue of the same strains were compared to analyse if gene expression is either increased or decreased by bruising and whether this could lead to new insights on genes involved in the bruising-tolerance trait. Data analyses showed that for most genes the expression level is not affected when the skin tissue is bruised (the boxes that colour yellow in Figure 2). This was found for both the white and brown cap coloured strains. Exceptions to this are the genes *CM*, *4ABS_1 & 2*, *4ABH_3*, *PAL_2*, *PPO_2*, *3*, *4*, *5 & 6*, *L-chain*, *PHRB* and *CPO*. In general, *CM* and *PAL_2* show an increased expression after bruising. CM is an enzyme at the beginning of the melanin pathway and can have an influence on the formation of DOPA-melanin and the part of the pathway leading to flavonoids. This last pathway also includes the PAL enzyme, of which *PAL_2* is increased after bruising and does not lead to coloured melanin, but to other compounds of which the influence on melanin formation is unknown.

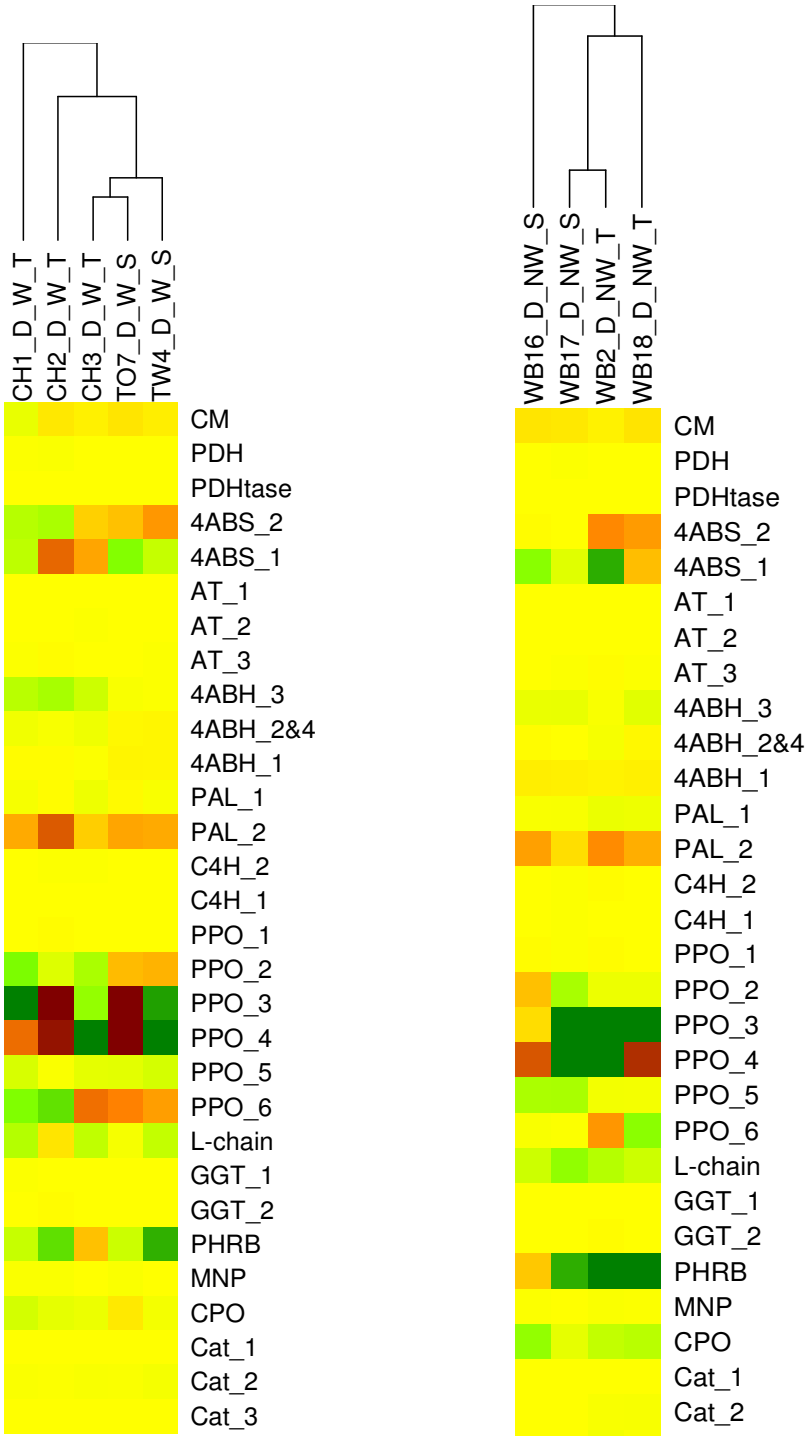
Figure 1. Relative gene expression of white and brown strains (next page). Expression is shown of non-bruised (C) samples. Left part of the figure are the white (W) strains, right part are the brown (non-white, NW) strains. Sensitive strains are indicated with an S, tolerant strains with a T. The relative gene expression was log2 transformed, the lowest expression is shown in dark green (-15), the highest expression in dark red (5). CM = chorismate mutase, 4ABS = 4-aminobenzoate synthase, 4ABH = 4-aminobenzoate hydroxylase, GGT = γ-glutamyltransferase, PDHtase = prephenate dehydratase, PDH = prephenate dehydrogenase, AT = (4-hydroxy) phenylpyruvate aminotransferase, PPO = polyphenol oxidase, PAL = phenylalanine ammonia-lyase, C4H = trans-cinnamate-4-monooxygenase, PHRB = photoregulator B, and Cat = catalase, CPO = chloroperoxidase, MNP = manganese peroxidase.


TW4_C_W_S
TO7_C_W_S
CH2_C_W_T
CH1_C_W_T
CH3_C_W_T
PAL_1
AT_3
PPO_5
AT_1
PPO_3
CM
PPO_1
Cat_1
4ABS_1
C4H_1
PPO_6
MNP
PDHtase
PDH
GGT_2
L-chain
CPO
C4H_2
PPO_4
4ABS_2
4ABH_1
PHRB
Cat_2
GGT_1
4ABH_3
PPO_2
4ABH_2&4
Cat_3
AT_2
PAL_2
low
average
high
WB18_C_NW_T
WB2_C_NW_T
WB16_C_NW_S
WB17_C_NW_S
AT_2
GGT_2
4ABS_2
4ABH_3
PDHtase
Cat_1
4ABH_2&4
PPO_3
GGT_1
C4H_2
PPO_2
PHRB
PPO_1
PAL_2
AT_3
PPO_5
L-chain
PAL_1
PPO_6
AT_1
4ABH_1
CPO
4ABS_1
Cat_2
C4H_1
MNP
PPO_4
PDH
CM
Cat_3

PAL is a wound and stress responding enzyme as identified in many species and crops (Chang *et al.*, 2008; Joos and Hahlbrock, 1992). The identification of increased expression of *PAL_2* indicates that mushrooms can respond within 60 minutes to bruising.

When comparing the response to bruising of bruising-sensitive and bruising-tolerant strains it appears that no consistent differences can be observed between these two classes of strains but that responses are rather strain specific. *4ABH_3* expression decreased upon bruising in the white tolerant strains; the expression level is the same before and after bruising in the sensitive white strains (left part Figure 2). *PPO_2* is higher expressed in white sensitive strains after bruising compared with the tolerant white strains. The expression of *4ABS_2* is only consistent in the brown strains, where it increased upon bruising in the sensitive strains (right part of Figure 2). *4ABH_3* slightly decreased in three of the brown strains. *PHRB* decreased in most strains except in CH3 (T) and WB16 (S), which both showed an increase of *PHRB*. *CPO* decreased in all strains, except TO7 (S), the decrease was higher in the brown strains. Expression of *PPO_1* is not influenced by bruising; the other PPO genes and the L-chain are either increased or decreased upon bruising as discussed above but do not show a correlation with the bruising-sensitivity level of the strains. Taken all this together leads to the conclusion that no clear candidates for key factors of the bruising-tolerance mechanism can be appointed. Different bruising-mechanisms are possibly involved in the trait of bruising-tolerance which can be cap colour or strain specific.

Figure 2. Difference in gene expression between non-bruised and bruised skin tissue (next page). Expression is shown as the difference (D) between 60 min bruised samples and 60 min non-bruised samples. Left part of the figure shows the white (W) strains, right part represents the brown (non-white, NW) strains. Sensitive strains are indicated with an S, tolerant strains with a T. The highest decrease in expression is shown in dark green, the highest increase in expression in dark red, the difference between these is a factor ten. Yellow means no difference between non-bruised and bruised sample.


CH1_D_W_T
CH2_D_W_T
CH3_D_W_T
TO7_D_W_S
TW4_D_W_S
WB16_D_NW_S
WB17_D_NW_S
WB2_D_NW_T
WB18_D_NW_T
CM
PDH
PDHtase
4ABS_2
4ABS_1
AT_1
AT_2
AT_3
4ABH_3
4ABH_2&4
4ABH_1
PAL_1
PAL_2
C4H_2
C4H_1
PPO_1
PPO_2
PPO_3
PPO_4
PPO_5
PPO_6
L-chain
GGT_1
GGT_2
PHRB
MNP
CPO
Cat_1
Cat_2
Cat_3

3.4 Genetic background of the sequenced strains and sequence alignments

As gene expression analysis did not result in conclusive key genes of the origin of the bruising-tolerance trait it was decided to study the genomes of haploid lines of which the bruising-sensitivity was determined. *A. bisporus* is a basidiomycete and in most basidiomycetes, two types of haploid nuclei exist side by side in the mycelium cells. The two constituent homokaryotic parental lines (homokaryons) can easily be separated because the fusion of the nuclei only occurs just before meiosis (Gao *et al.*, 2011). Both homokaryons were obtained and sequenced from the commercial strain Horst U1 (H97 and H39) that has shown to be bruising-tolerant. WB2A is one of the homokaryons of WB2, a high bruising-tolerant brown strain, and Z8 is obtained from a bruising-sensitive white strain. Besides these lines, *A. bisporus* var. *burnettii*, a tetrasporic line, was fully sequenced. All five genomes where used to study differences in the coding sequences of the genes involved in the melanin biosynthesis pathway. The results of the alignments showed that the coding sequence of each gene has on average 17 point mutations leading to an average of five mutations in the amino acid sequence per gene. The genomic sequence of *A. bisporus* var. *burnettii* is most deviant from the other four sequences. The genome of H39 still contained unreliable sequenced nucleotide bases which leads to unknown amino acids (indicated by an x in the amino acid sequence). None of the genes analysed had the same sequence in all five genomes. *CM*, *CPO* and *PAL_1* gave the least differences. H39, Bur and WB2A had one amino acid different from H97 and Z8 for CM. For CPO both H39 and Z8 had one amino acid different and for PAL_1 H39 had four changed amino acids and Bur five changed amino acids compared with the other sequenced genomes.

Most genes analysed did not show mutations for which a direct effect on the gene expression, gene translation, protein folding or stability, or activity of the enzyme was postulated. Of course DNA mutations have been identified, leading to amino acid changes but based on the current knowledge of protein sequences these

were not found in conserved domains. However, in the bruising-tolerant line WB2A four genes showed a very intriguing mutation which very likely will have an effect on the translation to protein and on protein function. Primers were designed based on the mutation (both for the wild type H97 and the mutated WB2A sequence) to check the mutation by RT-qPCR (section 3.5) in order to analyse the link between the presence of the mutation in the genome and the bruising-sensitivity of the nine *A. bisporus* strains.

3.4.1 An elongation at the C-terminus of AT_1 in WB2A

The enzyme (4-hydroxy) phenylpyruvate aminotransferase (AT) can be involved in both the melanin synthesis pathway leading to DOPA-melanin or/and can be active in the pathway leading to flavonoids. Three homologous genes for *AT* have been identified in the *A. bisporus* genome (Weijn *et al.*, 2012a). Possibly these three have slightly different functions and substrate specificity but such is hard to predict solely based on gene sequences.

An important difference between WB2A and the other sequenced homokaryons was identified at the C-terminus of AT_1 (Figure 3). In WB2A 15 extra nucleotides were identified leading to an extra five amino acids before the protein terminates. This sequence mutation was confirmed based on individual sequence reads of the genomic WB2A sequence. It is possible that this addition does not have an influence on the protein function however for other AT genes it has been shown that the C-terminus of AT is involved in protein folding (Ko *et al.*, 1999). Folding of the protein can be influenced by the amino acid cysteine (c). Cysteine is an amino acid that forms a sulphite bridge with another cysteine in a protein. The AT_1 sequence contains eight cysteines. As this is an even number, in total four di-sulphite bridges can be formed. An extra cysteine, as found at the C-terminus of WB2A, can possibly interfere with the original folding structure.

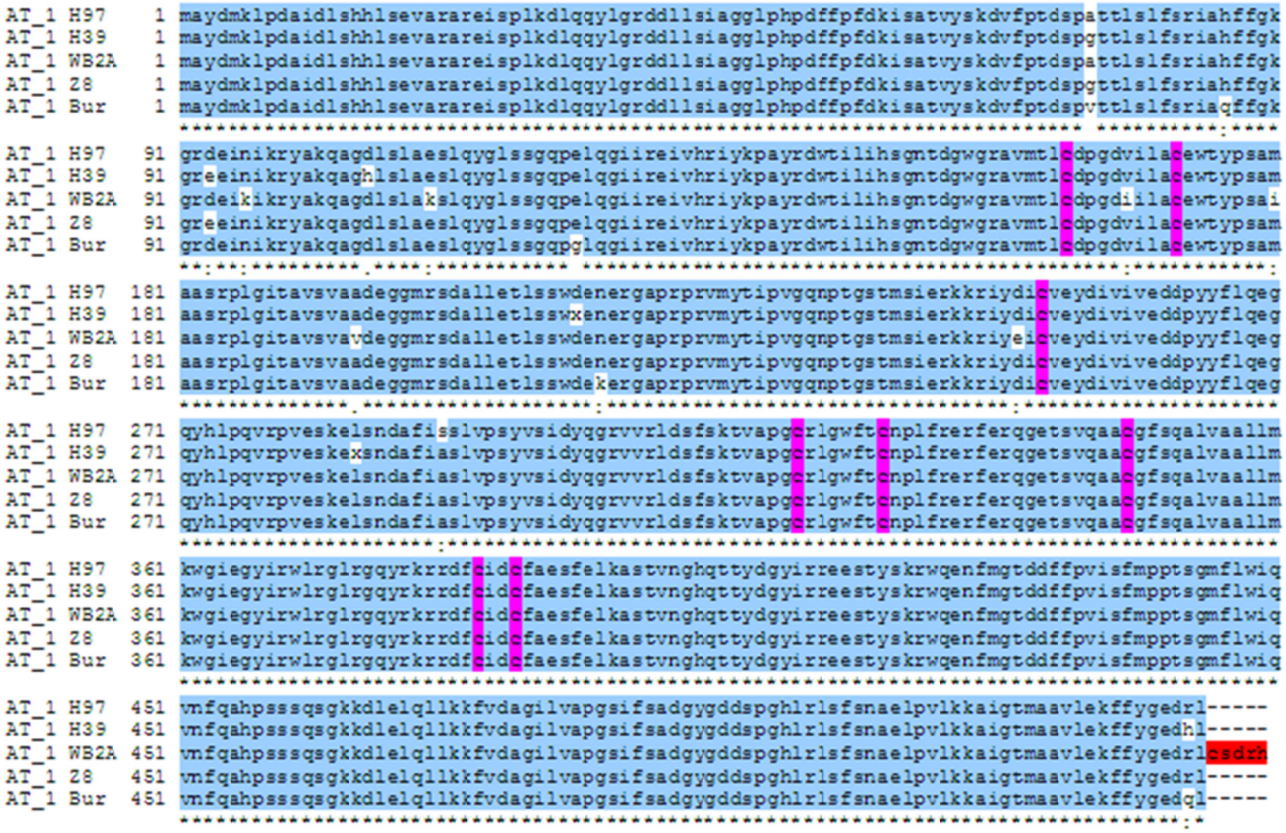


Figure 3. Amino acid sequence alignment of AT_1. In blue the homologous and conserved sequences, in white the mutations on the protein level and in red the mutation leading to a change at the C-terminus of the enzyme. The cysteine amino acids are indicated in pink.

3.4.2 A premature stop codon for WB2A in AT_3

As the *AT_1* gene described above, also *AT_3* can influence the same conversions of phenolic compounds. Expression of *AT_3* was the highest of the three AT genes analysed in the nine *A. bisporus* strains (section 3.2). Due to a point mutation in the coding sequence of the *AT_3* gene of homokaryon WB2A a premature stop codon was found at amino acid position 17 resulting in a non-functional enzyme (Figure 4). Lowered or lacking presence of this AT_3 protein and subsequent enzyme activity (when heterozygously present) in WB2 (the heterokaryotic strain which contains the WB2A genetic information) could lead to a decreased conversion of phenolic compounds towards the production of DOPA-melanin. Again sequence analysis of the genomic reads illustrated that it is not a sequence error but a consistent mutation found in all the reads of the WB2A homokaryon.

3.4.3 Delayed start codon in WB2A for GGT_1

Gene sequences start with an ATG triplet which codes for a methionine amino acid at the N-terminus of the protein. For the parental line WB2A a point mutation at the start of the gene was identified resulting in an isoleucine amino acid at the start of *GGT_1* instead of a methionine (Figure 5). This might lead to a protein start at the next ATG resulting in a three amino acid deletion at the N-terminus of the protein. The effect of the mutation on the function of the enzyme is unknown. The GGT enzyme is involved in the synthesis of GHB- and PAP-melanin. Changed proteins could result in potentially less production of melanin and by consequence lead to an increase in bruising-tolerance. The mutation was again verified to be consistent. The *GGT_1* gene was found to be expressed at a lower level in the two most sensitive white strains compared to the bruising-tolerant white strains, but the gene is also expressed at a low level in tolerant strain WB18.

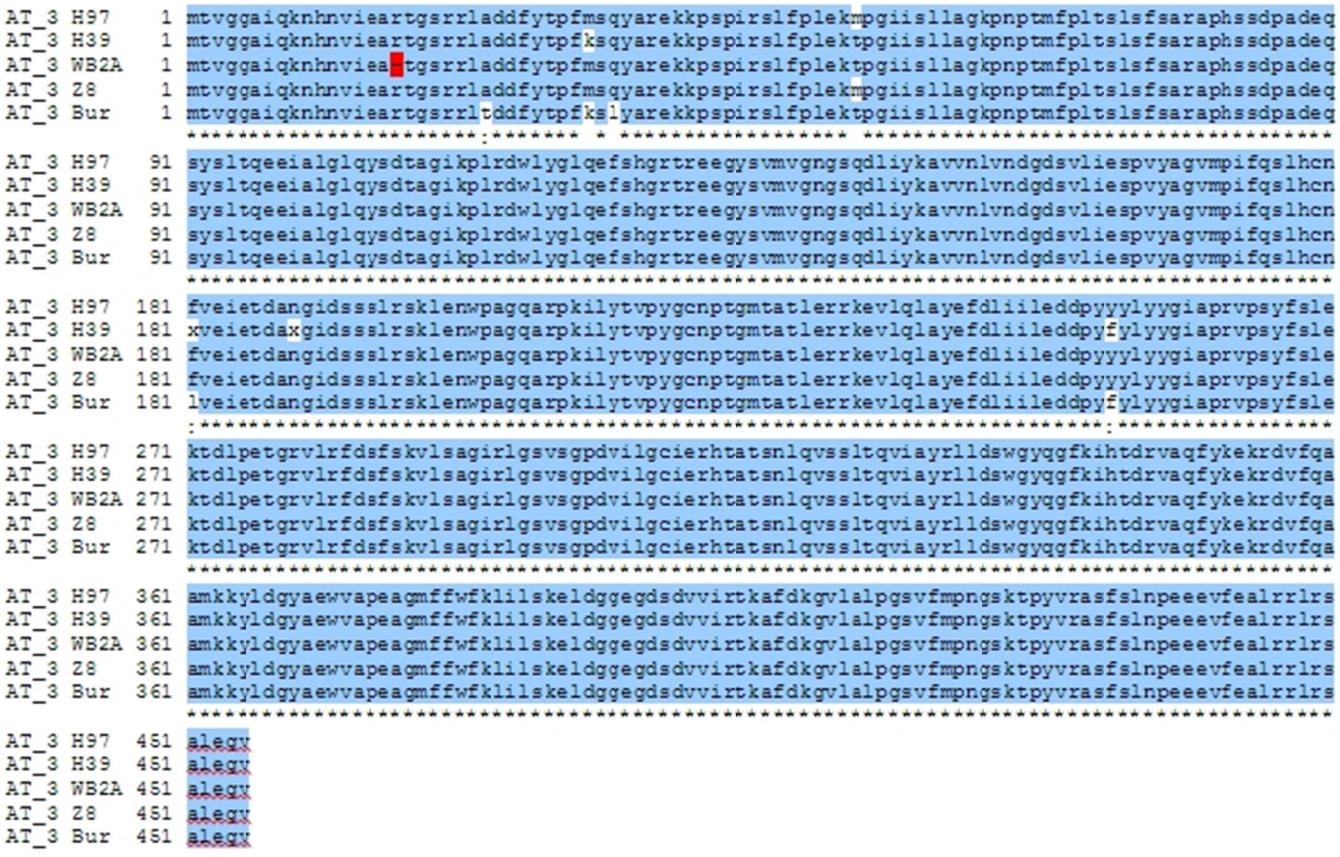


Figure 4. Amino acid sequence alignment of AT_3. In blue the homologous and conserved sequences, in white the mutations on the protein level and in red the mutation leading to a premature stop codon and abortion of the functional protein.

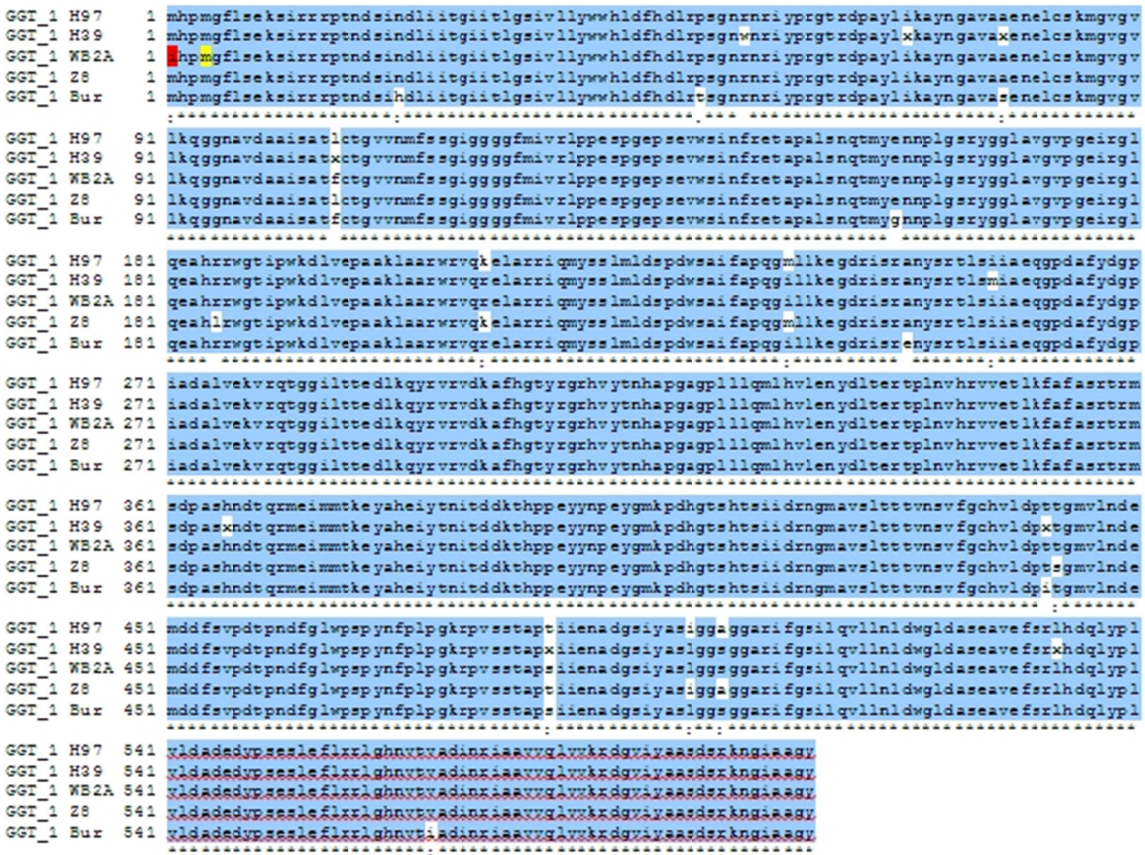


Figure 5. Amino acid sequence alignment of GGT_1. In blue the homologous and conserved sequences, in white the mutations on the protein level and in red the mutation leading to another codon for WB2A resulting in a protein start at the next methionine, which is indicated in yellow.

Chapter 6

3.4.4 A premature stop codon for WB2A in PPO_3

As discussed before PPO is a very important gene in the melanin pathway as it can convert several phenolic compounds into di-phenolic compounds and subsequently into quinones which can non-enzymatically polymerize into melanins. Out of the family of six PPOs identified in the genome *PPO_3* is one of the PPOs that is expressed most abundantly, specifically in skin tissue (Weijn *et al.*, 2012a). *PPO_3* is also the PPO that was found most abundantly in the commercial mushroom tyrosinase as can be obtained from Sigma (Ismaya *et al.*, 2011). When aligning the *PPO_3* sequences of the five different genomes, several mutations where detected which changed the amino acid sequence. The most interesting mutation was discovered in WB2A and introduces a premature stop codon at position 48, which leads to a truncated protein and therefore will no longer have PPO activity (Figure 6). A mutation in WB2A *PPO_3* could hypothetically result in less PPO enzyme in the skin and is possibly a key component of the high bruising tolerance level of WB2. The lowest expression of *PPO*_3 amongst the strains analysed was found in both the non-bruised and bruised skin tissue of WB2 (section 3.2). Again the consistence of the mutation was confirmed by single reads.

3.5 Identification of mutations with Real Time-qPCR

To analyse the mutations found in WB2A in the nine *A. bisporus* strains used for gene expression analysis RT-primer sets were designed (Table 3). Two forward RT-qPCR primers were designed, one fully matching the wild type (H97) and one matching the mutated sequence (WB2A) together with a reverse primer. Primers were tested on the homokaryons H97 and WB2A and analysis showed that each primer pair is efficient in discriminating the wild type or the mutated sequences in genomic DNA indicated by a difference of at least 7 C_T values (Table 4).

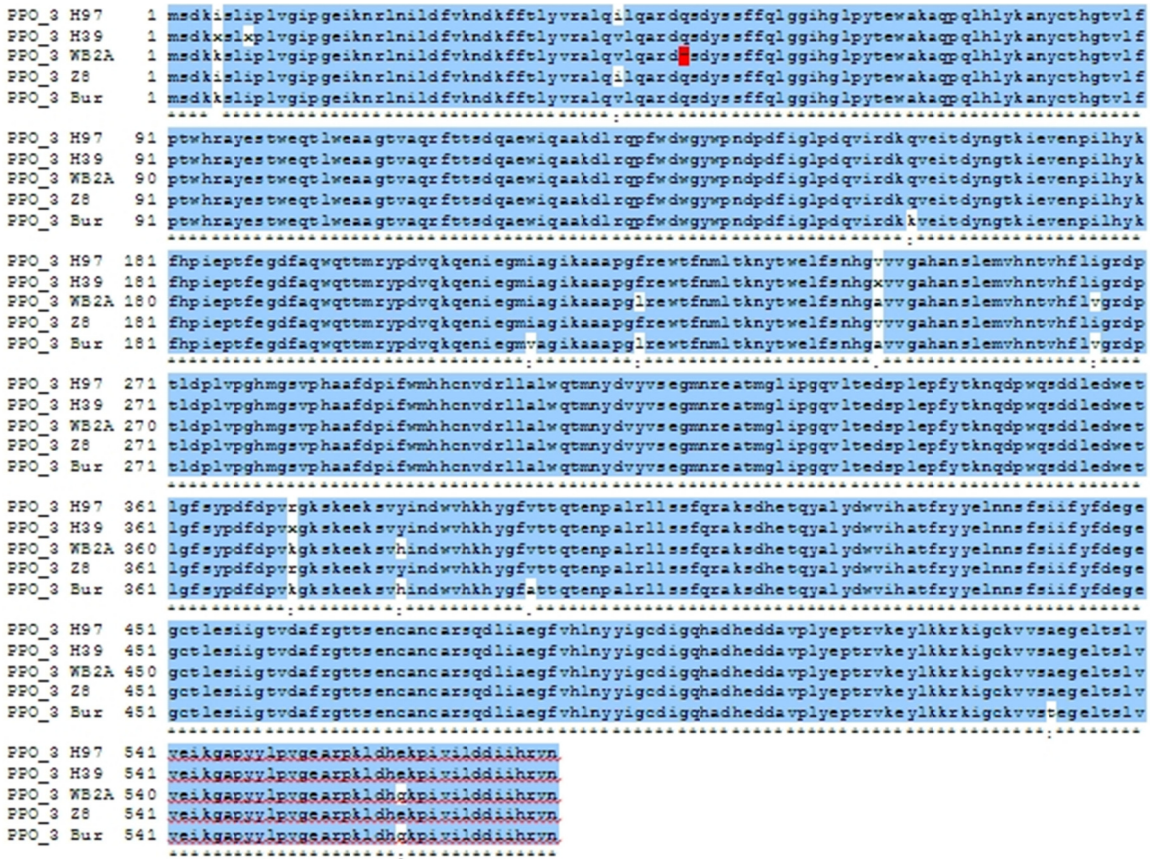


Figure 6. Amino acid sequence alignment of PPO_3. In blue the homologous and conserved sequences, in white the mutations on the protein level and in red the mutation leading to a premature stop codon in WB2A.

Chapter 6

Next, the nine strains were analysed for the presence of the mutations in the heterokaryotic genomic DNA in order to confirm that the mutations exist in WB2 (from which the homokaryon WB2A was recovered) and to investigate whether the other strains have this genetic background (Table 4). Analysis showed that for all four genes tested the H97 allele is present in the commercial hybrids CH1, CH2 and CH3. The results for *AT_3* are purely based on the formation of an amplification product or absence of a product (N/A in Table 4). Also the presence of the WB2A allele in WB2 was confirmed for all four mutations.

Table 4. Result of mutation analyses with RT-primers. Indicated are C_T values obtained for each primer set. In red is indicated when the strain contains the mutation of WB2A and in blue is indicated when the strain contains the wild type H97 allele. Chr = chromosome. W = white and NW = non-white strain. T = tolerant and S = sensitive.

Strain	***AT_1* (Chr.4)**		***AT_3* (Chr.5)**		***GGT_1* (Chr.2)**		***PPO_3* (Chr.5)**	
	WB2A	H97	WB2A	H97	WB2A	H97	WB2A	H97
H97	28.28	21.24	31.67	21.11	33.45	19.42	38.43	20.93
CH1_W_T	36.06	21.50	N/A	34.50	35.29	19.51	30.82	20.24
CH2_W_T	33.22	21.86	N/A	31.44	28.87	19.95	28.09	20.40
CH3_W_T	38.18	21.83	N/A	37.74	35.81	19.23	31.02	20.16
TW4_W_S	37.66	32.68	N/A	36.99	35.51	19.69	31.69	20.37
TO7_W_S	37.10	29.83	N/A	36.10	35.55	19.49	31.22	20.33
WB2A	20.96	34.40	20.86	34.27	19.99	32.01	20.55	36.39
WB2_NW_T	22.81	32.82	32.35	N/A	20.44	22.45	20.26	29.42
WB18_NW_T	36.16	32.16	N/A	N/A	32.76	19.58	32.73	19.32
WB16_NW_S	38.23	32.48	N/A	35.16	39.39	20.04	29.41	30.41
WB17_NW_S	37.59	31.28	N/A	37.83	35.45	19.59	29.46	29.03

The commercial hybrids do not contain the WB2A allele, as was expected and probably contain H39 as the other allele. The WB2B homokaryon of WB2 (containing homokaryons WB2A and WB2B) likely does not have the genetic background of H97 as the H97 primers do not amplify products with the same C_T values as found in the commercial hybrids. The exception is the GGT_1 allele for which an amplification product was detected with a C_T value of 22, which is slightly higher than those for the other H97 alleles (indicated in pink in Table 4).

This might indicate that the other homokaryon in WB2 is different from H97. It was surprising that some strains did not show an efficient amplification of *AT_1* for neither of the two primers sets indicating that more genetic variation can be found within this sequence. The same holds true for *PPO_3* for the two strains WB16 and WB17, which do not show efficient amplification of RT-PCR products using the two PPO_3 primer sets.

Based on these results no conclusive hypothesis can be postulated whether these mutations or gene loci are of key importance for the bruising-tolerance. However, it is tempting to speculate on the background of the high tolerance of WB2 for which a mutation early in the GBH-melanin pathway was postulated based on the analyses of phenolic compounds. These mutation analyses confirmed the presence of a change in *GGT_1* which is at least present in one homokaryon but might be present in the other homokaryon of WB2. Strain specific sequence analyses of the full genes together with sequence specific mRNA expression analysis will be needed to identify possible correlations between bruising-tolerance and the genes involved in the trait.

Discussion

In this investigation a molecular approach was used to identify key components of the bruising-tolerance trait in button mushrooms. Nine *A. bisporus* strains were selected based on bruising-sensitivity and used for a molecular analysis. Gene expression analyses showed that all genes previously identified in the melanin biosynthesis pathway and expressed in the strain Horst U1 (Weijn *et al.*, 2012a) are also expressed in the strains analysed here. Gene expression is based on primers developed on the gene sequences of H97. Primers were designed before the complete genomic sequences of other strains were obtained and therefore have not been developed on conserved sequences per se. Afterwards only for some primers single mismatches have been observed but not on crucial positions for

amplification. This could, however, not be checked for non-sequenced strains. Amplifications were performed at relatively low annealing temperature and melting curves of amplified products were checked and confirmed the amplification of one product with the same melting point as the product amplified from Horst U1. This however does not rule out that observed differences in gene expression are due to primer mismatches.

Gene expression analysis was performed on non-bruised and bruised skin tissue of both white and brown cap coloured strains in order to identify bruising responsive genes. It was found that for some genes the expression level is affected when the skin tissue is bruised. *PAL_2* expression increased after bruising in all strains. PAL has been identified as a wound responding enzyme of which increased expression was found in potato and tomato and many other species (Chang *et al.*, 2008; Joos and Hahlbrock, 1992). Also for PPO genes from other species it has been found that they are responsive to wounding or pathogen infection (Boss *et al.*, 1995; Kim *et al.*, 2001; Thipyapong *et al.,* 1995; 1997). It would be interesting to study *PAL_2*, *CM* and other bruising responsive genes of *A. bisporus* in relation to infections of button mushrooms and to study a possible correlation in mechanism between response to bruising and other stresses.

No genes were identified that were similar in response to bruising for either the tolerant or sensitive strains of both white and brown mushrooms. The expression of *4ABS_2* increased upon bruising in the sensitive brown strains. 4ABS is the first enzyme in the melanin pathway after chorismate leading to GHB- and PAP-melanin. A role of 4ABS in the higher bruising-sensitivity of brown strains is plausible because it was observed that WB16 and WB17 contain a higher concentration of GHB and GDHB upon bruising compared to WB2 and WB18 (Chapter 5). *PPO_2* was higher expressed in sensitive white strains after bruising compared with the tolerant white strains. *PPO_2* up-regulation during discoloration upon bruising is in agreement with the findings of Largeteau *et al.* (2010). They

found an increase of *PPO_2* during tissue discoloration in bubbles, which are caused by the fungus *L. fungicola*. It is currently not known to what degree the changes in gene expression contribute to the bruising-sensitivity as both are measured only at one time point (one hour after bruising). From studies with the *AtMYB15* gene it has been found that within 30 minutes RNA synthesis was increased upon wounding of *Arabisopsis* (Chen *et al*., 2006) and an increase in the amount of RecA protein in *E. coli* was observed 30 minutes after exposure to UV irradiation (Fridrichova *et al*., 1992). Therefore it might be expected that upon bruising both gene and protein levels are changing. This can be analysed with deep sequencing of the transcriptome (RNAseq) and proteomics. Besides these analyses it would be interesting to study the effect of less intense bruising or standard post-harvest conditions on gene expression of these genes in order to identify genes that might be related to these quality aspects as it was identified that these traits are unrelated (Weijn *et al.*, 2011).

Previous research indicated that bruising-tolerance was related to a blockage in the pathway from *p*-aminophenol to GBH and GDHB as these last two compounds were found at a low level in bruising-tolerant strains while *p*-aminophenol concentrations were relatively similar between bruising-sensitive and tolerant strains. Genes involved in this part of the pathway are GGT and PPOs. A lower expression of *GGT_1* was found in the non-bruised skin tissue of sensitive white mushrooms and bruising did not have an effect on the expression of *GGT_1*. Analyses of the phenolic compounds in the same strains (TW4 and TO7) showed a higher concentration of GHB and GDHB in the sensitive strains (Chapter 5). Several explanations are possible for this inverse correlation. The first option is the specific time point that was analysed. The analyses do not take into account the earlier gene activity differences, accumulated enzymes and the differences in phenolic compounds formed before the mushrooms were picked. The second option is that this lower *GGT_1* expression reflects a primer mismatch and reduced

RT-qPCR efficiency. In that case low expression of *GGT_1* might correlate with a different GGT_1 allele present in these strains. Analysis of the *GGT_1* mutation of WB2A in the strains indicated that this mutation was specific for WB2 but this does not exclude the possibility that the strains TW4 and TO7 have other mutations in *GGT_1* that also can influence the function and activity of this protein. The same applies for the homolog *GGT_2* which might have the same functional activity as *GGT_1*.

Low GHB and GDHB concentrations in bruising-tolerant strains might also be the result of changed PPO conversions of either these compounds or *p*-aminophenol which all can be substrates for PPO enzymes and can lead to quinones that can non-enzymatically polymerise in melanins. These different melanins not necessarily have the same brown colour as it has been observed that some strains have a more yellow-brown or red-brown type of discoloration, which might be a reflection of differences in the main melanins formed. To determine specific enzyme activity of different homologs the genes can be cloned and brought to expression in for instance *E. coli* or *Pichia pastoris* or another expression system. Lezzi *et al.* (2012) performed the heterologous expression of *PPO_2* of *A. bisporus* in *Saccharomyces cerevisiae* and obtained an active enzyme. This enzyme showed the highest affinity for L-tyrosine, compared to L-DOPA, pyrocatechol and pyrogallol. Transformation into *A. bisporus* can be used to determine the effect of overexpression of a gene on the bruising-sensitivity of that individual strain (Mikosch *et al.*, 2001; Stoop and Mooibroek, 1999).

The WB2 strain was analysed because of its extreme high bruising-tolerance. Phenolic compound analysis identified that this strain had a low concentration of GHB and GDBH and that it was the only strain that showed this low concentration after bruising (Chapter 5). Based on the hypothesised melanin synthesis pathway it was expected that this strain would have a change in the GGT genes, either in gene expression, or RNA stability or mutations leading to changes in the proteins. The

study here indeed underlined that in one of the homokaryons a mutation was found in GGT, namely in *GGT_1*. The impact on the enzyme function of the relative small change in the *GGT_1* sequence is however unknown. Possibly, also the other homokaryon in WB2 carries a mutation with an impact on enzyme function. Also the truncated PPO_3 mutation might be the cause of the lower GHB and GDHB concentrations but then resulting in changing fluxes in the pathway leading to other compounds and causing a reduction of GHB and GDHB accumulation. The role of the truncated PPO_3 might also be taken over by other PPOs. Another option is that due to the low content of GHB and GDHB in WB2 the *PPO_3* mutation does not show a phenotype in this background. Therefore it will be interesting to introduce the PPO_3 allele from WB2A in another background that still can form GBH and analyse the impact on bruising-sensitivity. Previous research showed that a wild potato species (*Solanum hjerntingii*) which does not exhibit enzymatic browning contained PPO that has no enzymatic activity (Sim *et al.*, 1997). Detailed analysis identified that this wild potato species contained a truncated PPO (Culley *et al.* 2000). Further research will be needed to investigate whether the spontaneous truncation of *PPO_3* in WB2A can lead to new commercially interesting strains.

Strategies for future research will be to analyse the full sequences of the genes and mRNAs that have been identified as possible candidates for bruising-tolerance in these strains with different bruising-sensitivity. Assuming that these genes remain the most interesting genes for introducing bruising-tolerance, then targeted gene knockouts or antisense silencing of genes will be the next step. Bachem *et al.* (1994) showed that the antisense based reduction of PPO expression in potato resulted in a significantly lower level of browning. Sonnenberg *et al.* (2006) isolated the parental lines of a wild strain with low sensitivity for *L. fungicola* and from a commercial strain with higher sensitivity. The parental lines were crossed and new hybrids were obtained with lower sensitivity than the wild strain. In alignment with that the next step in the analysis of the bruising-sensitivity trait is

the analysis of a population based on a cross between homokaryons recovered from bruising-tolerant strains to obtain potentially high bruising-tolerant mushrooms. Simultaneously, this population can be used to unravel the key components of the bruising-tolerance trait.

Acknowledgements

This project is supported by the governmental grant "TTI Green Genetics", Dutch Board for Horticulture, Bromyc, Lutèce, Sylvan, Banken Mushrooms, the Greenery and WeBe Engineering. The parental lines MES09143 and Z8 were obtained by Wei Gao (PRI, Wageningen University). The H39 genome sequence was a kind gift from Anton Sonnenberg (PRI, Wageningen University) via Technology Foundation STW grant number 6698.

References

Bachem, C.W.B., Speckmann, G.-J., van der Linde, P.C.G., Verheggen, F.T.M., Hunt, M.D., Steffens, J.C., Zabeau, M. (1994). Antisense expression of polyphenol oxidase genes inhibits enzymatic browning in potato tubers. *Nature Biotechnology*, *12*(11), 1101-1105.

Boss, P.K., Gardner, R.C., Janssen, B.J., Ross, G.S. (1995). An apple polyphenol oxidase cDNA is up-regulated in wounded tissues. *Plant Molecular Biology*, *27*(2), 429-33.

Burton, K.S. (2004). Cultural factors affecting mushroom quality - cause and control of bruising. *Proceedings of the XVIth International Congress of Science and Cultivation of Edible and Medicinal Fungi,* Maimi (pp. 397-402).

Chang, A., Lim, M.-H., Lee, S.-W., Robb, E.J., Nazar, R.N. (2008). Tomato phenylalanine ammonia-lyase gene family, highly redundant but strongly underutilized. *The Journal of Biological Chemistry*, *283*(48), 33591-33601.

Chen, Y., Zhang, X., Wu, W., Chen, Z., Gu, H., Qu, L.-J. (2006). Overexpression of the wounding-responsive gene *AtMYB15* activates the shikimate pathway in *Arabidopsis*. *Journal of Integrative Plant Biology*, *48*(9), 1084-1095.

Dixon, R.A., Paiva, N.L. (1995). Stress-Induced Phenylpropanoid Metabolism. *The Plant Cell*, *7*(7), 1085-1097.

Fridrichova, I., Kovarik, A., Rosskopfov, O. (1992). Immunological quantification of RecA protein in cell extracts of *E. coli* after exposure to chemical mutagens or UV radiation. *Folia Micorbiology*, *37*(1), 24-30.

Gao, W., Baars, J.J.P., Sonnenberg, A.S.M., Visser, R.G.F. (2011). Inheritance pattern of bruising sensitivity trait in *Agaricus bisporus*. *Proceedings of the 7th International Conference on Mushroom Biology and Mushroom Products,* INRA Bordeaux (pp. 43-51).

Ismaya, W.T., Rozeboom, H.J., Weijn, A., Mes, J.J., Fusetti, F., Wichers, H.J., Dijkstra, B.W. (2011). Crystal structure of *Agaricus bisporus* mushroom tyrosinase: identity of the tetramer subunits and interaction with tropolone. *Biochemistry*, *50*(24), 5477-5486.

Jolivet, S., Arpin, N., Wichers, H.J., Pellon, G. (1998). *Agaricus bisporus* browning : a review. *Mycological Research*, *102*(12), 1459-1483.

Joos, H.-J., Hahlbrock, K. (1992). Phenylalanine ammonia-lyase in potato (*Solanum tuberosum L.*) Genomic complexity, structural comparison of two selected genes and modes of expression. *European Journal of Biochemistry*, *629*(2), 621-629.

Kerrigan, R.W. (1996). Characteristics of a large collection of edible wild mushroom germ plasm: the *Agaricus* Resource Program. R.A. Samson, Ed. *Culture collections to improve the quality of life*. Centraalbureau voor Schimmelcultures P.O. Box 273, 3470 AG Baarn, The Netherlands.

Kim, J.Y., Seo, Y.S., Kim, J.E., Sung, S.-K., Song, K.J., An, G., Kim, W.T. (2001). Two polyphenol oxidases are differentially expressed during vegetative and reproductive development and in response to wounding in the Fuji apple. *Plant Science*, *161*(6), 1145-1152.

Ko, T.-P., Wu, S.-P., Yang, W.-Z., Tsai, H., Yuan, H.S. (1999). Crystallization and preliminary crystallographic analysis of the *Escherichia coli* tyrosine aminotransferase. *Acta Crystallographica Section D Biological Crystallography*, *55*(8), 1474-1477.

Largeteau, M., Latapy, C., Minvielle, N., Regnault-Roger, C., Savoie, J.-M. (2010). Expression of phenol oxidase and heat-shock genes during the development of *Agaricus bisporus* fruiting bodies, healthy and infected by *Lecanicillium fungicola*. *Applied Microbiology and Biotechnology*, *85*(5), 1499-1507.

Lezzi, C., Bleve, G., Spagnolo, S., Perrotta, C., Grieco, F. (2012). Production of recombinant *Agaricus bisporus* tyrosinase in *Saccharomyces cerevisiae* cells. *Journal of Industrial Microbiology and Biotechnology*, *39*(12), 1875-1880.

Mikosch, T.S.P., Lavrijssen, B., Sonnenberg, A.S.M., van Griensven, L.J.L.D. (2001). Transformation of the cultivated mushroom *Agaricus bisporus* (Lange) using T-DNA from *Agrobacterium tumefaciens*. *Current Genetics*, *39*(1), 35-39.

Morin, E., Kohler, A., Baker, A.R., Foulongne-Oriol, M., Lombard, V., Nagy, L G., Ohm, R.A, Patyshakuliyeva, A., Brun, A., Aerts, A.L., Bailey, A.M., Billette, C., Coutinho, P.M., Deakin, G., Doddapaneni, H., Floudas, D., Grimwood, J., Hildén, K., Kües, U., Labutti, K.M., Lapidus, A., Lindquist, E.A., Lucas, S.M., Murat, C., Riley, R.W., Salamov, A.A., Schmutz, J., Subramanian, V., Wösten, H.A.B., Xu, J., Eastwood, D.C., Foster, G.D., Sonnenberg, A.S.M., Cullen, D., de Vries, R.P., Lundell, T., Hibbett, D.S., Henrissat, B., Burton, K.S., Kerrigan, R.W., Challen, M.P., Grigoriev, I.V., Martin, F.

(2012). Genome sequence of the button mushroom Agaricus bisporus reveals mechanisms governing adaptation to a humic-rich ecological niche. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(43), 17501-17506.

Sim, S. K., Ohmann, S.M., Tong, C.B.S. (1997). Comparison of polyphenol ixidase in tubers of *Solanum tumberosum* and the non-browning tubers of *S. hjertingii*. *American Potato Journal*, *74*(1), 1-13.

Soler-Rivas, C., Möller, A.C., Arpin, N., Olivier, J.-M., Wichers, H.J. (2001). Induction of a tyrosinase mRNA in *Agaricus bisporus* upon treatment with a tolaasin preparation from *Pseudomonas tolaasii*. *Physiological and Molecular Plant Pathology*, *58*(2), 95-99.

Sonnenberg, A.S.M., Baars, J.J.P., Hendrickx, P.M., Kerrigan, R.W. (2006). Breeding mushrooms: state of the art. *Fifth International Conference on MushroomBbiology and MushroomPproducts, 12* (pp. 163-173).

Stoop, J.M.H., Mooibroek, H. (1999). Advances in genetic analysis and biotechnology of the cultivated button mushroom, *Agaricus bisporus*. *Applied Microbiology and Biotechnology*, *52*(4), 474-483.

Thipyapong, P., Hunt, M.D., Steffens, J.C. (1995). Systemic wound induction of potato (*Solanum tuberosum*) polyphenol oxidase. *Phytochemistry*, *40*(3), 673-676.

Thipyapong, P., Hunt, M.D., Steffens, J.C. (2004). Antisense downregulation of polyphenol oxidase results in enhanced disease susceptibility. *Planta*, *220*(1), 105-117.

Thipyapong, P., Steffens, J.C. (1997). Tomato polyphenol oxidase: differential response of the polyphenol oxidase F promoter to injuries and wound signals. *Plant physiology*, *115*(2), 409-418.

Tomas-Barberan, F.A., Espin, J.C. (2001). Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. *Journal of the Science of Food and Agriculture*, *81*(9), 853-876.

Vandesompele, J., de Preter, K., Pattyn, F., Poppe, B., van Roy, N., de Paepe, A., Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, *3*(7), 1-12.

Weijn, A., Bastiaan-Net, S., Wichers, H.J., Mes, J.J. (2012a). Melanin biosynthesis pathway in Agaricus bisporus mushrooms. *Fungal Genetics and Biology*. doi:http://dx.doi.org/10.1016/j.fgb.2012.10.004

Weijn, A., Tomassen, M.M.M., Bastiaan-Net, S., Hendrix, E.A.H.J., Baars, J.J.P., Sonnenberg, A.S.M., Wichers, H.J., Mes, J.J., (2011). Browning sensitivity of button mushrooms. *Proceedings of the 7th International Conference for Mushroom Biology and Mushroom Products*, INRA Bordeaux (pp. 208-216).

Weijn, A., Tomassen, M.M.M., Bastiaan-Net, S., Wigham, M.L.I., Boer, E.P.J., Hendrix, E.A.H.J., Baars, J.J.P., Sonnenberg, A.S.M., Wichers, H.J., Mes, J.J. (2012b). A new method to apply and quantify bruising sensitivity of button mushrooms. *LWT - Food Science and Technolgy*, *47*(2), 308-314.

Chapter 7

Molecular and biochemical analysis of bruising-tolerance based on *Agaricus bisporus* strains selected from a segregating population

A. Weijn[a,b], W. Gao[c], D.S.M. Somhorst-van den Berg[a], M.M.M. Tomassen[a], A.S.M. Sonnenberg[c], H.J. Wichers[a,b] and J.J. Mes[a]

a Food & Biobased Research, Wageningen University and Research Centre, Bornse Weilanden 9, 6708 WG Wageningen, The Netherlands
b Laboratory of Food Chemistry, Wageningen University and Research Centre, Bomenweg 2, 6703 HD Wageningen, The Netherlands
c Plant Research International – Mushrooms, Wageningen University and Research Centre, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands

Abstract

The button mushroom is by far the most cultivated edible mushroom species but unfortunately is prone to develop discoloration upon handling and storage. A segregating population based on a cross between two bruising-tolerant strains was analysed for bruising-sensitivity inheritance. In-depth analysis was performed on a selection of extremes from the offspring. Expression analysis of the genes from and related to the melanin biosynthesis pathway showed that the L-chain, a polyphenol oxidase associated peptide with co-factor-like properties, was more abundantly expressed in both white and non-white bruising-tolerant offspring together with polyphenol oxidase 3 and 5. Analysis of the phenolic compounds from the melanin pathway showed a high correlation between the total concentration of phenolic compounds and the bruising-sensitivity of the white offspring. For several, so far mainly unidentified, phenolic compounds a higher concentration was found in the bruising-sensitive offspring among them agaritine and L-DOPA. Analysis of previously identified genomic mutations in a high tolerant wild brown strain showed intriguing results which resulted in a new hypothesis on the key parameters of bruising-sensitivity and possible strategies towards obtaining a high bruising-tolerance trait.

1 Introduction

Agaricus bisporus, commonly known as the button mushroom, is the most cultivated species of edible fungi. The Netherlands is the fourth largest producer in the world of these mushrooms (Ministerie van Landbouw, 2010). For the fresh market, mushrooms are exclusively hand-picked, to minimize bruising-related discoloration. Mechanical harvesting results in bruising of the mushrooms which causes discoloration and leads to a lower quality of the mushrooms (Burton, 2004). Discoloration of mushrooms can also be initiated by handling and storage of the mushrooms or through microbial infections and leads to decreased yield and economic losses of the crop (Burton, 2002). Discoloration is caused by the oxidation of phenolic compounds catalysed by the enzyme polyphenol oxidase (PPO) (Jolivet *et al.*, 1998). Oxidized phenolic compounds can react further into quinones, which non-enzymatically react into coloured pigments named melanin. Different types of melanin can be formed based on the initial mono-phenolic compounds that polymerise. These phenolic compounds are hypothesized to be derived from chorismate and converted via different enzymatic steps into γ-L-glutaminyl-3-hydroxybenzene (GHB), *p*-aminophenol (PAP) and L-3,4-dihydroxyphenylalanine (L-DOPA) which in turn can be converted into GHB-, PAP- and DOPA-melanin. Besides the PPOs, other enzymatic steps in this pathway are probably responsible for blocking the melanin synthesis or can be rate limiting factors in the discoloration process.

Discoloration reactions can occur in many types of fruits and vegetables. A few examples are banana, potato, and lettuce (Adams and Brown, 2007). The discoloration in potato was analysed with a combined approach including quantitative trait locus (QTL) mapping for enzyme discoloration and the subsequent substrates, candidate gene mapping and expression assays for PPO (Werij *et al.*, 2007). Werij *et al.* identified three QTLs for enzymatic discoloration and two QTLs that correlated with the compound levels of chlorogenic acid and

tyrosine. One of the QTLs identified for enzymatic discoloration was found on chromosome 8 and co-localized with a PPO (POT32). A clear correlation between allele combinations, POT32 gene expression and degree of discoloration was observed. Sim *et al.* (1997) identified a wild potato species (*Solanum hjerntingii*) which does not exhibit enzymatic browning, probably resulting from truncation of a PPO gene (Culley *et al.*, 2000).

A. bisporus strains have been identified with a very high level of bruising-tolerance, very likely due to different mechanisms. One strain, wild brown strain 2 (WB2), contained a low concentration of the phenolic compounds GHB and GDHB (Chapter 5) possible caused by genetic changes in γ-glutamyltransferase (GGT) or variation in other enzymes downstream of GHB synthesis. This is supported by the analysis of Jolivet *et al.* (1995) who also identified GHB and GDHB as the main phenolic compounds involved in bruising sensitivity.

A. bisporus var. *bisporus* is a heterokaryotic fungus which fruiting body is composed of cells having two separated genomes (Sonnenberg *et al.*, 2011). Using protoplasting, homokaryons can be recovered containing only the genome of one of the parental lines (Gao *et al.*, 2011). One of the parental lines of WB2 (WB2A), which has a dominant inheritance pattern for bruising tolerance when combined with other homokaryons, was selected and fully sequenced. The sequenced genome was compared with genomic sequences of other parental lines for the genes involved and related to the melanin pathway. This revealed possible differences on the genetic level that might be responsible for the high level of bruising-tolerance of this WB2 strain (Chapter 6).

The first hybrid varieties of the white button mushroom were introduced on the market in 1981 and were developed in the Netherlands (Fritsche, 1986). New button mushroom varieties released afterwards were either identical or very similar to these first hybrids (Sonnenberg *et al.*, 2011). Currently breeding is used to develop new strains with higher yield, natural cap colour, and resistance against

Lecanicilium fungicola (Foulongne-Oriol *et al.*, 2012a; 2012b). Besides that genetic maps have been constructed (Foulongne-Oriol *et al.*, 2010; 2011; Kerrigan *et al.*, 1993; Moquet *et al.*, 1999) and some traits have been mapped like cap colour which was mapped on chromosome 8 (Callac *et al.*, 1998).

Here the analysis of offspring from a population based on a cross between two bruising-tolerant strains is described. In order to unravel the bruising-tolerance mechanism a molecular and biochemical approach was used. To this aim several differential lines from the population were selected and analysed for gene expression of the genes involved in the melanin biosynthesis pathway, the phenolic compounds present in cap skin tissue were determined and possible mutations in the genes of the melanin pathway were analysed. Next to that QTL analysis is performed on the population which will be presented elsewhere (Wei Gao *et al.*, in preparation).

2 Materials and Methods

2.1 Production of segregating population

Homokaryotic parental lines were obtained by protoplasting the vegetative mycelium and were identified using PCR (Gao *et al.*, 2011). Both parental lines from the white commercial hybrid 2 (CH2), named H39 and H97, were recovered and used to make hybrid crosses. Analysis of hybrid crosses with either H39 or H97 as a parental line gave variation in bruising-sensitivity, which depended on the partner in the cross (Gao *et al.*, 2011). Gao *et al.* (2011) also succeeded in selecting one of the parental lines of the tolerant wild brown 2 strain (WB2) and showed that this line, WB2A (WB2A is identical to MES09143 from Gao *et al.*, 2011, the terminology WB2A is used for convenience in this report), can pass on bruising-tolerance when combined with other parental lines. A segregating population based on bruising-tolerance was generated with a cross between H97 and WB2A and

after meiosis 200 homokaryons were selected. H39 was used as a white tester line to cross the population and produce mushrooms.

2.2 Mushroom growth and sample collection

Mushrooms were grown as described in Weijn *et al.* (2012b). Mushrooms were grown in boxes of 0.02 m^3 filled with 8 kg phase II compost. 200 Crosses were grown at the same time in one growing room. Reference strains were included to be able to scale the bruising-sensitivity in relation to other strains of which bruising-sensitivity was analysed repeatedly in the past. The following strains were used as a reference; the white tolerant CH2, white moderately sensitive commercial hybrid 6 (CH6), the sensitive wild white strain 7 (WW7), brown tolerant strain WB2, and the brown moderately sensitive cross between WB2AxH97.

Mushroom bruising and discoloration analysis were performed as described in Weijn *et al.* (2012b). Medium sized closed or veiled mushrooms from the middle of the first flush of each strain were picked randomly. Mushrooms were bruised within 4 h after harvest. Pictures of the bruised mushrooms were taken 60 min after bruising and analysed with a computer image analysis system. The bruised skin tissue was collected directly after taking the picture and immediately frozen in liquid nitrogen. Due to limitation in mushroom production of the correct developmental stage, non-bruised skin tissue was collected adjacent to the bruised area on the cap of the bruised mushrooms. Samples were stored at -80 °C until use. The samples were always handled with liquid nitrogen and were ground with mortar and pestle. For each experiment the amount of mushroom powder needed was weighed and stored at -80 °C until use.

2.3 Gene expression analysis

Gene expression analysis was performed as described in Chapter 4 (Weijn *et al.*, 2012a). RNA was extracted from 40 mg mushroom powder using the RNeasy mini kit (Qiagen) according to the Qiagen Plants and Fungi protocol. All samples

were treated with DNase I to remove the genomic DNA, according to the protocol of Sigma (AMP-D1). 200 ng DNase I treated RNA was used to make cDNA with the iScript cDNA synthesis kit (Biorad). 20 ng cDNA was used in each RT-qPCR reaction with 10 µl iQ SYBR Green Supermix (Biorad) and forward and reversed primers (primer sequences and final concentrations are indicated in Chapter 4 and Chapter 6) in a total volume of 20 µl. Each sample was measured in duplicate. The relative gene expression is determined as the relative expression to housekeeping genes actin and protein kinase inhibitor α (PkiA), calculated as $\Delta C_T = 2^{\wedge}(Ct_{geomean(actin:PkiA)} - Ct_{sample})$. Relative gene expression data were log2 transformed. Squared Pearson correlation was used for similarity and UPGMA for clustering in GeneMaths XT (Applied Maths, version 2.12).

2.4 Phenolic compound analysis

Phenolic compounds were extracted from 10 mg mushroom powder with 500 µl sulphite containing extraction solution (0.5 % (w/v) sodium metabisulphite ($Na_2S_2O_5$) in 1 % (v/v) acetic acid solution) as described in Chapter 5. Samples were injected in an Accela UHPLC system (Thermo Scientific, San Jose, CA) equipped with a pump, autosampler and photo-diode array detector (PDA) using a Hypersil gold aQ column (Thermo Scientific 150 mm x 2.1 mm i.d.; particle size 1.9 µm) at 20 °C. MS^n analysis was performed on a Thermo Scientific LTQ-XL using electrospray ionisation (ESI) and detection in the positive and negative mode. The complete analysis method is described in Chapter 5. Results are means of four determinations; the data were combined from two independent extracts which both were analysed in duplicate. *p*-Aminophenol, catechol, *p*-coumaric acid, L-DOPA, GHB, L-phenylalanine and L-tyrosine were used as standard compounds and dissolved in the sulphite solution. Calibration curves were run at 254 nm. Data analysis was performed with Xcalibur (Thermo Scientific, version 2.1.0) as described in Chapter 5.

2.5 Mutation analysis

Four mutations were identified in WB2A (homokaryon of WB2). RT-qPCR primer sets were developed to analyse the mutations and are described in Chapter 6 (Table 3). For each mutation a primer set was developed on the H97 sequence (wild type) and the WB2A sequence (mutation). DNA was isolated from the selection of the population (Table 1) according to the standard protocol of the DNeasy Plant Mini kit (Qiagen). RT-qPCR was performed as described in Chapter 6 with 5 µl (1 ng/µl) DNA with 10 µl iQ SYBR Green Supermix (Biorad) and 0.25 µM forward and reversed primers in a total volume of 20 µl. Each sample was measured in duplicate. The RT-qPCR was preheated at 95 °C for 1.5 min. This was followed by 40 cycles with a denaturing temperature of 95 °C for 10 s, annealing temperature of 70 °C (60 °C was used for GGT_1) for 10 s, and elongation temperature of 72 °C for 15 s. For each sample a melting curve was determined to verify a single amplification product.

3 Results

3.1 Bruising-sensitivity of the segregating population

The 200 individuals of the population were grown, picked, bruised and the bruising sensitivity was analysed. Only one offspring (OS), OS142, did not produce mushrooms and was excluded. The offspring was based on cap colour divided into a white group (107 individual lines, labelled with W) and a non-white group (92 individual lines, labelled with NW). Thirteen non-white lines gave a negative value as Whiteness Index difference (WI difference, OS134 until OS171 in Figure 1). The pictures of these lines were checked by eye to ascertain that negative values did not occur through scraping of the skin tissue by the spatula, which means that the white flesh tissue underneath the skin becomes visible. This was not the case and therefore the WI difference values can be interpreted as being zero and no discoloration is measured upon bruising. The offspring gave a range of

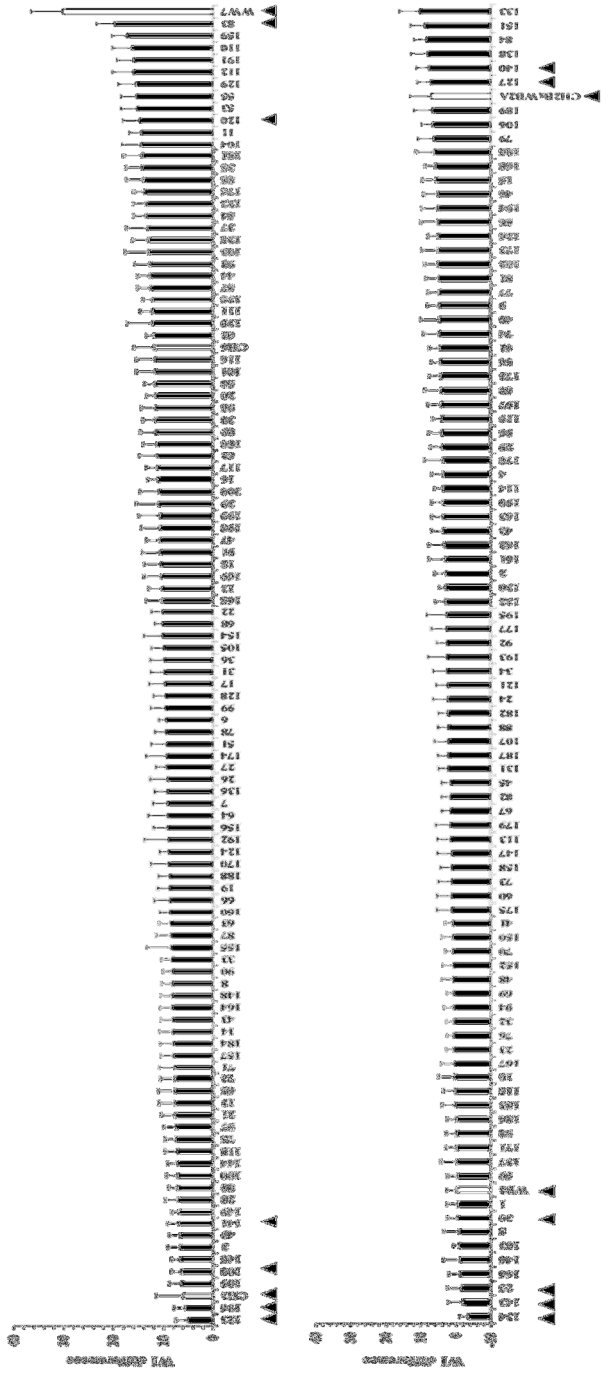


Figure 1. Bruising-sensitivity of *A. bisporus* population. Top graph is the white offspring and the bottom graph is the non-white offspring (which includes off-white, light brown and brown coloured cap). Five reference strains were added to the experiment (indicated by a white bar with black border in the graphs). Offspring selected for in-depth melanin pathway analysis are indicated by a ▲.

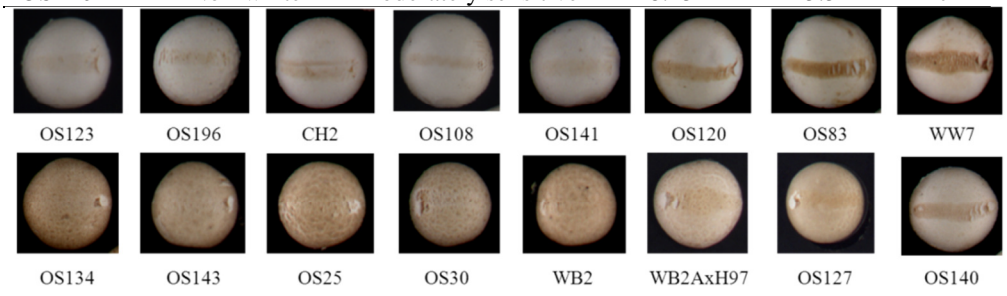
bruising-sensitivity, in general the white offspring has a higher WI difference value (ranging from a WI 5.23-18.94) than the non-white offspring (WI of 0-10.50). The WI difference of the five reference strains resulted in the expected classification into bruising-tolerant, moderate or sensitive (indicated with a white bar in Figure 1). The white reference strain CH2 was previously identified as one of the most bruising-tolerant strains; in the population only two lines (OS123 and OS196) had a lower WI difference and therefore have a slightly higher bruising-tolerance. The segregating population also resulted in sensitive white offspring but not as sensitive as WW7, which is one of the most sensitive white strains identified previously (Weijn *et al.*, 2012b). The group of non-white offspring contained nine tolerant lines that have a slightly lower WI difference than reference strain WB2 and all show a very high bruising-tolerance as the bruised area was hardly distinguished by eye. Six non-white offspring are more sensitive than the moderately sensitive reference cross WB2AxH97 based on WI difference. Previously, more sensitive brown strains were identified (Weijn *et al.*, 2012a), which were not included in this research and therefore the strains with almost equal WI difference as cross WB2AxH97 are classified as moderately sensitive.

Lines with contrasting bruising-sensitivity were selected from the population to perform in-depth analysis of the melanin pathway. Selection of the offspring was based on bruising-sensitivity but also if sufficient skin tissue was collected from mushrooms in the same developmental stage. In total sixteen strains were selected (indicated with ▲ in Figure 1 and more in detail in Table 1). Eight white strains, including four tolerant offspring (OS108, OS123, OS141, and OS196), two sensitive offspring (OS83, and OS120) and two reference strains (CH2, and WW7), were selected. Next to that eight non-white strains were selected, including four tolerant offspring (OS25, OS30, OS134, and OS143), two moderately sensitive offspring (OS127, and OS140) and two reference strains (WB2, and cross

WB2AxH97). LSD analysis confirmed the division into bruising-tolerant, moderately sensitive or sensitive strains.

Table 1. Offspring selection of the population. Offspring is indicated by number. WI diff = WI difference. Least significant difference (LSD) was calculated for the white strains separately from the non-white strains, which is indicated by #. Below the table a picture is shown of a bruised mushroom for each selected offspring.

Strain	Cap colour	Classification	WI diff	Std. Dev.	LSD
OS123	White	Tolerant	5.23	2.23	a
OS196	White	Tolerant	5.64	2.19	a
CH2	White	Tolerant	6.13	5.38	a
OS108	White	Tolerant	6.54	1.94	a
OS141	White	Tolerant	6.74	2.22	a
OS120	White	Sensitive	16.38	3.68	b
OS83	White	Sensitive	18.94	3.22	b
WW7	White	Sensitive	30.79	6.05	c
OS134	Non-white	Tolerant	-3.89	2.21	a#
OS143	Non-white	Tolerant	-2.67	3.83	ab#
OS25	Non-white	Tolerant	-2.29	3.18	ab#
OS30	Non-white	Tolerant	-0.68	2.97	b#
WB2	Non-white	Tolerant	-0.23	2.93	b#
WB2AxH97	Non-white	Moderately sensitive	6.23	4.81	c#
OS127	Non-white	Moderately sensitive	7.51	4.09	c#
OS140	Non-white	Moderately sensitive	8.25	3.32	c#



A genetic affiliation analysis of the selected offspring was performed based on single nuclear polymorphism (SNP) analysis of the homokaryons (Figure 2, performed by Wei Gao, PRI Wageningen UR). The analysis was performed to identify possible isogenic lines which have a different bruising-sensitivity as such lines might facilitate research to unravel the mechanism of bruising-tolerance. The

analysis did not reveal clear candidates with very high genetic similarities, only offspring OS120 and OS141 showed to have some genetic similarities while having contrasting bruising-sensitivity. SNP analysis revealed that these two OS lines had differences on at least four chromosomes (Chr. 4, 7, 9 and 10; data not shown). More detailed analysis of chromosomal inheritance and QTL analysis based on this population will be presented elsewhere (Wei Gao *et al.*, in preparation).

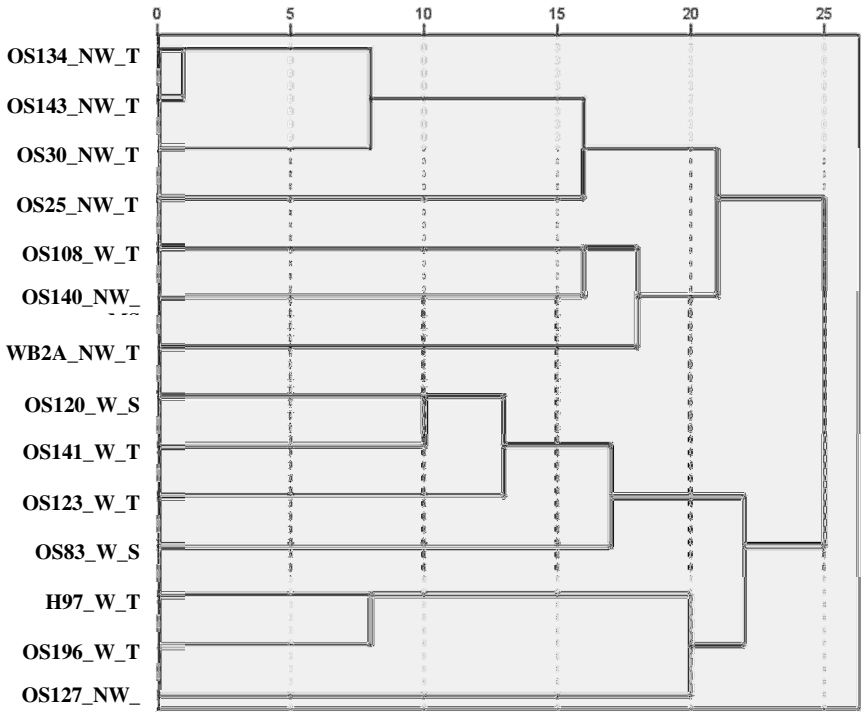


Figure 2. Dendrogram of selected offspring from the population. Comparison is performed based on SNP analysis of the homokaryons making use of average linkage between groups. Rescaled distance cluster combination was used. Offspring is indicated with a number followed by a W for white lines or NW for non-white lines and a T for tolerant, MS for moderately sensitive or S for sensitive. The parental lines used to make the initial cross of the population were included (H97 and WB2A).

3.2 Phenolic compound analysis of the offspring selection

3.2.1 Total phenolic compounds and bruising-sensitivity

Phenolic compound analysis was performed on the cap skin tissue of the selected offspring and reference strains. For the white capped strains a high correlation was found between the total concentration of phenolics (calculated as total peak area) and the WI difference for both the non-bruised and bruised tissue (R^2 = 0.94 and R^2 = 0.96, respectively, Figure 3). This correlation was not found for the non-white offspring for which the total phenolic concentration is closer to each other (data not shown).

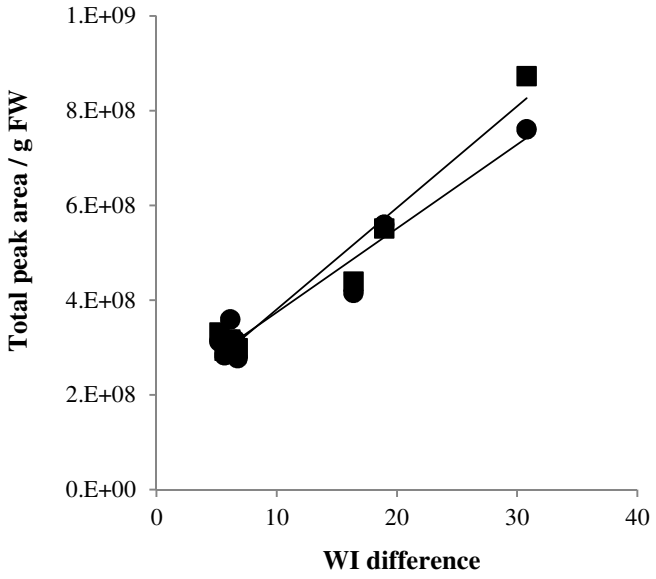


Figure 3. Correlation between WI difference and total phenolic compound concentration for the selection of white strains. Non-bruised samples are indicated with a circle and bruised samples with a square.

3.2.2 Phenolic compound analysis of non-bruised tissue of the offspring

Based on the analysis of nine *A. bisporus* strains it was described that the bruising-tolerance can be predicted based on low concentrations of unidentified compound 9, GHB and GDHB (Chapter 5). This indicates that GHB-melanin will be the major melanin responsible for bruising based discoloration and that mutations early in the pathway towards GHB can result in high bruising tolerance. The population was generated by using a homokaryotic parental line of the non-white tolerant WB2 (WB2A) combined with the white tolerant CH2 (H39 and H79) which both showed low levels of compound 9, GHB and GDHB. First analysis was focus on the presence of these three compounds in the selected offspring. Offspring with a low concentration of compound 9, GHB and GDHB (OS123W, OS25NW, OS30NW, and OS134NW) indeed all were identified as bruising-tolerant (Figure 4). This confirms the major role of these phenolic compounds. OS140NW is a bruising moderately sensitive line that has a low concentration of GHB and GDHB (although slightly higher than the lines mentioned above) but this line showed an intriguing high presence of agaritine. This high level of agaritine was also identified in the bruising-sensitive lines OS83W and OS120W, and moderately sensitive OS127NW. The results indicate a link of agaritine to the melanin synthesis pathway and showed a correlation with bruising-sensitivity. The exact relation of agaritine with the melanin pathway is unknown. It can be hypothesised that the γ-glutamyl group of agaritine can be transferred by GGT to *p*-aminophenol leading to GHB. Besides the compounds described above also DOPA and some other unidentified compounds (8, 9, and 27) show a higher concentration in the non-bruised skin tissue of sensitive offspring than the tolerant (Figure 4). Currently it will be difficult to speculate how these can contribute to bruising-sensitivity other than that they are possible depending on fluxes within the pathways and inter connections between phenolic compounds.

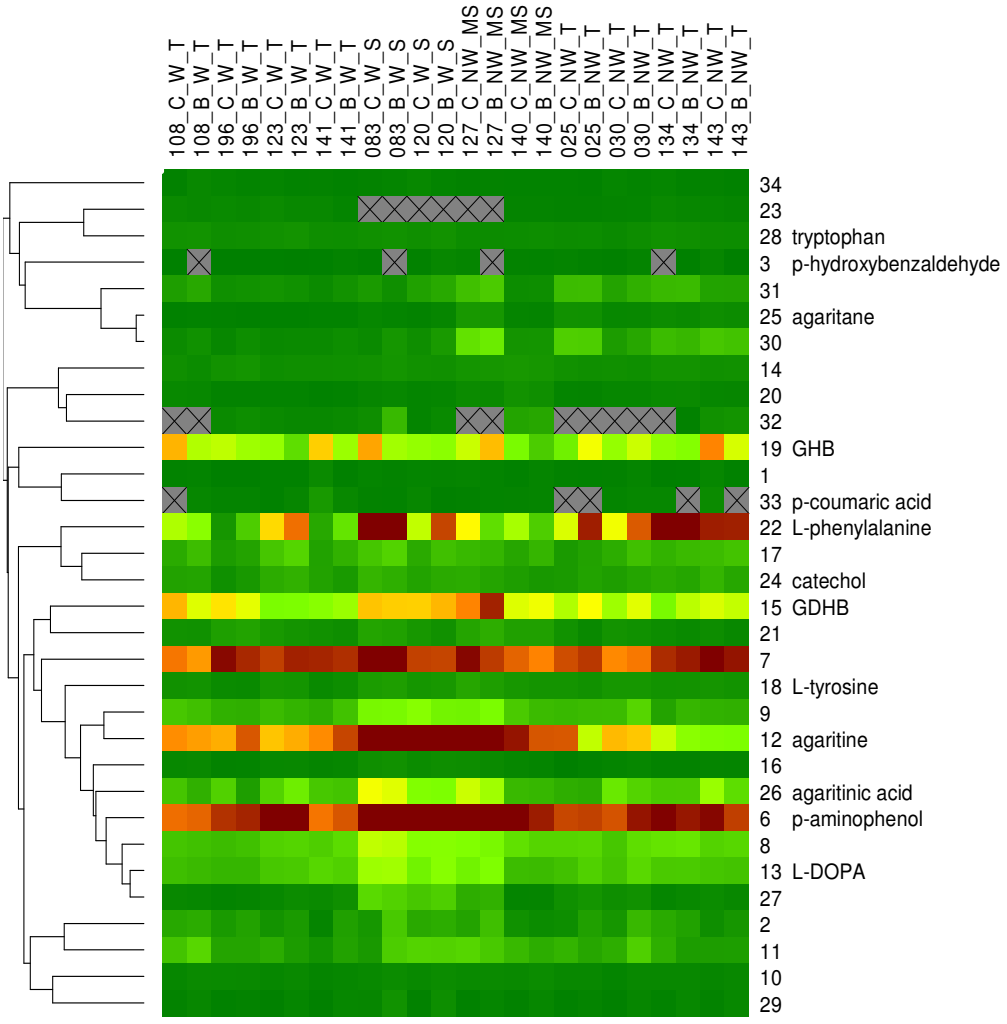


Figure 4. Heat map of phenolic compounds identified in the non-bruised (C) and bruised (B) skin tissue of the offspring. White (W) lines and non-white (NW) lines are indicated with a T for tolerant, MS for moderately sensitive, and a S for sensitive. Phenolic compounds are indicated by peak number and when possible with compound name.

It is also clear from these analyses that the high bruising-tolerance of some lines could not be explained by the phenolic compounds. In these lines it is postulated that enzymatic steps upstream or downstream of GHB and the conversions in the

GHB-melanin and PAP-melanin pathway might be the critical factor for bruising-tolerance. Therefore the next step in the analysis was to study the expression of genes involved in this melanin synthesis pathway.

Phenolic compound analysis was also performed on the bruised skin tissue (Figure 4, samples labelled with B) and was compared with the concentrations of phenolic compounds in the non-bruised skin tissue (labelled with C). Samples before and after bruising were almost similar therefore comparing the phenolics will not lead to new leads for key components of the bruising tolerance trait.

3.3 Gene expression analysis of the skin tissue

Gene expression analysis of the non-bruised skin tissue of the offspring showed that the expression does not differ for most genes analysed when comparing tolerant and sensitive offspring (Figure 5). Several genes have a low expression in all offspring, such as *PPO_1*, *C4H_1*, *C4H_2*, *GGT_1*, *GGT_2*, *PDHtase*, and *Cat_1*, which is in consensus with the analysis of nine *A. bisporus* strains with different bruising-sensitivity (Chapter 6). *PHRB*, *PPO_3* and *PPO_4* have a high expression in the non-bruised tissue as found before in several *A. bisporus* strains (Chapter 6).

Small differences in gene expression were observed between the white offspring for *PAL_1*, *C4H_1*, *4ABS_1*, and *PDH* but this did not relate with the bruising-sensitivity (left part of Figure 5). The main difference is found for the *L-chain*, *PPO_3* and *PPO_5*, which are more abundantly expressed in the tolerant OS108 and OS196 than in the bruising-sensitive OS83 and OS120, and in the tolerant OS123 and OS141. Gene expression analysis in the skin tissue of the non-white offspring showed a 100 % correlation between the expression of *L-chain*, *PPO_3* and, to a lesser extent *PPO_5,* and bruising-sensitivity.

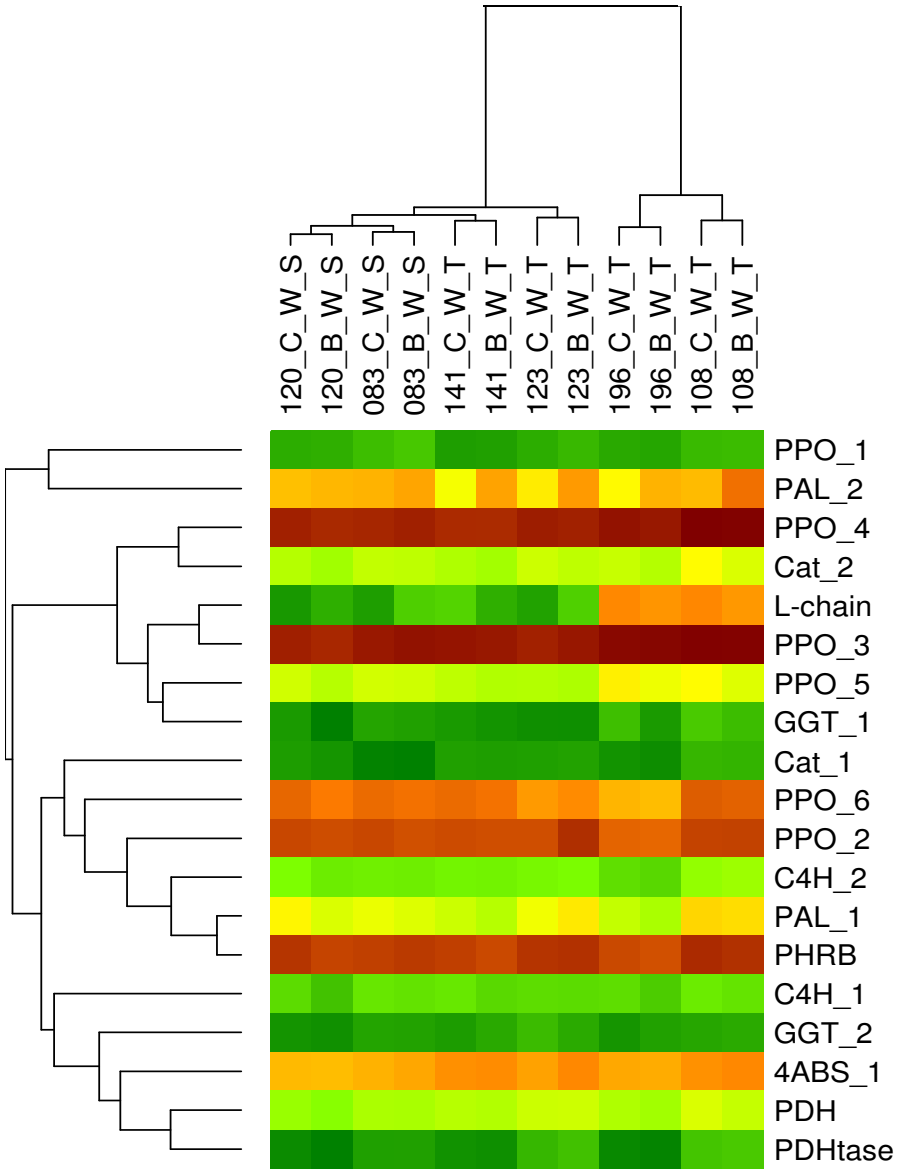


Figure 5A. Heat map of gene expression analysis in non-bruised (C) and bruised (B) skin tissue of selected white (W) offspring from the population. The lowest gene expression is shown in dark green, the highest expression in dark red. Tolerant offspring is indicated with a T and sensitive offspring with an S. 4ABS = 4-aminobenzoate synthase, GGT = γ-glutamyltransferase, PDHtase = prephenate dehydratase, PDH = prephenate dehydrogenase, PPO = polyphenol oxidase, PAL = phenylalanine ammonia-lyase, C4H = trans-cinnamate-4-monooxygenase, PHRB = photoregulator B, and Cat = catalase.

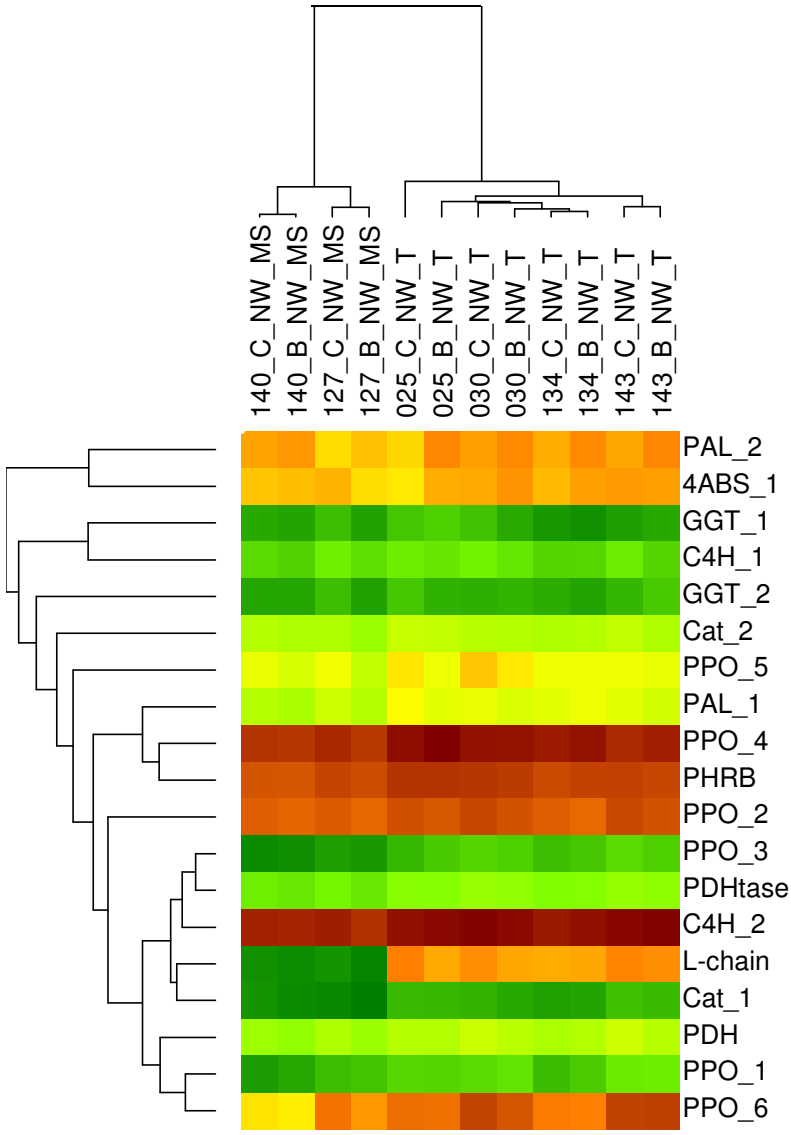


Figure 5B. Heat map of gene expression analysis in non-bruised (C) and bruised (B) skin tissue of selected non-white (NW) offspring from the population. The lowest gene expression is shown in dark green, the highest expression in dark red. Tolerant offspring is indicated with a T and moderately sensitive offspring is indicated with MS. 4ABS = 4-aminobenzoate synthase, GGT = γ-glutamyltransferase, PDHtase = prephenate dehydratase, PDH = prephenate dehydrogenase, PPO = polyphenol oxidase, PAL = phenylalanine ammonia-lyase, C4H = trans-cinnamate-4-monooxygenase, PHRB = photoregulator B, and Cat = catalase.

This is in line with the expectation that bruising tolerance, when not based on the GHB synthesis (identified as the low availability of GHB and GDHB) is probably regulated downstream of GHB in the conversion into melanin. The three genes, *L-chain*, *PPO_3*, and *PPO_5*, are located on the lower end of chromosome 5 and it can be postulated that a factor for bruising-tolerance is located on this locus. When combining the results of the phenolic analysis and the gene expression analysis it becomes clear that the bruising-tolerance can be obtained by either a low concentration of GHB and GDHB or a higher expression of *L-chain*, *PPO_3* and or *PPO_5*. The only exception to this mechanism is OS141 in which a higher GHB concentration is present and *L-chain* and *PPO_3* have a low expression. However, this line does not contain a high level of GDHB which might indicate the presence of a change in one of the PPO enzymes that otherwise converts GHB into GDHB. Another possibility is to postulate an influence of an enzyme that converts phenolic compounds into non-colouring compounds, which is illustrated by the high content of phenylalanine in all NW tolerant strains and a higher expression level of *PAL_2* in these strains compared to the sensitive strains.

The gene expression profiles of non-bruised and bruised tissue were quite similar. As identified before *PAL_2* is one of the genes that responded to bruising (Chapter 6) which was confirmed in all offspring lines. On average the increase of *PAL_2* was 3.5 times higher in the white tolerant offspring than the white sensitive offspring, for the non-white offspring a two times higher increase was found comparing the tolerant with the sensitive offspring. Whatever the background might be it is interesting to study the genomic mutations and how these might influence enzymatic functions.

3.5 Mutation analysis of population selection

Sequence analysis of the genes related to and involved in the melanin biosynthesis pathway identified four mutations in WB2A (parental line of tolerant strain WB2). Analysis with RT-qPCR primer sets (Chapter 6) showed that the mutations only existed in the WB2 strain and not in the other eight *A. bisporus* strains analysed. For the offspring the same mutations were analysed to check whether these mutations related with bruising-tolerance. Unfortunately, the primer set used for the mutation in *AT_3* did not show consistent results probably due to homology of the primers with *AT_2*. Based on chromosome inheritance (based on the data of Wei Gao *et al.*, in preparation) and low recombination frequencies in *A. bisporus* (Sonnenberg *et al.*, 2011) it is expected that both the PPO_3 and the AT_3 mutated genes will be coherently inherited on the same allele and are therefore correlated.

The first thing to keep in mind is that *A. bisporus* is heterokaryotic and contains two nuclei of different origin. The hybrid between WB2AxH97 will result in the presence of all alleles of both WB2A and H97 which was confirmed by RT-qPCR (Table 2). The population was generated by crossing the homokaryons obtained after meiosis of WB2AxH97 with H39. As GGT_1 H97 primers give RT-qPCR products with all OS at a low C_T value it can be concluded that this primer set also is able to amplify the H39 allele of GGT_1. However, the other primers for H97 (AT_1 and PPO_3) do not show amplification products for all lines and therefore it can be concluded that these primers sets do not amplify the H39 allele for these genes. Surprisingly, amplified products of OS30 showed that both WB2A and H97 alleles are present which is not possible having H39 as one of the homokaryons. The amplification of all alleles might result from a mistake in sampling or a contamination with another strain at time of inoculation of the spawn. Based on the slightly higher C_T value obtained by the AT_1 H97 primers and PPO_3 WB2A primers we hypothesize that OS30 will have the AT_1 and GGT_1 alleles inherited

from WB2A and the PPO_3 (and AT_3) locus of H97 (which was confirmed by the data of Wei Gao *et al*., personal communication). For all other OS the presence of either the WB2A mutated allele or the H97 allele was detected with high certainty. When combining the presence of the alleles with the expression analysis it is observed that the presence of the PPO_3 H97 allele correlated with the higher expression of the *L-chain* and *PPO_3*. As these are both located on chromosome 5 (and close to AT_3) it can be concluded that the bruising-tolerant OS196, OS108, OS25, OS30, OS134, and OS143 will have inherited this part of chromosome 5 of H97. All four sensitive lines OS83, OS120, OS127, and OS140, have inherited the PPO_3 truncated form of WB2A. It is surprising that this mutation (a truncated PPO) can result in a higher sensitivity but this can be explained by hypothesising that a functional PPO_3 (as found in some of the tolerant offspring) converts phenolic compounds into other compounds that do not polymerise into dark coloured melanin. Some strains do not follow this consensus, such as the white tolerant strain OS123 and OS141 that have inherited PPO_3 of WB2A but are not bruising-sensitive; the same can be concluded for WB2. However, for WB2 it was found this strain forms low concentrations of GBH and GDHB very likely due to a mutation early in the synthesis pathway. A possible candidate for this is GGT_1 for which a mutation was found in WB2A. In order to have a full effect this GGT_1 should be mutated in both homokaryotic genomes of WB2. Based on the RT-qPCR it was found that the mutation in GGT_1 was present but also that the H97 genome was present (although a small mutation can be postulated based on the difference of two C_T values, indicated in pink in Table 2). However, when in WB2 the WB2A genome would be more active than the other genome it can be that this WB2A allele in the offspring is responsible for a blockage of GHB synthesis. In line with this hypothesis the offspring with a low concentration of both GHB and GDHB might have the mutated GGT_1 allele inherited from WB2A. For the strains OS123, OS134 and OS30 this is correct but for line OS25 this is not the case.

Table 2. Result of mutation analyses of the offspring. Indicated are C_T values obtained for each primer set. Indicated in red is when the strain contains the mutation of WB2A and indicated in blue is when the strain contains the wild type H97 allele. Chr = chromosome. W = white and NW = non-white strain. T = tolerant, MS = moderately sensitive and S = sensitive. N/A = not available.

Strain	*AT_1* (Chr. 4)		*GGT_1* (Chr. 2)		*PPO_3* (Chr. 5)	
	WB2A	H97	WB2A	H97	WB2A	H97
H97	28.28	21.24	33.45	19.42	38.43	20.93
CH2_W_T	33.22	21.86	28.87	19.95	28.09	20.40
OS123_W_T	23.33	32.67	20.34	20.24	20.24	29.23
OS196_W_T	23.65	33.14	32.67	19.46	30.68	20.39
OS108_W_T	22.26	31.53	29.70	19.30	29.15	20.53
OS141_W_T	22.81	29.72	20.50	20.30	20.27	27.42
OS120_W_S	34.49	22.03	20.50	20.62	20.38	30.28
OS083_W_S	34.45	21.70	20.63	20.69	20.48	30.43
WB2A	20.96	34.40	19.99	32.01	20.55	36.39
WB2_NW_T	22.81	32.82	20.44	22.45	20.26	29.42
WB2AxH97	23.48	22.52	20.65	20.47	20.47	20.26
OS134_NW_T	22.82	31.43	20.67	20.75	28.75	20.37
OS143_NW_T	23.38	33.36	20.56	20.87	30.53	20.36
OS025_NW_T	32.22	21.71	28.95	19.45	29.24	20.29
OS030_NW_T	23.12	24.48	20.55	20.46	22.46	20.99
OS127_NW_MS	34.93	21.63	31.24	19.41	20.24	30.77
OS140_NW_MS	25.21	32.91	32.61	19.53	20.87	31.07

Surprisingly, the AT_1 allele of WB2A (which is located on chromosome 4) was identified in all white tolerant offspring while the H97 allele was solely present in the sensitive white offspring. For the non-white offspring this correlation between tolerance and the presence of the AT_1 allele of WB2A did not withstand as two lines deviate from this: OS25 (tolerant but lacking the AT_1 allele of WB2A) and OS140 (moderately sensitive and containing the WB2A allele).

Based on these results it should be concluded that no straightforward explanation for the bruising-tolerance and sensitivity of the offspring can be given. As the genes were only studied by RT-qPCR on a single mutation it might be that other mutations in these loci have been overlooked and have an equal or more important contribution to the trait. This might have happened for OS25 because it is a tolerant non-white offspring but lacks the four mutations analysed. OS123 is

also an interesting line for further analysis because this offspring contains the four mutations of WB2A. Next to that OS123 is a tolerant line with a slightly lower WI difference than CH2 and has a white cap colour, which is more applicable for the commercial mushroom market than a non-white cap.

4 Discussion

A segregating populating based on a cross between two tolerant strains was analysed with respect to the bruising-sensitivity trait. It was possible to distinguish between bruising-tolerant and more sensitive individual lines for both the white and non-white offspring. The bruising-sensitivity of the reference strains correlated with previous analysis (Weijn *et al.,* 2012b) and indicated that the bruising method is reproducible. A selection of the offspring based on bruising-sensitivity was made for in-depth analysis of the melanin pathway to identify possible key parameters related to the trait. The bruise was applied to the top of the cap and non-bruised and bruised samples were taken from the same mushroom cap. The effect of the bruising-discoloration on adjacent cells is not known and might have an influence on the molecular and biochemical analysis of the offspring.

For a selection of the offspring the bruising-tolerance trait was analysed based on the phenolic compounds and the gene expression in the skin tissue and mutations in four genes of the melanin biosynthesis pathway. A combined overview of the results of these analyses is given in Table 3. A higher gene expression of the *L-chain*, *PPO_3*, and *PPO_5* correlated with high tolerance in the offspring. The tolerant white offspring OS123 and OS141, however, did not follow this pattern and the gene expression profiles of this offspring showed higher similarity with the sensitive white offspring. However, a full correlation can be found between the low expression of the *L-chain*, *PPO_3*, and *PPO_5* and the presence of the WB2A allele on chromosome 5. As all sensitive and moderately sensitive lines have inherited the WB2A allele of chromosome 5 it can be

concluded that for chromosome 5 it is better to have inherited both the H97 and the H39 allele, as is the case in the genetic background of CH2. A possibility is that in offspring which contains a truncated *PPO_3*, which is linked to a truncated *AT_3*, other enzymes become more active or compensate for these functions and as a consequence phenolics are converted into melanin. Through the clustering of *AT_3* and *PPO_3* on the same chromosome it is not possible to predict which mutation is more important. At this stage, it can not even be excluded if this correlates with the *L-chain* and *PPO_5* or even with other genes like *AT_2*, *PPO_2*, or *PPO_4* which are also found on chromosome 5.

Another conclusion is based on the offspring that obtained chromosomes 2, 4 and 5 from WB2A and H39 (middle part Table 3) and which are bruising-tolerant. Possibly, through the mutations found in WB2A, the phenolic fluxes in the melanin pathway are diminished and consequently a higher tolerance level is obtained. A combination of WB2A with H39 leads to a higher tolerance than a combination of WB2AxH97. It is interesting to analyse the other homokaryon of WB2 to investigate whether the identified mutations are either homo- or heterozygotic. Possibly, if mutations are homozygous, a population based on a cross between WB2AxH97 or WB2AxH39 and the back cross with the other homokaryon of WB2 might lead to a higher bruising-tolerance. SNP analysis revealed that OS120 and OS141 have a difference on at least four chromosomes, of which chromosome 4 is probably the most relevant for the difference in bruising-sensitivity based on the obtained result (Table 3).

Strain	Cap colour	Sensitivity	WI diff	*GGT_1* (Chr. 2)	*AT_1* (Chr. 4)	*PPO_3* & *AT_3* (Chr. 5)	Expression *L-chain* & *PPO_3*	Phenolics (ug/g FW)	Phenolics % of total peak area
WB2	NW	T	-0.23	WB2A/?	WB2A/?	WB2A/?	High[1]	10.31	29.14
CH2	W	T	6.13	H97/H39	H97/ H39	H97/ H39	High	15.99	26.70
OS134	NW	T	-3.89	WB2A/ H39	WB2A/ H39	H97/ H39	High	9.66	16.78
OS143	NW	T	-2.67	WB2A/ H39	WB2A/ H39	H97/ H39	High	8.37	13.25
OS25	NW	T	-2.29	H97/ H39	H97/ H39	H97/ H39	High	13.39	25.97
OS196	W	T	5.64	H97/ H39	WB2A/ H39	H97/ H39	High	10.26	21.70
OS108	W	T	6.54	H97/ H39	WB2A/ H39	H97/ H39	High	11.95	22.70
OS30	NW	T	-0.68	WB2A/ H39	WB2A/ H39	WB2A/ H39	High[2]	11.19	23.03
OS123	W	T	5.23	WB2A/ H39	WB2A/ H39	WB2A/ H39	Low	11.16	21.41
OS141	W	T	6.74	WB2A/ H39	WB2A/ H39	WB2A/ H39	Low	12.22	26.43
WB2AxH97	NW	MS	6.23	WB2A/H97	WB2A/ H97	WB2A/ H97	High[1]	16.93	29.33
OS127	NW	MS	7.51	H97/ H39	H97/ H39	WB2A/ H39	Low	22.26	27.66
OS140	NW	MS	8.25	H97/ H39	WB2A/ H39	WB2A/ H39	Low	15.31	27.84
OS120	W	S	16.38	WB2A/ H39	H97/ H39	WB2A/ H39	Low	23.09	33.34
OS83	W	S	18.94	WB2A/ H39	H97/ H39	WB2A/ H39	Low	23.91	26.65

Table 3. Combined overview of bruising-tolerance trait. Indicated for each strain is the strain name, the cap colour, the bruising-sensitivity classification, the WI difference (WI diff), and the genetic background based on the analysed mutations. Phenolics is the total concentration in non-bruised skin tissue of compounds 8, 9, 12 (agaritine), 13 (DOPA), and 27 as equivalents of the DOPA standard or as percentage of the total peak area. [1] High expression for *L-chain* , *PPO_3* level is lower than in the other strains indicated with high. [2] High expression found probably due to contamination of the sample.

The population segregated in almost equal numbers of white and non-white OS which we analysed separately because it is not known whether the image based tolerance quantification is influenced by the difference in natural cap colour. It is also not known whether there is a link between this cap colour and bruising-sensitivity. Therefore, it should be kept in mind that the cap colour might have an influence on the phenolic fluxes of the melanin pathway. Previous research identified GHB and GHDB as the main phenolics involved in bruising-sensitivity but is not known which phenolic compounds are involved in the generation of the natural brown cap colour. The analyses of the population did not reveal phenolic compounds or gene mutations or gene expressions that were different between white and non-white OS. When differences were found this might had led to identification of the mechanism involved in the formation of the brown cap colour. In the past QTL analysis identified a QTL for cap colour on chromosome 8 (Callac *et al.*, 1998). Notably, on chromosome 8 *PPO_1*, *PPO_6* and *4CL_9* were identified (Weijn *et al.*, 2012a). Based on the currently available data we could not rule out a possible involvement of one of these genes in cap colour but they also do not show any positive correlation.

The phenolic compounds that were more abundant in the sensitive offspring were agaritine, DOPA and compounds 8, 9, and 27. A good correlation was found between the concentration of these phenolics and the bruising sensitivity for both the white and non-white strains ($R^2 = 0.91$ for the white strains and $R^2 = 0.70$ for the non-white strains). A high correlation was also found between the total concentration of phenolic compounds and the bruising-sensitivity in both the non-bruised and bruised skin tissue of the white offspring. The phenolic compounds give a better prediction for the bruising-sensitivity of the white than for the non-white offspring.

Upon bruising, gene expression of *PAL_2* increased, as was found before in nine *A. bisporus* strains (Chapter 6). The increase of PAL_2-expression was higher in the tolerant than in the sensitive offspring of both white and non-white cap coloured lines. Based on the current analysis it is difficult to correlate the increased expression of *PAL_2* with phenolic compounds in the skin tissue after bruising. It is unknown whether the induction of gene expression results in an increase of enzymes and their activity. The time point between applying the bruise and analysis is only 60 minutes. This was sufficient to identify an increase in gene expression but translational effects might become apparent later in time.

The truncated PPO_3 gene appeared to be a promising parameter as also a truncated PPO gene was identified in potato with low discoloration (Culley *et al.*, 2000; Sim *et al.*, 1997). However, analysis of the occurrence of the mutations, previously identified in WB2A, in the offspring showed that the mutations did not correlate with bruising tolerance. Urbany *et al.* (2012) analysed the natural variety in the proteome of twenty potato varieties with either low or high bruising-sensitivity and showed that seven genes or gene families were differentially expressed resulting in differences on the protein level. As novel factors involved in bruising they identified lipases (putatively involved in cell shape regulation and lipid signalling), patatins (perhaps involved in membrane modification) and annexins (probably in mediating Ca^{2+}-dependent events involving interactions of the cytoskeleton and cellular membranes). As these proteins react to mechanical damage they can also be important in mushrooms. Possibly upon bruising by a slip-shear force the membrane is damaged and enzymes and phenolic compounds are brought into contact with each other. Therefore it is plausible that also other parameters than those involved in phenolic synthesis and conversion can be important for the bruising tolerance trait.

It can be concluded that the, in this report, presented approach of molecular research combined with a biochemical approach and further supported by proteome analysis of the bruising-sensitivity is a promising strategy to build hypotheses on the mechanism of bruising-sensitivity and how to obtain new strains with a high bruising-tolerance. Gene silencing or knock out lines as developed for *A. bisporus* (Costa *et al.*, 2009; Mikosch *et al.*, 2001) can be used to confirm the involvement of certain key parameters in discoloration. The strains and OS from the population together with the here developed hypothesis on how to further increase the bruising-tolerance level and introducing this in a white cap colour background will have a high economic potential.

Acknowledgements

This project is supported by the governmental grant "TTI Green Genetics", Dutch Board for Horticulture, Bromyc, Lutèce, Sylvan, Banken Mushrooms, the Greenery and WeBe Engineering. The similarity matrix was performed by Wei Gao from Plant Research International, Wageningen UR. The colleagues from Food and Biobased Research, Plant Research International - Mushrooms and Unifarm are acknowledge for their help during picking of the mushrooms, bruising the mushrooms and collecting the bruised and non-bruised skin material of the offspring.

References

Adams, J.B., Brown, H.M. (2007). Discoloration in raw and processed fruits and vegetables. *Critical Reviews in Food Science and Nutrition*, *47*(3), 319-333.

Burton, K.S. (2002). Bruising means lost mushroom sales. *Mushroom Journal*, *624*, 23-24.

Burton, K.S. (2004). Cultural factors affecting mushroom quality - cause and control of bruising. *Proceedings of the XVIth International congress of Science and Cultivation of Edible and Medicinal Fungi* (pp. 397-402). Maimi.

Callac, P., Moquet, F., Imbernon, M., Guedes-Lafargue, M.R., Mamoun, M., Olivier, J.-M. (1998). Evidence for PPC1, a determinant of the pilei-pellis color of *Agaricus bisporus* fruitbodies. *Fungal Genetics and Biology*, *23*(2), 181-188.

Costa, A.S.M.B., Thomas, D.T.I., Eastwood, D., Cutler, S.B., Bailey, A.M., Foster, G.D., Mills, P.R., Challen, M.P. (2009). Quantifiable downregulation of endogenous genes in *Agaricus bisporus* mediated by expression of RNA hairpins. *Journal of Microbiology and Biotechnology*, *19*(3), 271-276.

Culley, D., Yang, C.P., Dean, B., Brown, C. (2000). Characterization of the polyphenol oxidase gene family from the low browning Solanum species, *S. hjertingii*. *American Potato Journal Research*, *77*, 395-396.

Foulongne-Oriol, M., Dufourcq, R., Spataro, C., Devesse, C., Broly, A., Rodier, A., Savoie, J.-M. (2011). Comparative linkage mapping in the white button mushroom *Agaricus bisporus* provides foundation for breeding management. *Current Genetics*, *57*(1), 39-50.

Foulongne-Oriol, M., Rodier, A., Rousseau, T., Savoie, J.-M. (2012). Quantitative trait locus mapping of yield-related components and oligogenic control of the cap color of the button mushroom, *Agaricus bisporus*. *Applied and Environmental Microbiology*, *78*(7), 2422-2434.

Foulongne-Oriol, M., Rodier, A., Savoie, J. (2012). Relationship between yield components and partial resistance to *Lecanicillium fungicola* in the button mushroom, *Agaricus bisporus*, assessed by quantitative trait locus mapping. *Applied Environmental Microbiology*, *78*(7), 2435-2442.

Foulongne-Oriol, M., Spataro, C., Cathalot, V., Monllor, S., Savoie, J.-M. (2010). An expanded genetic linkage map of an intervarietal *Agaricus bisporus* var. *bisporus* x *A. bisporus* var. *burnettii* hybrid based on AFLP, SSR and CAPS markers sheds light on

the recombination behaviour of the species. *Fungal Genetics and Biology*, *47*(3), 226-236.

Fritsche G. (1986). Breeding mushrooms. *Mushroom Journal, 157*, 4-17.

Gao, W., Baars, J.J.P., Sonnenberg, A.S.M., Visser, R.G.F. (2011). Inheritance pattern of bruising sensitivity trait in *Agaricus bisporus*. *Proceedings of the 7th International Conference on Mushroom Biology and Mushroom Products,* INRA Bordeaux (pp. 43-51).

Jolivet, S., Arpin, N., Wichers, H.J., Pellon, G. (1998). *Agaricus bisporus* browning : a review. *Mycological Research*, *102*(12), 1459-1483.

Jolivet, S., Voiland, A., Pellon, G., Arpin, N. (1995). Main factors involved in the browning of *Agaricus bisporus. Mushroom Science*, *14*, 695-702.

Kerrigan, R.W., Royer, J.C., Baller, L.M., Kohli, Y., Horgen, P.A., Anderson, J.B. (1993). Meiotic behavior and linkage relationships in the secondarily homothallic fungus *Agaricus bisporus*. *Genetics*, *133*(2), 225-236.

Matoba, Y., Kumagai, T., Yamamoto, A., Yoshitsu, H., Sugiyama, M. (2006). Crystallographic evidence that the dinuclear copper center of tyrosinase is flexible during catalysis. *The Journal of Biological Chemistry*, *281*(13), 8981-8990.

Mikosch, T.S.P., Lavrijssen, B., Sonnenberg, A.S.M., van Griensven, L.J.L.D. (2001). Transformation of the cultivated mushroom *Agaricus bisporus* (Lange) using T-DNA from *Agrobacterium tumefaciens*. *Current Genetics*, *39*(1), 35-39.

Ministerie van Landbouw, N. en V. (2010). Sector Special Champignons. *Berichten Buitenland Special Champignons*, *36*(3), 1-29.

Moquet, F., Desmerger, C., Mamoun, M., Ramos-Guedes-Lafargue, M., Olivier, J.-M. (1999). A quantitative trait locus of *Agaricus bisporus* resistance to *Pseudomonas tolaasii* is closely linked to natural cap color. *Fungal Genetics and Biology*, *28*(1), 34-42.

Sim, S.K., Ohmann, S.M., Tong, C.B.S. (1997). Comparison of polyphenol ixidase in tubers of *Solanum tumberosum* and the non-browning tubers of *S. hjertingii*. *American Potato Journal*, *74*(1), 1-13.

Sonnenberg, A.S.M., Baars, J.J.P., Hendrickx, P.M., Lavrijssen, B., Gao, W., Weijn, A., Mes, J.J. (2011). Breeding and strain protection in the button mushroom Agaricus

bisporus. *Proceedings of the 7th International Conference on Mushroom Biology and Mushroom Products*, 7-15.

Urbany, C., Colby, T., Stich, B., Schmidt, L., Schmidt, J., Gebhardt, C. (2012). Analysis of natural variation of the potato tuber proteome reveals novel candidate genes for tuber bruising. *Journal of Proteome Research*, *11*(2), 703-16.

Weijn, A., Bastiaan-Net, S., Wichers, H.J., Mes, J.J. (2012a). Melanin biosynthesis pathway in *Agaricus bisporus* mushrooms. *Fungal Genetics and Biology*. doi:http://dx.doi.org/10.1016/j.fgb.2012.10.004

Weijn, A., Tomassen, M.M.M., Bastiaan-Net, S., Wigham, M.L.I., Boer, E.P.J., Hendrix, E.A.H.J., Baars, J.J.P., Sonnenberg, A.S.M., Wichers, H.J., Mes, J.J. (2012b). A new method to apply and quantify bruising sensitivity of button mushrooms. *LWT - Food Science and Technolgy*, *47*(2), 308-314.

Werij, J.S., Kloosterman, B., Celis-Gamboa, C., de Vos, C.H.R., America, T., Visser, R.G.F., Bachem, C.W.B. (2007). Unravelling enzymatic discoloration in potato through a combined approach of candidate genes, QTL, and expression analysis. *TAG. Theoretical and Applied Genetics. Theoretische und Angewandte Genetik*, *115*(2), 245-252.

Chapter 8

General Discussion

General Discussion

Contact-based discoloration, or bruising, is caused by a mechanical process known as 'slip-shear' (Burton, 2004), a downwards force and a sideways movement. This bruising based discoloration of mushrooms can occur during picking, handling, and storage and leads to lowering of quality. Due to production of mushrooms in European countries with lower labour costs the mushroom market in the Netherlands is challenged. Labour costs can be lowered by automatic harvesting of the mushrooms but the critical success factor is the availability of strains that are less sensitive for mechanical damage. Breeding for bruising-tolerant mushroom strains will be more efficient when the key components of bruising-discoloration are known. A start was made in this thesis by in-depth analysis of the melanin biosynthesis pathway.

1 Developed bruising device and method

To be able to analyse bruising-sensitivity of button mushrooms a new bruising device and method were developed (Chapter 2). The bruising method was optimised using a limited number of strains and with this method it was possible to distinguish between bruising-tolerant and bruising-sensitive strains. A larger collection of strains was analysed and a selection was used for further investigation of the bruising-tolerant trait. Bruising-sensitivity analysis was repeated for various *A. bisporus* strains and reproducible results were obtained (Chapter 3, 5 and 7). A high correlation was found between discolouration upon bruising by a conveyor belt and the bruising device (Weijn *et al.*, 2011). This showed that the developed bruising device induces a bruising-discoloration that is comparable with industrially used techniques.

2 Molecular and biochemical analysis of the bruising-tolerance trait

The research in this study was focused on the genes of the melanin pathway or genes closely related to the pathway and the phenolic compounds available in the skin tissue. Only the skin tissue of the mushrooms was analysed because this tissue shows fastest and most pronounced discoloration after bruising. Analyses were performed 60 minutes after applying the bruise. This time point was chosen because it allowed to distinguish bruising-tolerant and bruising-sensitive strains (Weijn *et al.*, 2012b). Another reason to analyse only one time point is the fact that the gene expression and phenolic compounds analysed should not be influenced by post-harvest senescence of the mushroom but should be solely based on bruising-discoloration. A low correlation was found between discoloration upon storage of undamaged mushrooms and discoloration initiated with the bruising-device (Weijn *et al.*, 2011). Possibly different mechanisms are involved in storage or bruising-related discoloration and, in addition, the loss of water, membrane damage and enzyme activation during storage might cause a different response. Long term storage should be included during the analysis of advanced breeding stock to ascertain that new hybrids are tolerant for both bruising and storage-discoloration. As different mechanisms might be involved it is not known at the moment whether either the DOPA- or catechol-melanin pathways are more abundant during storage. Therefore strains with low levels of GHB and GDHB might still discolour during storage which is less appealing for consumers.

2.1 Genetic analysis of the bruising-tolerant trait

At the start of this research only a few genes coding for the enzymes of the melanin pathway were known (Tsuji *et al.*, 1996; Wichers *et al.*, 2003; Wu *et al.*, 2010). With the availability of the genomic sequence of *A. bisporus* (Morin *et al.*, 2012) it was possible to identify 30 genes that are involved or related to the melanin pathway (Weijn *et al.*, 2012a, Figures at the end of this chapter). Only

small differences were found for the expression of the genes analysed in the skin tissue of bruising-tolerant and sensitive strains (Chapter 6, Figures at the end of this chapter). Analysis of the offspring from a segregating population based on a cross between two tolerant strains showed a possible involvement of the *L-chain*, *PPO_3*, and *PPO_5* genes in the bruising-tolerance (Chapter 7). These three genes are found on a locus on the lower end of chromosome 5 which also includes the AT_3 gene (this locus will be discussed in more detail later on in this chapter). Probably differences in gene expression are already established during the development of the mushroom as phenolic compounds have already been formed during the development. Therefore, additional analysis of gene expression should be performed on earlier developmental stages, which might result in more abundant differences and was so far not included in this research. Time series might be included to analyse the long term effect of bruising on gene expression. Differences in gene expression between strains might be diminished through the thickness of the peeled skin tissue. A difference between strains was observed in the convenience of peeling off the skin tissue. This resulted in skin tissue samples that contained some underlying flesh for mushroom which were less easy to peel. This might hamper gene expression analysis as the response of the flesh tissue on bruising not necessarily is the same as the response of the skin tissue. Another explanation for low gene expression levels is that the proteins are not formed in the skin tissue but in another part of the fruiting body or in the mycelium. Woolston *et al.* (2011) showed long-distance translocation of the β-glucuronidase protein from the compost-borne mycelium into the developing fruiting body, a finding which strongly suggests that transport of native proteins is also possible.

Transcription factors are sequence-specific DNA-binding proteins that interact with the promoter regions of target genes and modulate the rate of initiation of transcription (Petersen, 2007). Genes that are regulated co-ordinately often have similar binding sites in their promoters. In plants, genes from the shikimate

pathway, aromatic amino acid biosynthesis and phenylpropanoids are regulated by MYB-like and basic-loop-helix (bHLH) transcription factors (Tzin *et al.*, 2012). *AtMYB15* is an activator gene of the shikimate pathway in *Arabidopsis* and is a wound-inducible gene that responds earlier than *PAL* in *Arabidopsis*. Overexpression of *AtMYB15* in transgenic plants resulted in increased expression of almost all the genes involved in the shikimate pathway. These genes were also found to be induced by wounding (Chen *et al.*, 2006). Similar DNA binding sites were found in the promoter regions of the PPOs and the L-chain (Li *et al.*, 2011; unpublished data from Weijn *et al.*). In the current study only one putative transcription factor, PHRB was studied. It will be interesting to analyse more transcription factors and study their involved in the melanin biosynthesis pathway. Perhaps, as found in *Arabidopsis*, one single transcription factor might regulate the shikimate pathway in button mushrooms.

Another strategy is to analyse the position of the nuclei in *A. bisporus*. Analysis of the positioning of nuclei in the dikaryon of *Schizophyllum commune* showed that there is a relation between the distance of nuclei and differential gene expression (Schuurs *et al.*, 1998). It might be possible that this phenomenon also occurs between the different homokaryons of *A. bisporus* and has an influence on the regulation of important key genes of the bruising-discoloration trait.

It was decided to only analyse gene expression based on the knowledge of the melanin pathway at the start of this research. With the diverse bruising-tolerant strains that are now available at the end of the research, deep RNA sequencing might be used to further analyse the trait and identify additional new genes. With gene expression all identified homologs of a certain gene can be analysed. Unfortunately, the correlation between gene expression and enzyme activity is unknown. Enzyme activity was not analysed because activity can not be measured for individual enzymes without being able to discriminate between specific isoforms, e.g. with immunochemical methods. The PPO_1 and PPO_2 genes were

cloned and expressed in *E. coli* as (respectively GSH- and His-tagged) fusion proteins. ~64 kDa proteins were obtained but enzyme activity was not described (Wichers *et al.*, 2003). Recently, PPO_2 was cloned in *E. coli* by Ismaya *et al.* (2011) and a very low enzyme activity was observed. Wu *et al.* (2010) analysed the expression of PPO_3 and PPO_4 in *E. coli* and did obtain expressed proteins that, however, did not show activity. They suggested that a eukaryotic expression system should be used to obtain active PPO, as in a prokaryotic expression system not all modifications, in particular post-translational modifications, can take place that are required to produce fully functional eukaryotic proteins. This might explain why the recombinant protein did not form an active center. Lezzi *et al.* (2012) heterologously expressed *PPO_2* in *Saccharomyces cerevisiae* and indeed an active enzyme was obtained. We tried to express *PPO_5* and the *L-chain* both in *E. coli* and *Pichia pastoris*. For *P. pastoris* two different vectors were used to express the genes and subsequently the proteins were obtained from the cells or were targeted to the medium. Only small amounts of protein were obtained which lacked activity. The exact reason why we did not obtain an active enzyme is not known. Perhaps targeting to the medium results in attachment of PPO to the membrane. Another reason might be that too mild techniques were used to lyse the cells to obtain the proteins from the *P. pastoris*. Lezzi *et al.* used a French press to lyse the *S. cerevisiae* cells and were able to obtain an active enzyme. It seems worthwhile to apply the same expression system for *PPO_3* or *PPO_5* in an attempt to produce also active enzymes with these PPO genes. A good alternative for cloning and expression of an enzyme is the transformation of genes of interest into *A. bisporus* itself and initiate overexpression or silencing of the gene. The transformation of *A. bisporus* has been performed before (Mikosch *et al.*, 2001; Stoop and Mooibroek, 1999), but is still under development.

2.2 Phenolic compounds

The main phenolic precursors of melanin in *A. bisporus* are L-tyrosine, L-DOPA, GHB and GDHB (Jolivet *et al.*, 1995). L-Tyrosine and L-DOPA on the one hand, and GHB and GDHB on the other, lead to the formation of DOPA-melanin and GHB-melanin, respectively. It is not possible to analyse the melanin structure due to insolubility of melanin (Eisenman and Casadevall, 2012) and therefore the precursors were analysed and quantified. A high correlation was found between the concentration of the phenolic substrates GHB and GDHB in the skin tissue and the bruising-sensitivity of the strains (Chapter 5), which means that a higher concentration was found in both white and brown sensitive strains compared with tolerant strains (Figures at the end of this chapter). The involvement of GHB and GDHB in bruising-sensitivity indicated that the GHB-melanin pathway is mainly involved in bruising-discoloration as was found before (Hanotel, 1994; Jolivet *et al.*, 1995; Pierce and Rast, 1995). Agaritine is an aromatic compound characteristic for *A. bisporus* containing a γ-glutamyl group which can be removed in a reaction catalysed by GGT (Walton *et al.*, 2001). Baumgartner *et al.* (1998) showed that agaritine is not formed via the shikimate pathway and was synthesized in the vegetative mycelium and proposed that via translocation the compound is available in the fruiting body. The relation between agaritine and the melanin pathway is not known and therefore the involvement of agaritine in the bruising-discoloration is uncertain. However, a correlation was found in the analysed population between a higher agaritine content and bruising-sensitivity. Therefore it is proposed that there is a direct link between *p*-aminophenol and agaritine, possibly the γ-glutamyl group of agaritine can be transferred to *p*-aminophenol resulting in the formation of GHB.

The research in this thesis was focused on the skin tissue of the mushroom, whereas in most other research the phenolic compounds in the whole mushroom are analysed. Therefore it was difficult to identify unknown compounds as some phenolic compounds might be skin specific and have not been identified before in

literature. Phenolic compounds in the button mushroom are mainly analysed for their involvement in either discoloration or in the antioxidant properties of the phenolic compounds (Robards *et al.*, 1999). It is proposed to make a general database for fungi in which quantified and identified data of metabolites from literature is combined including molecular weight, modifications, UV/Vis data and other obtained results.

2.3 Mutations in gene sequences

Based on full genomic sequence alignments, naturally occurring mutations were identified in the coding regions of the genes involved and related to the melanin biosynthesis pathway. In total four mutations that can have an effect on the function of the enzyme were identified in the parental line WB2A of a bruising-tolerant wild brown strain. The mutations comprise a modification at the start in *GGT_1*, an elongation of 15 nucleotides at the C-terminus of *AT_3* and a nucleotide mutation resulting in a premature stop codon in both *AT_1* and *PPO_3* leading to truncated proteins. Mutations were confirmed with RT-qPCR and were specific for the sequenced wild brown bruising-tolerant strain. These mutations were also analysed in the offspring of a segregating population, which was based on homokaryons obtained from a cross between WB2AxH97. This showed that the mutations were not specific for bruising-tolerant lines, but indicated that a combination of mutations and low availability of phenolic compounds is needed to obtain a high bruising-tolerance trait. This was shown for the offspring lines 30, 123, and 141 which obtained the four mutations identified and were classified as bruising-tolerant (Chapter 7).

Mutation breeding can be used to analyse the effect of randomly induced mutations on a specific trait of interest and might be combined with naturally occurring mutations. TILLING or sequencing of PCR products might be used to analyse preferred mutations in hybrids. However, button mushrooms have a low

recombination frequency (Sonnenberg *et al.*, 2011) and next to that potentially unwanted negative mutations might be coupled which are difficult to select against. Transposon mutagenesis was used in *Marinomonas mediterranea*, a Gram-negative marine bacterium, and resulted in a mutant affected in the regulation of PPO activity and melanin formation (Lucas-Elío *et al.*, 2002). The disrupted gene was sequenced and identified as a sensor histidine kinase belonging to a two-component regulatory system. These systems control many different cellular processes and have been described in prokaryotic micro-organisms and in some eukaryotic organisms (Lucas-Elío *et al.*, 2002). Recently Lavín *et al.* (2012) identified eight genes coding for two-component systems in the genome of *A. bisporus* (H97), which included four hybrid histidine kinases, a single histidine-containing phototransfer protein and three response regulators. The genes are spread over the genome and not found in the cluster between *PPO_3* and *PPO_5* but might be interested to study in relation to bruising-sensitivity.

2.4 Polyphenol oxidase, the main enzyme of the melanin biosynthesis pathway

PPO is the main enzyme involved in the melanin biosynthesis pathway and a gene family of six genes and a gene coding for an associated peptide (L-chain) were identified in *A. bisporus*. Based on the gene expression, involvement of *PPO_1* in bruising-discoloration can probably be excluded because it is not induced by bruising (Chapter 6 and 7) and expression is low in different mushroom tissues during development (Chapter 4). *PPO_2* expression, upon bruising, increased in sensitive white strains and was identified to be skin specific and therefore might be relevant in bruising-discoloration of the skin. *PPO_3* is expressed in all tissues during mushroom development, except in the gills. Although a mutation was identified for *PPO_3* in one of the parental lines of WB2 also a high expression level of this gene was found, because the mutation is only effective on the protein level. In addition, inheritance of the mutated *PPO_3* (or

linked *AT_3*) resulted in higher bruising-sensitivity in the analysed offspring of the segregating population. Therefore, it might be that *PPO_3* is involved in the pathway but leads to melanins that have a less abundant colour. *PPO_4* was the highest expressed of the six PPO genes in the gills and therefore it is hypothesized that *PPO_4* is involved in the discoloration of the spores and lamellae, as expression upon bruising did not correlate with bruising-sensitivity. *PPO_5* expression was comparable with the expression of *PPO_3* and the *L-chain* in the analysed offspring (Chapter 7). *PPO_5* is therefore probably involved in bruising-discoloration. The involvement of *PPO_6* in brown discoloration of the skin is less easy to explain. *PPO_6* was identified to be more specific for the skin than for other mushroom tissues (Chapter 4), but expression in non-bruised and bruised skin tissue did not correlate with the bruising-sensitivity of the strains analysed (Chapter 6 and 7). Perhaps *PPO_6* is involved in the natural cap colour of button mushrooms as Callac *et al.* (1998) showed that the white cap colour was determined by a recessive allele at a single locus on chromosome 8. *PPO_6* was also identified on chromosome 8 (Chapter 4). The cap colour is visible during the maturation of the mushroom. It is therefore possible to analyse the involvement of *PPO_6* through comparison of mushrooms from different developmental stages from strains with a different cap colour. The sequence of *PPO_6* is most deviant from the other PPO sequences as obtained from H97, it is the largest of the six PPOs and contains two starting codons and it was initially annotated as two different tyrosinases (Chapter 4). Sequence comparison of *PPO_6* of white and brown strains did not reveal obvious differences, which were postulated to have an effect on the function of the PPO_6 protein. However, it can not be excluded that small changes, alternative splicing of *PPO_6* or other changes of *PPO_6* have a relation with cap colour.

The function of the L-chain is until now unknown, but from the crystal structure of *A. bisporus* PPO it is apparent that the L-chain is positioned too far

away from the active site of PPO_3 to have the same function as the caddie protein from *Streptomyces*. The latter, is postulated to serve as a chaperone to insert the cupric ions into tyrosinase (Matoba *et al.*, 2006). It can be hypothesized that the L-chain functions as an (allosteric) inhibitor of PPO limiting the accessibility of the active site to compounds or preventing the enzyme from becoming active. Another hypothesis might be that the L-chain is involved in the functioning of PPO but has a specific affinity for certain PPO homologs. The function of the L-chain can be studied more in-depth using overexpression, gene silencing or knock out lines. If this L-chain is indeed involved in regulating PPO-activity, it may be an appealing target in breeding programs and based on the correlation between a higher expression of the *L-chain* and bruising-tolerance in the analysed offspring (Chapter 7) overexpression of the *L-chain* might lead to an increase in tolerance.

3 Location and function of melanin

In plants it is known that phenolic compounds are present in vacuoles and the PPO enzyme is located in the plastids (chloroplasts and amyloplasts) (Mcgarry *et al.*, 1996; Urbany *et al.*, 2011). Cell disintegration caused by external damage results in the release of PPO from its subcellular compartment and PPO can come into contact with its phenolic substrates (Stevens and Davelaar, 1997). In several fungal species internal melanosomes, vesicles containing melanin, are observed. In *Cryptococcus neoformans* laccase containing vesicles are synthesized intracellularly and transported to the cell wall (Figure 1, (Eisenman and Casadevall, 2012)). At the cell wall the vesicles interact with phenolic substrates, such as L-DOPA, to produce melanin granules. Cell wall polysaccharides such as chitin serve as a scaffold to which the melanin granules are cross-linked.

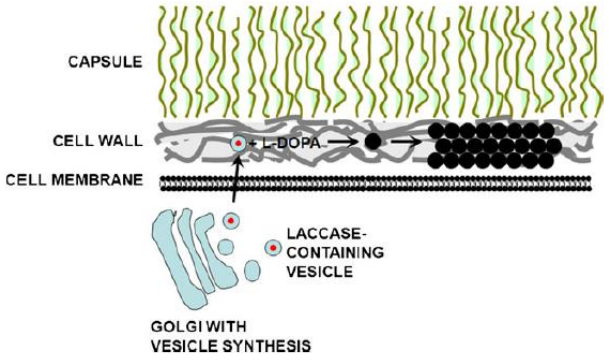


Figure 1. Model depicting melanin formation in *Cryptococcus neoformans.* Retrieved from Eisenman and Casadevall (2012).

The location of the phenolic substrates and PPO in button mushrooms is so far not determined exactly. Melanin is found in the cell walls of *A. bisporus* and indications are found that next to soluble and membrane-bound isoforms of PPO also cell wall-bound isoforms of PPO exist (Sassoon and Mooibroek, 2001). Rast *et al.* (2003) performed the same kind of research and also found that PPO is both a soluble and a cell wall-associated enzyme. In this thesis it was identified that the PPO gene family consist of six members and an associated peptide, the L-chain. Expression differences of PPO were found in different tissues of the sporophore and in different developmental stages, but exact locations of the individual enzymes are unknown (Chapter 4). An approach to analyse the PPO locations in the sporophore is to use green fluorescent protein linked to each individual gene combined with microscopic analysis during the development of the mushroom or histochemical analysis with antibodies against PPO and the L-chain.

In an attempt to identify other genes related to the bruising-discoloration the genes clustering around and in between *PPO_3* and *PPO_5* on chromosome 5 were analysed. This resulted in the identification of genes containing conserved NACHT and WD40 domains or a P-loop nucleoside triphosphate hydrolase, which are domains involved in diverse cellular functions. Next to that vacuole-related genes

were identified. These suggested gene functions support the hypothesis that melanin formation might be a consequence of a release of mono-phenolic compounds from the vacuole, perhaps via dedicated membrane channels. Mechanosensitive ion channels open in response to physical stimuli that affect the membrane (Kumamoto, 2009). The changes in ion flux that result from channel opening cause changes in cellular physiology and a biological response is initiated as a result of a mechanical stimulus. In general not much is known yet about mechanosensing in plants. Despite evidence for a central role of Ca^{2+}-dependent mechanical signalling in plants, there are no molecularly identified mechanoresponsive receptors or channels known in plants (Monshausen and Gilroy, 2009). Kinases might act as mechanosensors, as an *Arabidopsis* plasma-membrane-localized histidine kinase can complement yeast lines containing a deletion in their own putative osmosensing histidine kinase (Urao *et al.*, 1999). The cell-wall integrity pathway of *Saccharomyces cerevisiae* is a MAP kinase signal-transduction pathway that is activated when the fungal cells experience cell-wall or plasma-membrane damage (Kumamoto, 2009). Perhaps a mechanosensing pathway is involved in the button mushroom as a response to wounding and genes coding for this are located together with the PPOs on this cluster. More in-depth analysis of this whole cluster should be performed to identify the functions of the genes and their relation to the bruising-tolerance trait.

Fungi can produce appressoria; structures that penetrate plant tissue, allowing the organism to invade the host. Melanin in the cell wall of these structures provides mechanical strength to the appressoria that aids in tissue penetration (Eisenman and Casadevall, 2012). Based on this principal, melanin formation might be expected in mycelium or only in the tip of the mycelium that penetrates through the compost. This hypothetical function for melanin is sustained based on the high expression level of the *L-chain* and *PPO_5* in the mycelium of Horst U1 (Chapter 4).

Melanin functions as a defence system in plants and fungi and is important to confer resistance to microbial attack (Bell and Wheeler, 1986). *PPO_2* expression increased in mushrooms infected with *Lecanicillium fungicola* and also upon infection with *Pseudomonas tolaassi* (Largeteau *et al.*, 2010; Soler-Rivas *et al.*, 2001). Thipyapong *et al.* (2004) achieved antisense down-regulation of PPO expression in tomato. In this way all members of the tomato PPO gene family were down-regulated. Growth, development, or reproduction of the plant were not affected. However, transformants were hypersensitive to pathogen infections. Together this indicates the important role of PPO in the formation of melanin during pathogen defence. Therefore it is possible that plants engineered to minimize PPO-mediated enzymatic browning may require additional input to compensate for increased pathogen sensitivity. Okanagan Specialty Fruits Inc. (http://www.okspecialtyfruits.com/) has developed non-browning apples, termed Arctic apples. They claim that a targeted and specific gene modification is used that silences the PPO enzyme. Gene silencing was performed with genes isolated from apples and resulted in apples that produce less than 10 % of the PPO amount that is produced in the original cultivars. Brown discoloration was not observed anymore in apples with this low level of PPO enzyme. It is stated that the low level of PPO enzyme does not change any other aspect of the cultivar. Therefore, a low level of PPO enzyme might be needed to prevent hypersensitivity for pathogens. The sensitivity for infection with *L. fungicola* or *P. tolaasii* of the bruising-tolerant strains was not tested in this thesis. This should be performed with the segregating population as bruising-tolerant strains are developed for the mushroom market and therefore pathogen sensitivity should be at least the same as the currently used strains and preferably better.

4 Analysis of samples obtained during different growing experiments

It is known that mushroom properties vary from batch to batch depending on the production technique, variety and season (Mohapatra *et al.*, 2008). This in turn affects the enzyme levels as the mushroom physiological state varies from batch to batch, lot to lot and flush to flush. This is important to keep in mind when comparing research results from different studies. The discrimination of strains into tolerant and sensitive was reproducible during different growing experiments. A comparison was made between a Horst U1 skin sample from 2010 and 2012 for the phenolic compounds and the gene expression in the skin tissue. In general similar peaks and peak areas were observed for the available phenolics of both the non-bruised and the bruised skin tissue. Gene expression analysis showed slight differences between the 2010 and 2012 sample. The slight differences in both phenolic compounds and gene expression did not lead to other conclusions on the relation between these parameters and bruising-sensitivity. All though care was taken that mushrooms were picked form the same developmental stage and treated the same way after picking until sample collection, small differences can be caused by several factors. One such factor is that the mushrooms were not picked on exactly the same day after the start of the flush. It is known that both substrate content and enzyme activity change during development (Robards *et al.*, 1999). Both growing experiments were performed in the same way; the only difference was the size of the boxes that were used for cultivation of the mushrooms. Another factor that might have an influence on observed differences is the composition of the compost used. The amount of available nutrients in the compost can differ from batch to batch and has an influence on bruising-discoloration (Burton, 2004). The amount of copper sulphate in the compost is claimed to affect the content of GHB and PPO activity in the mushroom (Mamoun *et al.*, 1999). The total amount of mushrooms on the bed might be relevant for the final amount of available metabolites per individual mushroom. Experiments should be conducted on the

same samples to ascertain comparability, or specific strains should be included in different experiments as a kind of internal standard. The analyses described in Chapter 5 and 6 were performed on exactly the same samples. Differential lines from the segregating population were analysed and several reference strains were included.

5 Future research

Combined proteomic and genomic analyses are suitable techniques to further investigate the key factors involved in bruising-discoloration. Urbany *et al.* (2012) used a proteomics approach to analyse the natural variation between potato cultivars with different bruising-sensitivity. They showed that seven genes or gene families were differentially expressed on the protein level. As a novel factor contributing to the natural variation of bruising, a putative class III lipase was identified, which hydrolyses ester linkages of triglycerides and is involved in lipid metabolic processes (http://www.ebi.ac.uk/interpro/Entry?ac=IPR002921). Urbany *et al.* (2012) also analysed the changes of the tuber proteome upon mechanical impact of potato cultivars with low and high bruising sensitivity. Differences were found in the expression of lipases (putatively involved in cell shape regulation and lipid signalling), patatins (perhaps involved in membrane modification) and annexins (probably in mediating Ca^{2+}-dependent events involving interactions of the cytoskeleton and cellular membranes). The sequenced genomes of *A. bisporus* strains can be used to identify naturally occurring mutations in the button mushroom for these enzymes. As these proteins react to mechanical damage they can also be important key components in mushrooms and show similarities with the factors involved in mechanosensing (as discussed above). An untargeted proteomics approach might be used to analyse the melanin pathway more in-depth, to identify candidate proteins and genes involved in the bruising trait outside the

melanin biosynthesis pathway. In particular for the mutations identified in *GGT_1* and *AT_3* a proteomic analysis might shed light on the formed enzymes.

Deep sequencing can be used to simultaneously obtain information about mutations and the expression of RNA sequences which might be used to analyse the natural variation amongst strains with different bruising-sensitivity. These results can be used to perform quantitative trait locus (QTL) analysis on the expression level and can be combined with QTL analysis based on bruising-sensitivity (Wei Gao *et al.*, personal communication). Expression QTL analysis can also be performed on the whole segregating population for the genes involved in the melanin pathway. Analysis of a selection of the offspring indicated gene expression differences between tolerant and sensitive lines for the *L-chain*, *PPO_3*, and *PPO_5*. Natural antisense transcripts (NATs) are endogenous RNA molecules with regions complementary to a sense transcript. When the gene involved is protein coding, the sense transcript is the mRNA (Donaldson and Saville, 2012). The shs13 gene was identified as a NAT in *A. bisporus*, but it is not known in which mechanism this NAT is involved (http://getentry.ddbj.nig. ac.jp/getentry/na/AJ565923). Naturally occurring antisense transcripts might be involved in the high bruising-tolerant trait of WB2 and sequencing of both homokaryons might aid in the analysis of NATs in WB2 and their functions.

Antisense and sense technology can be used to establish conclusive roles of enzymes involved in the bruising-tolerance trait. Functional analysis of individual gene members whose sequences are highly similar is a more challenging task and might require additional technology such as RNAi for inhibitions of specific genes or a knock-out strategy can be used (Thipyapong *et al.*, 2007). Especially the physiological function of the L-chain and its role in the functioning of PPO might be investigated with these techniques. Crop improvement via genetic engineering is a subject of public concern that has led to the development of evaluation steps before a genetic engineered crop can be regarded as safe (Llorente *et al.*, 2011).

Llorente *et al.* engineered potato plants to down-regulate the expression of PPO transcripts. Three lines with reduced PPO activity and diminished tuber browning were analysed according to the safety evaluation steps. They showed that differences between genetically engineered potato lines and the wild type plants were minimal and no adverse effects were found in physiological parameters analysed during *in vivo* intake by mice.

In general, in breeding programs lines with the highest difference for a specific trait are combined to analyse inheritance. In this thesis a segregating population was developed based on a cross between two tolerant strains (WB2AxH97) to obtain stacking of bruising-tolerance and possibly increase bruising-tolerance. Analysis of the bruising-sensitivity showed that not many lines were obtained with a higher bruising tolerance than WB2, which is already at the extreme end of tolerance. Therefore this bruising-tolerant background should be combined to improve the overall quality such as good production, firm mushrooms, absence of cap scaling, and of early senescence. The homokaryons obtained from WB2AxH97 were also crossed with Z6, homokaryon of the wild white strain 7 (WW7), which was found to be recessive for bruising sensitivity (Gao *et al.*, 2011). Another population was based on a cross between homokaryons obtained from H39xZ8 which were crossed with H97. Z8 is the other homokaryon of WW7, which was found to be dominant for bruising sensitivity (Gao *et al.*, 2011). The offspring of these populations were analysed for bruising-sensitivity and used for QTL analysis which will be discussed elsewhere (Wei Gao *et al.*, in preparation). Unfortunately, these populations were not analysed with the molecular and biochemical analyses described in this thesis, but are interesting lines to further analyse the difference between bruising-tolerant and sensitive strains. The cross based on homokaryons obtained from WB2AxH97 which were crossed with H39 resulted for most of the analysed offspring in a low content of the main phenolics GHB and GDHB (Chapter 7), which is desirable because GHB-melanin is the main melanin pathway

involved in discoloration (Jolivet *et al.*, 1995, Chapter 5). One homokaryon (WB2A) of WB2 already showed a high bruising-tolerance inheritance. The inheritance trait of the other homokaryon (WB2B) should be analysed and studied for natural mutations. A combination of WB2B with chromosome 4 of WB2A (containing a mutated *GGT_1*) together with chromosome 5 of H97 (correlating with a high gene expression of the *L-chain*, *PPO_3*, and *PPO_5* in tolerant offspring) might be used to obtain a high tolerant trait. Breeding with chromosomal substitution lines can be used to obtain the preferred genetic background and can include the desired chromosome 8 to obtain the white cap colour (Foulongne-Oriol *et al.*, 2012). All together this will result, within a few years, in the availability of a new hybrid strain with a high bruising-tolerance for the commercial mushroom market.

Legend for Figures 2, 3 and 4 (on the next pages).

In Figures 2, 3, and 4 an overview is given of the obtained results from Chapter 5 and 6. This was performed to show the differences between bruising-sensitive and bruising-tolerant strains in a pathway overview. The figures were obtained with the program MapMan (version 3.5.1R2, Thimm *et al.*, 2004).

In each melanin pathway overview figure the gene expression and phenolic compounds were included of a specific strain of a non-bruised sample (the data is used from Chapter 5 and 6). Gene expression is shown in squares and each homolog is indicated with a square. The gene expression was log2 transformed, low expression is indicated in green (-5) and high expression in red (5).The phenolic compounds are shown in circles. The data of the phenolics, obtained as peak area per gram fresh weight, were normalized based on the standard score ((obtained value - mean of all samples)/ standard deviation of all samples). Low peak area is indicated in green (-1) and high peak area in red (1). In Figure 3 are the mutations found in WB2A (homokaryon of WB2) indicated with a black diamond. Enzymes involved in the pathway are indicated by Roman symbols. I = chorismate mutase, II = 4-aminobenzoate synthase, III = 4-aminobenzoate hydroxylase, IV = γ-glutamyltransferase, V = prephenate dehydratase, VI = prephenate dehydrogenase, VII / VIII = (4-hydroxy) phenylpyruvate aminotransferase, IX = polyphenol oxidase, X = phenylalanine ammonia-lyase, XI = trans-cinnamate-4-monooxygenase, XII = 4-coumarate CoA ligase. GHB = γ-L-glutaminyl-4-hydroxybenzene. GDHB = γ-L-glutaminyl-3,4-dihydroxybenzene. GBQ = γ-L-glutaminyl-3,4-benzo-quinone.

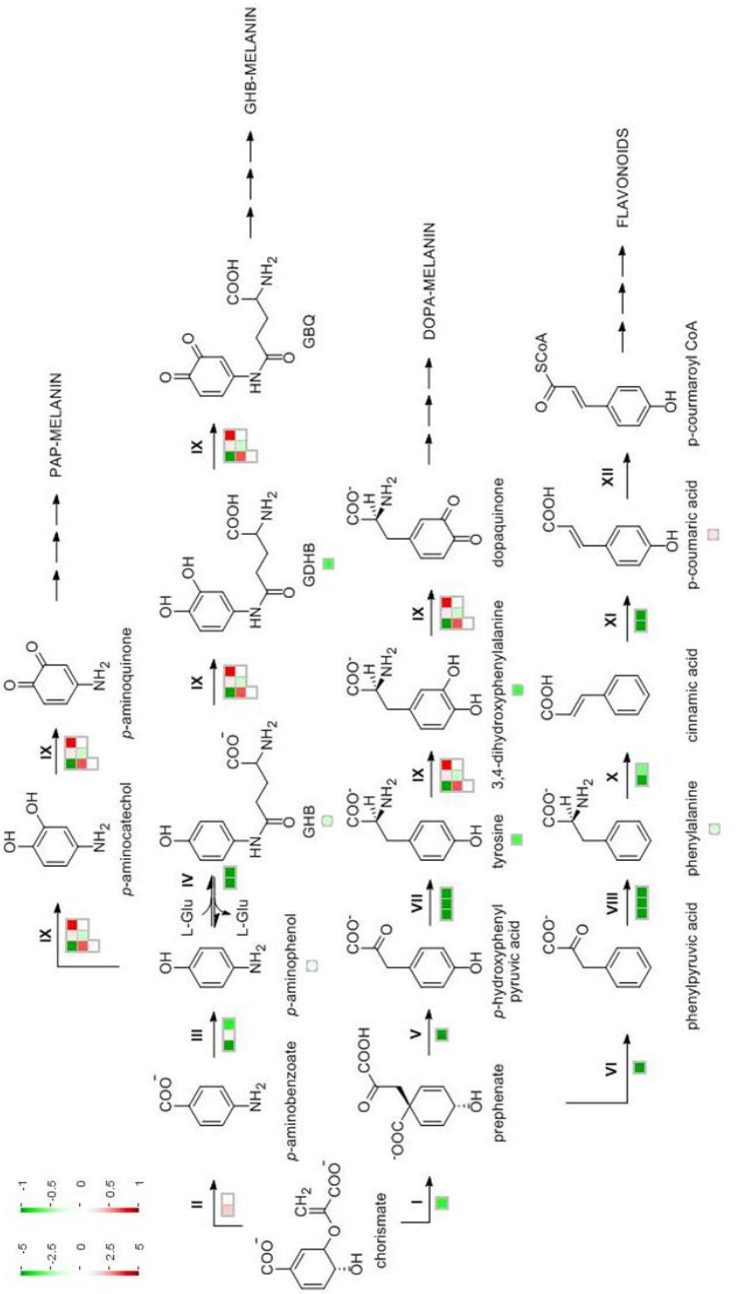


Figure 2. Overview of the melanin biosynthesis pathway for commercial hybrid 2 (CH2), a bruising-tolerant strain.

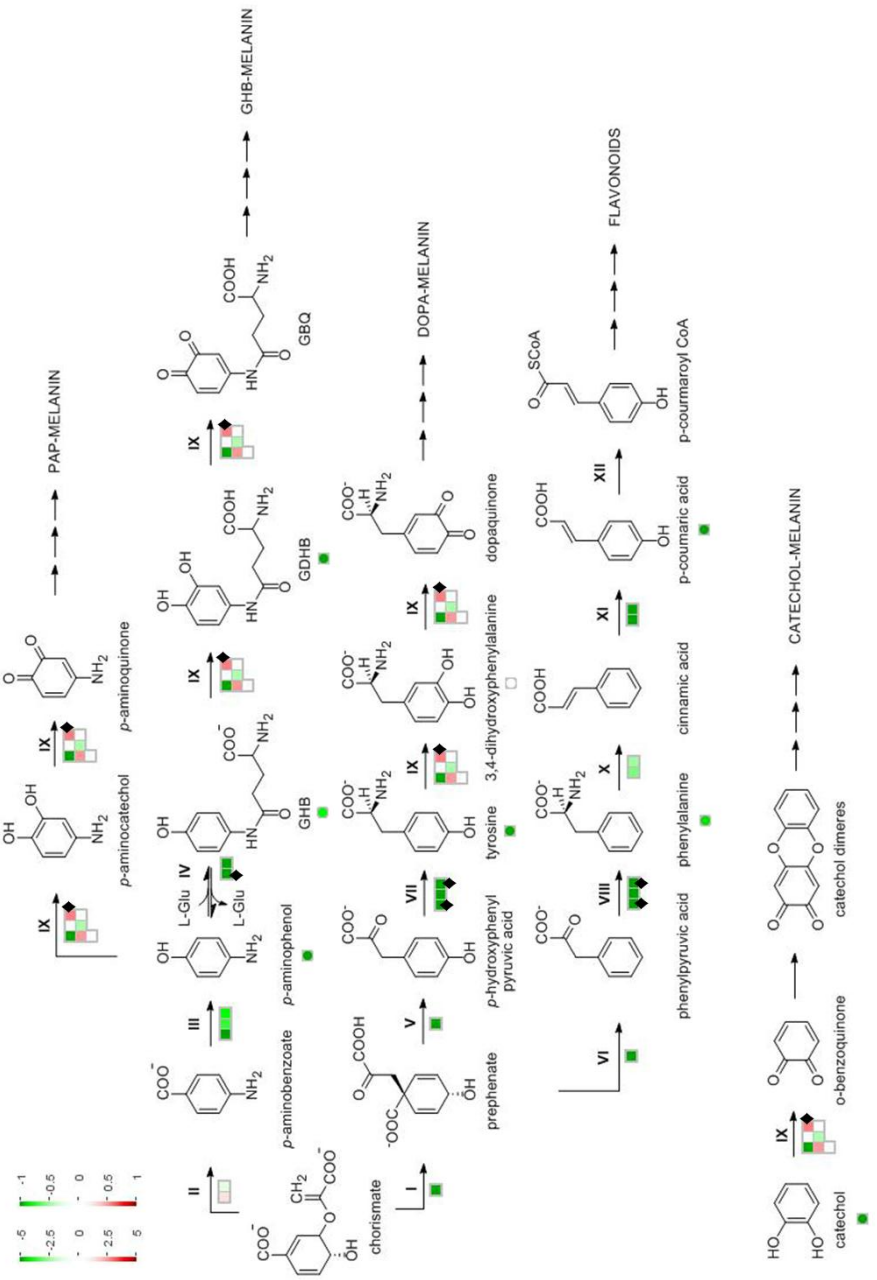


Figure 3. Overview of the melanin biosynthesis pathway for wild brown strain 2 (WB2), a high bruising-tolerant strain.

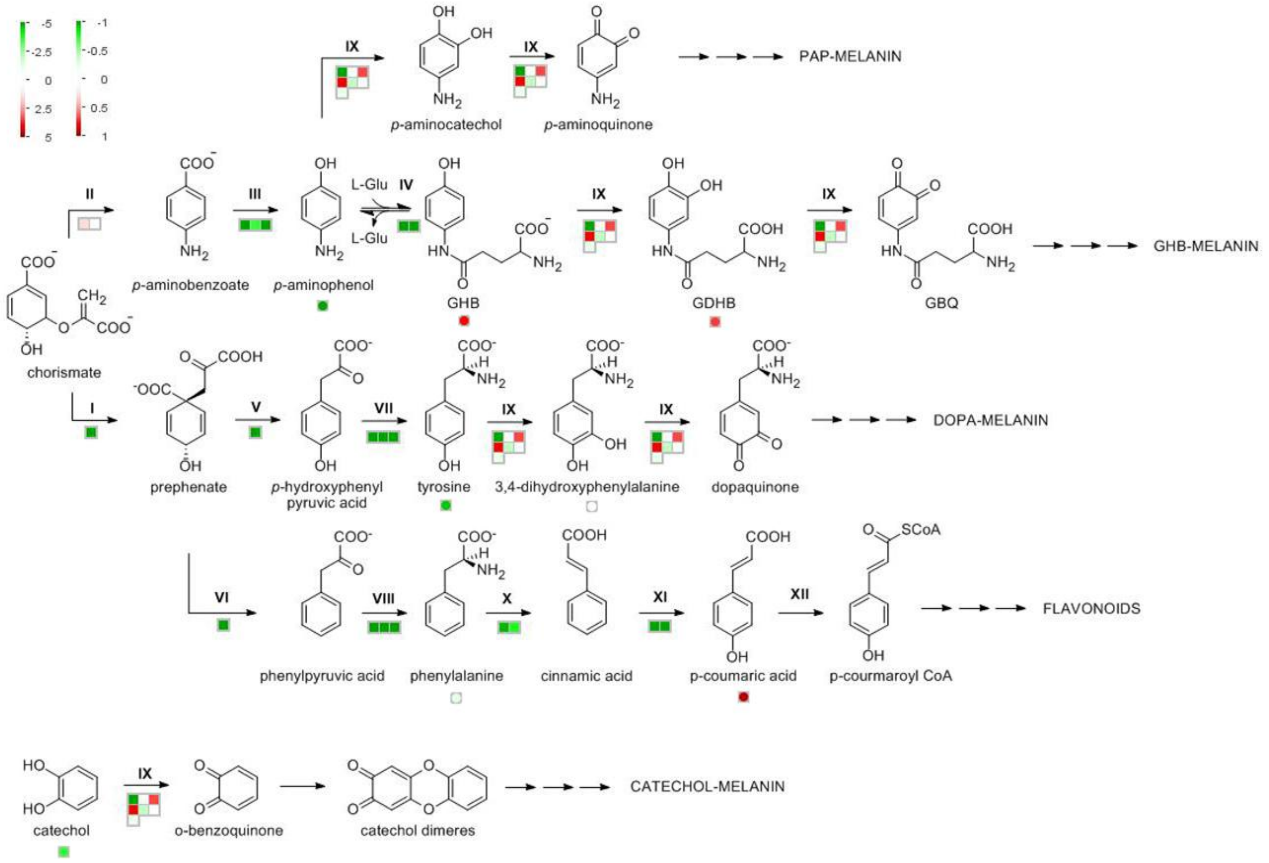


Figure 4. Overview of the melanin biosynthesis pathway for traditional white strain 4 (TW4), a bruising-sensitive strain.

Chapter 8

References

Baumgartner, D., Hoesch, L., Rast, D.M. (1998). The biogenesis of beta-N-(gamma-glutamyl)-4-hydroxymethylphenylhydrazine (agaritine) in *Agaricus bisporus*. *Phytochemistry*, *49*(2), 465-474.

Bell, A.A., Wheeler, M.H. (1986). Biosynthesis and functions of fungal melanins. *Annual Reviews Phytopathology*, *24*, 411-451.

Burton, K.S. (2004). Cultural factors affecting mushroom quality - cause and control of bruising. *Proceedings of the XVIth International Congress of Science and Cultivation of Edible and Medicinal Fungi* (pp. 397-402). Maimi.

Callac, P., Moquet, F., Imbernon, M., Guedes-Lafargue, M.R., Mamoun, M., Olivier, J.-M. (1998). Evidence for PPC1, a determinant of the pilei-pellis color of *Agaricus bisporus* fruitbodies. *Fungal Genetics and Biology*, *23*(2), 181-188.

Chen, Y., Zhang, X., Wu, W., Chen, Z., Gu, H., Qu, L.-J. (2006). Overexpression of the wounding-responsive gene *AtMYB15* activates the shikimate pathway in *Arabidopsis*. *Journal of Integrative Plant Biology*, *48*(9), 1084-1095.

Donaldson, M.E., Saville, B.J. (2012). Natural antisense transcripts in fungi. *Molecular Microbiology*, *85*(3), 405-417.

Eisenman, H.C., Casadevall, A. (2012). Synthesis and assembly of fungal melanin. *Applied Microbiology and Biotechnology*, *93*(3), 931-940.

Foulongne-Oriol, M., Rodier, A., Rousseau, T., Savoie, J.-M. (2012). Quantitative trait locus mapping of yield-related components and oligogenic control of the cap color of the button mushroom, *Agaricus bisporus*. *Applied and Environmental Microbiology*, *78*(7), 2422-2434.

Gao, W., Baars, J.J.P., Sonnenberg, A.S.M., Visser, R.G.F. (2011). Inheritance pattern of bruising sensitivity in *Agaricus bisporus*. *Proceedings of the 7th International Conference on Mushroom Biology and Mushroom Products*, INRA Bordeaux (pp. 43-51).

Hanotel, L. (1994). Effets de l'ionisation sur le bruissement enzymatique de végétaux frais prédécoupés. Cas de l'endive (*Cichorium intybus* L.) et du champignon de Paris (*Agaricus bisporus* (Lange) Imbach). PhD thesis, Université de Montpellier.

Ismaya, W.T., Boeria, C.G., Wichers, H.J., Dijkstra, B.W. (2011). Overproduction, purification and refolding of recombinant mushroom tyrosinase. PhD thesis, University of Groningen (pp. 25-40).

Jolivet, S., Voiland, A., Pellon, G., Arpin, N. (1995). Main factors involved in the browning of *Agaricus bisporus*. *Mushroom Science*, *14*, 695-702.

Kumamoto, C.A. (2009). Molecular mechanisms of mechanosensing and their roles in fungal contact sensing. *Nature Reviews Microbiology*, *6*(9), 667-673.

Largeteau, M., Latapy, C., Minvielle, N., Regnault-Roger, C., Savoie, J.-M. (2010). Expression of phenol oxidase and heat-shock genes during the development of *Agaricus bisporus* fruiting bodies, healthy and infected by *Lecanicillium fungicola*. *Applied Microbiology and Biotechnology*, *85*(5), 1499-1507.

Lavín, J.L., García-Yoldi, A., Ramírez, L., Pisabarro, A.G., Oguiza, J.A. (2012). Two-component signal transduction in *Agaricus bisporus*: A comparative genomic analysis with other basidiomycetes through the web-based tool BASID2CS. *Fungal Genetics and Biology*, doi:10.1016/j.fgb.2012.09.012.

Lezzi, C., Bleve, G., Spagnolo, S., Perrotta, C., Grieco, F. (2012). Production of recombinant *Agaricus bisporus* tyrosinase in *Saccharomyces cerevisiae* cells. *Journal of Industrial Microbiology & Biotechnology*, *39*(12), 1875-1880.

Li, N.-Y., Cai, W.-M., Jin, Q.-L., Qin, Q.-P., Ran, F.-L. (2011). Molecular cloning and expression of polyphenoloxidase genes from the mushroom, *Agaricus bisporus*. *Agricultural Sciences in China*, *10*(2), 185-194.

Llorente, B., Alonso, G.D., Bravo-Almonacid, F., Rodríguez, V., López, M.G., Carrari, F., Torres, H.N., Flawiá, M.M. (2011). Safety assessment of nonbrowning potatoes: opening the discussion about the relevance of substantial equivalence on next generation biotech crops. *Plant Biotechnology Journal*, *9*(2), 136-150.

Lucas-Elío, P., Solano, F., Sanchez-Amat, A. (2002). Regulation of polyphenol oxidase activities and melanin synthesis in *Marinomonas mediterranea*: identification of *ppoS*, a gene encoding a sensor histidine kinase. *Microbiology*, *148*(8), 2457-2466.

Mamoun, M., Moquet, F., Savoie, J.-M., Devesse, C., Ramos-Guedes-Lafargue, M., Olivier, J.M., Arpin, N. (1999). *Agaricus bisporus* susceptibility to bacterial blotch in

relation to environment: biochemical studies. *FEMS microbiology letters*, *181*(1), 131-136.

Mcgarry, A., Hole, C.C., Drew, R.L.K., Parsons, N. (1996). Internal damage in potato tubers : a critical review. *Postharvest Biology and Technology*, *8*(4), 239-258.

Mikosch, T.S.P., Lavrijssen, B., Sonnenberg, A.S.M., van Griensven, L.J.L.D. (2001). Transformation of the cultivated mushroom *Agaricus bisporus* (Lange) using T-DNA from *Agrobacterium tumefaciens*. *Current Genetics*, *39*(1), 35-39.

Mohapatra, D., Frias, J.M., Oliveira, F.A.R., Bira, Z.M., Kerry, J. (2008). Development and validation of a model to predict enzymatic activity during storage of cultivated mushrooms (*Agaricus bisporus* spp.). *Journal of Food Engineering*, *86*(1), 39-48.

Monshausen, G.B., Gilroy, S. (2009). Feeling green: mechanosensing in plants. *Trends in Cell Biology*, *19*(5), 228-235.

Morin, E., Kohler, A., Baker, A.R., Foulongne-Oriol, M., Lombard, V., Nagy, L G., Ohm, R.A, Patyshakuliyeva, A., Brun, A., Aerts, A.L., Bailey, A.M., Billette, C., Coutinho, P.M., Deakin, G., Doddapaneni, H., Floudas, D., Grimwood, J., Hildén, K., Kües, U., Labutti, K.M., Lapidus, A., Lindquist, E.A., Lucas, S.M., Murat, C., Riley, R.W., Salamov, A.A., Schmutz, J., Subramanian, V., Wösten, H.A.B., Xu, J., Eastwood, D.C., Foster, G.D., Sonnenberg, A.S.M., Cullen, D., de Vries, R.P., Lundell, T., Hibbett, D.S., Henrissat, B., Burton, K.S., Kerrigan, R.W., Challen, M.P., Grigoriev, I.V., Martin, F. (2012). Genome sequence of the button mushroom Agaricus bisporus reveals mechanisms governing adaptation to a humic-rich ecological niche. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(43), 17501-17506.

Petersen, M. (2007). Current status of metabolic phytochemistry. *Phytochemistry*, *68*(22-24), 2847-2860.

Pierce, J.A., Rast, D.M. (1995). A comparison of native and synthetic mushroom melanins by Fourier transform infrared spectroscopy. *Phytochemistry*, *39*(1), 49-55.

Rast, D.M., Baumgartner, D., Mayer, C., Hollenstein, G.O. (2003). Cell wall-associated enzymes in fungi. *Phytochemistry*, *64*(2), 339-366.

Robards, K., Prenzler, P.D., Tucker, G., Swatsitang, P., Glover, W. (1999). Phenolic compounds and their role in oxidative processes in fruits. *Food Chemistry*, *66*(4), 401-436.

Sassoon, J., Mooibroek, H. (2001). A system of categorizing enzyme-cell wall associations in *Agaricus bisporus*, using operational criteria. *Applied Microbiology and Biotechnology*, *56*(5-6), 613-622.

Schuurs, T.A., Dalstra, H.J., Scheer, J.M.J., Wessels, J.G.H. (1998). Positioning of nuclei in the secondary mycelium of *Schizophyllum commune* in relation to differential gene expression. *Fungal Genetics and Biology*, *23*(2), 150-161.

Soler-Rivas, C., Möller, A.C., Arpin, N., Olivier, J.-M., Wichers, H.J. (2001). Induction of a tyrosinase mRNA in *Agaricus bisporus* upon treatment with a tolaasin preparation from *Pseudomonas tolaasii*. *Physiological and Molecular Plant Pathology*, *58*(2), 95-99.

Sonnenberg, A.S.M., Baars, J.J.P., Hendrickx, P.M., Lavrijssen, B., Gao, W., Weijn, A., Mes, J.J. (2011). Breeding and strain protection in the button mushroom *Agaricus bisporus*. *Proceedings of the 7th International Conference on Mushroom Biology and Mushroom Products*, INRA Bordeaux (pp. 7-15).

Stevens, L.H., Davelaar, E. (1997). Biochemical potential of potato tubers to synthesize blackspot pigments in relation to their actual blackspot susceptibility. *Journal of Agricultural and Food Chemistry*, *45*(11), 4221-4226.

Stoop, J.M.H., Mooibroek, H. (1999). Advances in genetic analysis and biotechnology of the cultivated button mushroom, *Agaricus bisporus*. *Applied Microbiology and Biotechnology*, *52*(4), 474-483.

Thipyapong, P., Hunt, M.D., Steffens, J.C. (2004). Antisense downregulation of polyphenol oxidase results in enhanced disease susceptibility. *Planta*, *220*(1), 105-117.

Thipyapong, P., Stout, M.J., Attajarusit, J. (2007). Functional analysis of polyphenol oxidases by antisense/sense technology. *Molecules*, *12*(8), 1569-1595.

Tsuji, H., Oka, T., Kimoto, M., Hong, Y.-M., Natori, Y., Ogawa, T. (1996). Cloning and sequencing of cDNA encoding 4-aminobenzoate hydroxylase from *Agaricus bisporus*. *Biochimica et Biophysica Acta*, *1309*(1-2), 31-36.

Tzin, V., Galili, G., Aharoni, A. (2012). Shikimate pathway and aromatic amino acid biosynthesis. John Wiley & Sons Ltd., *eLS* (pp. 1-10). Chichester.

Urbany, C., Stich, B., Schmidt, L., Simon, L., Berding, H., Junghans, H., Niehoff, K.-H., Braun, A., Tacke, E., Hofferbert, H.-R., Lübeck, J., Strahwald, J., Gebhardt, C. (2011).

Association genetics in *Solanum tuberosum* provides new insights into potato tuber bruising and enzymatic tissue discoloration. *BMC genomics*, *12*(7), 1-14.

Urbany, C., Colby, T., Stich, B., Schmidt, L., Schmidt, J., Gebhardt, C. (2012). Analysis of natural variation of the potato tuber proteome reveals novel candidate genes for tuber bruising. *Journal of Proteome Research*, *11*(2), 703-716.

Walton, K., Coombs, M.M., Walker, R., Ioannides, C. (2001). The metabolism and bioactivation of agaritine and of other mushroom hydrazines by whole mushroom homogenate and by mushroom tyrosinase. *Toxicology*, *161*(3), 165-177.

Weijn, A., Bastiaan-Net, S., Wichers, H.J., Mes, J. J. (2012a). Melanin biosynthesis pathway in *Agaricus bisporus* mushrooms. *Fungal Genetics and Biology*, doi:http://dx.doi.org/10.1016/j.fgb.2012.10.004.

Weijn, A., Tomassen, M.M.M., Bastiaan-Net, S., Hendrix, E.A.H.J., Baars, J.J.P., Sonnenberg, A.S.M., Wichers, H.J., Mes, J.J., (2011). Browning sensitivity of button mushrooms. *Proceedings of the 7th International Conference on Mushroom Biology and Mushroom Products*, INRA Bordeaux (pp. 208-216).

Weijn, A., Tomassen, M.M.M., Bastiaan-Net, S., Wigham, M.L.I., Boer, E.P.J., Hendrix, E.A.H.J., Baars, J.J.P., Sonnenberg, A.S.M., Wichers, H.J., Mes, J.J. (2012b). A new method to apply and quantify bruising sensitivity of button mushrooms. LWT - Food Science and Technology, 47(2), 308-314.

Wichers, H.J., Recourt, K., Hendriks, M., Ebbelaar, C.E.M., Biancone, G., Hoeberichts, F.A., Mooibroek, H., Soler-Rivas, C. (2003). Cloning, expression and characterisation of two tyrosinase cDNAs from *Agaricus bisporus*. *Applied Microbiology and Biotechnology*, *61*(4), 336-341.

Woolston, B.M., Schlagnhaufer, C., Wilkinson, J., Larsen, J., Shi, Z., Mayer, K.M., Walters, D.S., Walters, D.S., Curtis, W.R., Romaine, C.P. (2011). Long-distance translocation of protein during morphogenesis of the fruiting body in the filamentous fungus, *Agaricus bisporus*. *PloS one*, *6*(12), 1-9.

Wu, J., Chen, H., Goa, J., Liu, X., Cheng, W., Ma, X. (2010). Cloning, characterization and expression of two new polyphenol oxidase cDNAs from *Agaricus bisporus*. *Biotechnology Letters*, *32*(10), 1439-1447.

Summary

Mushrooms are prone to brown discoloration upon bruising, which can occur during picking by hand or by robotic picking equipment and leads to lowering of quality. Mechanical harvesting cannot be applied yet to serve the fresh market, as commercial strains are too sensitive to bruising. When strains with less sensitivity to bruising are available an increase in quality for both the fresh and the preserved mushroom industry is possible. A general introduction on the button mushroom cultivation and production, mushroom bruising, and discoloration caused by the melanin synthesis pathway for both the enzymes and phenolic substrates involved is given in **Chapter 1**.

In **Chapter 2** the development of a new bruising device to damage mushrooms in a fast and reproducible way is described and a computer image analysis system was used to quantify bruising-discoloration. A protocol was developed to obtain the most reliable and reproducible method to compare bruising-sensitivity of different *Agaricus bisporus* strains. For this purpose, the extent of damage applied, time between harvest and bruising, developmental stage of the mushrooms and flushes were taken into account. Distinction between sensitive and tolerant *A. bisporus* strains is possible with the developed bruising-method and quantification analysis.

Chapter 3 describes the comparison between the developed bruising protocol and industrial practise. A correlation was found between discoloration applied with the bruising device and discoloration caused by transportation of mushrooms on a conveyor belt. A lower correlation was found between post-harvest storage-discoloration of undamaged mushrooms and the bruising device. The results of the bruising device are comparable to commercial circumstances and possibly a different discoloration mechanism is involved in storage discoloration.

The genes involved in the melanin biosynthesis pathway of button mushrooms were analysed in the genome sequence of *Agaricus bisporus* and were described in **Chapter 4**. Gene expression was determined for most gene homologs (26 genes)

involved in the melanin pathway during the development of the mushroom and in different tissue types. Of the analysed genes, those encoding polyphenol oxidase (PPO), a PPO associated peptide the L-chain (unique for *Agaricus bisporus*), and a putative transcription factor (photoregulator B) were among the highest expressed in the skin tissue. An in-depth look was taken at the clustering of several PPO genes and the L-chain on chromosome 5.

In **Chapter 5** a UHPLC-PDA-MS method was used to quantify and identify the phenolic compounds involved in the melanin biosynthesis pathway. Nine *A. bisporus* strains with a different level of bruising sensitivity were analysed for the available phenolics in non-bruised and bruised skin tissue. A higher content of GHB and GDHB was found in the non-bruised skin tissue and also remained more abundant in bruised skin tissue of both white and brown capped bruising-sensitive strains compared to tolerant strains. The concentrations of these phenolic compounds in the skin tissue might provide a good indication for the bruising-sensitivity of an *A. bisporus* strain.

After the identification of the phenolics involved in bruising-sensitivity the same strains were analysed on a molecular level using genomic sequences and transcriptional analysis (**Chapter 6**). A limited set of genes was found to respond to bruising, among them phenylalanine ammonia-lyase 2 and some of the polyphenol oxidases. Genomic sequence comparison resulted in the identification of at least four genes in a wild brown bruising-tolerant strain for which the sequence showed alteration resulting in truncated or mutated proteins. Mutations were confirmed with RT-qPCR and were specific for the sequenced wild brown bruising-tolerant strain.

The molecular and biochemical analysis of bruising-tolerance based on *A. bisporus* offspring selected from a segregating population is described in **Chapter 7**. Analysis of an offspring selection showed that the *L-chain* was more abundantly expressed in both white and non-white bruising-tolerant lines together with

polyphenol oxidase 3 and 5. A high correlation was found between the total concentration of phenolics and the bruising-sensitivity of the selected white offspring. Mutation analysis of the previously identified mutations did not correlate with the bruising-sensitivity of the offspring but intriguing results were obtained. These results were combined with the phenolic and gene expression analysis which resulted in a new hypothesis on the key parameters of bruising sensitivity and possible strategies towards obtaining a high bruising-tolerance trait.

The last chapter, **Chapter 8**, discusses the important results in each chapter of this thesis, combining the results obtained and giving future research perspectives. We propose that bruising-tolerant strains can be obtained by combining the genetic background of both homokaryons from wild brown strain 2 with a commercial hybrid.

Samenvatting

Bruinverkleuring van champignons door kneuzing kan veroorzaakt worden door het plukken van de champignons met de hand of met een plukmachine en leidt tot verminderde kwaliteit. Mechanisch oogsten kan op dit moment nog niet gebruikt worden voor de verse champignonmarkt, omdat commerciële rassen te gevoelig zijn voor kneuzing. Indien er rassen beschikbaar zijn die minder gevoelig zijn voor kneuzing is een betere kwaliteit voor zowel de vers- als de conservenmarkt mogelijk. Een algemene introductie over de kweek en productie van champignons, champignonkneuzing en verkleuring als gevolg van de activiteit van enzymen op fenolische substraten, wat leidt tot de aanmaak van het bruine pigment melanine, wordt gegeven in **Hoofdstuk 1**.

In **Hoofdstuk 2** is de ontwikkeling beschreven van een nieuw kneusapparaat waarmee champignons op een snelle en reproduceerbare manier gekneusd kunnen worden. Een computer-gebaseerd foto-analysesysteem is ontwikkeld en gebruikt om de verkleuring veroorzaakt door de kneuzing te kwantificeren. Een protocol werd ontwikkeld voor de meest betrouwbare en reproduceerbare methode om kneusgevoeligheid van verschillende *Agaricus bisporus*-rassen te vergelijken. Om dit te bereiken werden de mate van de toegediende beschadiging, de tijd tussen oogsten en kneuzen, het ontwikkelingsstadium van de champignon en verschillende vluchten met elkaar vergeleken. De ontwikkelde kneuzings-methode en kwantificeringsanalyse maakten het mogelijk om een collectie van champignonstammen te screenen met betrekking tot hun kneusgevoeligheid. Dit heeft geleid tot de identificatie van gevoelige en zeer interessante kneus-tolerante *A. bisporus* rassen.

Hoofdstuk 3 beschrijft de vergelijking tussen de ontwikkelde kneuzings-methode en industriële toepassingen. Een correlatie werd gevonden tussen verkleuring toegebracht met het kneusapparaat en verkleuring veroorzaakt door verplaatsing van de champignons met een transportband. Een lagere correlatie werd gevonden tussen verkleuring veroorzaakt door na-oogst koude bewaring van

onbeschadigde champignons en het kneusapparaat. De resultaten van het kneusapparaat waren vergelijkbaar met commerciële omstandigheden en mogelijk is er een ander verkleuringsmechanisme betrokken bij verkleuring veroorzaakt door bewaring.

Zeer recent is het volledige genoom van *A. bisporus* bepaald en automatisch geannoteerd. De genen betrokken bij de melaninebiosyntheseroute van champignons werden door ons geïdentificeerd, geanalyseerd en annotaties werden verbeterd. In totaal zijn meer dan 26 genen die waarschijnlijk zijn betrokken bij de melanine biosynthese beschreven in **Hoofdstuk 4**. Vervolgens werden voor deze genen RT-qPCR-primers ontwikkeld om de expressie van de genen te analyseren tijdens de ontwikkeling van de champignon en in verschillende weefseltypes. De genen coderend voor polyphenoloxidase (PPO), een PPO-geassocieerd peptide de L-chain (uniek voor *A. bisporus*) en een vermeende transcriptiefactor (photoregulator B) kwamen onder andere het hoogst tot expressie in de laag aan de buitenkant van de hoed van de champignon. Om additionele genen te identificeren die mogelijk betrokken zijn bij het verkleuringsmechanisme werd een uitgebreide analyse gedaan op de clustering van verschillende PPO-genen en de L-chain op chromosoom 5.

In **Hoofdstuk 5** is een UHPLC-PDA-MS-methode ontwikkeld en gebruikt om de fenolische stoffen betrokken bij de melaninebiosynthese te identificeren en te kwantificeren. Negen *A. bisporus*-rassen met een verschillende kneusgevoeligheid werden geanalyseerd met betrekking tot de beschikbare fenolen in zowel gekneusd als niet-gekneusd hoedepidermisweefsel (een laag aan de buitenkant van de hoed van de champignon die relatief gemakkelijk loslaat). Een hogere hoeveelheid van GHB en GDHB werd gevonden in het niet-gekneusde weefsel van gevoelige rassen. Dit verschil was zowel waarneembaar in monsters van gekneusd weefsel en was waarneembaar bij zowel witte als bij bruine rassen. De concentratie van deze fenolen in het hoedweefsel is waarschijnlijk een goede indicatie voor de

kneusgevoeligheid van een ras. In één kneustolerant ras waren GHB en GDHB nauwelijks meer aanwezig wat het ras interessant maakt voor verdere veredeling op kneustolerantie.

Na de analyse van de fenolische verbindingen die betrokken zijn bij de melaninesynthese werden dezelfde rassen gebruikt voor de moleculaire analyse door gebruik te maken van genomische sequenties en transcriptie-analyse (**Hoofdstuk 6**). Slechts van een aantal genen werd waargenomen dat de expressie verandert als reactie op de kneuzing, zoals phenylalanine ammonia-lyase 2 (waarvan de expressie omhoog ging) en sommige PPOs (waarvan de expressie omhoog of omlaag ging). Er waren geen duidelijke correlaties waarneembaar tussen genexpressie van de geanalyseerde genen en de kneusgevoeligheid van het ras. Vergelijking van genomische sequenties van een kneusgevoelig en kneustolerant ras resulteerde in de identificatie van ten minste vier genen van een wild-type bruin kneus-tolerant ras waarin de sequentie een verandering bevatte. Dit resulteerde in een verkort eiwit voor AT_3 en PPO_3 of een gemuteerd eiwit voor AT_1 en GGT_1 met een mogelijke veranderde functionaliteit. Met behulp van RT-qPCR werd de aanwezigheid van de mutaties bevestigd en werd aangetoond dat die specifiek waren voor het betreffende wild-type bruine kneus-tolerante ras.

Een kruisingspopulatie die werd gegenereerd uit een redelijk tolerant wit commercieel ras en het zeer kneustolerante bruine wilde ras. Uit de nakomelingen van deze populatie werden vervolgens kneusgevoelige en kneustolerante nakomelingen geselecteerd. De moleculaire en biochemische analyse van deze nakomelingen is beschreven in **Hoofdstuk 7**. Deze analyse toonde aan dat het gen, coderend voor de L-chain, hoger tot expressie kwam in zowel witte als niet-witte kneus-tolerante lijnen, samen met een iets verhoogde expressie van polyphenoloxidase-3 en -5-genen. Een hoge correlatie werd gevonden tussen de totale concentratie van fenolen en de kneusgevoeligheid van de geselecteerde witte nakomelingen. Analyse van de geïdentificeerde mutaties correleerde niet volledig

met de kneusgevoeligheid van de nakomelingen maar heeft wel geleid tot nieuwe inzichten die gebruikt kunnen worden om rassen met een hoge kneus-tolerantie te verkrijgen.

In het laatste hoofdstuk, **Hoofdstuk 8**, worden de belangrijkste resultaten van ieder hoofdstuk van dit proefschrift besproken door alle verkregen resultaten te combineren en toekomstige onderzoeksmogelijkheden te bespreken. Wij voorspellen dat kneus-tolerante rassen verkregen kunnen worden door de genetische achtergrond van beide homokaryons van het wilde bruine ras 2 te combineren met een commercieel ras.

Dankwoord/Acknowledgements

Eindelijk aanbeland bij het schrijven van het laatste stuk van mijn thesis. Het stuk waarvan je zeker weet dat het als eerste en door iedereen gelezen wordt. Het stuk waarin je op zoek gaat naar je eigen naam en wat bij mij altijd een glimlach op mijn gezicht tovert als ik mijn naam gevonden heb, hopelijk bij jullie ook!

Ten eerste wil ik natuurlijk de mensen bedanken die het mogelijk hebben gemaakt om mijn promotie te doen. Harry en Jurriaan bedankt voor het vertrouwen dat jullie mij hebben gegeven om aan het promotietraject te beginnen. Tijdens mijn stage heb ik prettig met jullie samengewerkt en ik ben blij dat we daar nog 4 jaar aan vast geknoopt hebben. Jullie hebben mij op alle wetenschappelijke vlakken geholpen en mijn kennis verbreed: wees kritisch op je eigen werk en stel je jezelf die extra vraag of je nog iets moet controleren in het lab en wat jouw onderzoek vernieuwend en uniek maakt.

Onderzoek doe je nooit alleen en daarom ben ik alle collega's van FBR, FCH en PRI, waarmee ik de afgelopen jaren heb samengewerkt, erg dankbaar. Vooral tijdens de grote champignonproeven waar af en toe maar geen einde leek te komen aan de aanvoer van nog meer champignons die allemaal gekneusd en gesampled moesten worden. Shanna, Monic, Dianne, Renata, Robert, Harmannus, Els, Coen, Jurriaan en Erika bedankt voor jullie hulp, voornamelijk tijdens de weekenden, want tja die champignons stoppen nauw eenmaal niet met groeien in het weekend. Shanna, Monic, en Dianne bedankt voor jullie hulp in het lab, jullie hebben veel samples voor mij geanalyseerd en daarmee hebben we een heleboel mooie data verkregen voor het proefschrift, wat heeft geleid tot een aantal mooie publicaties. Shanna, super bedankt dat je mijn paranimf wilt zijn! Giovanni and Dzmitry thanks for all your help during the busiest time of my PhD project. Dzmitry good luck in Germany with your own PhD project! Giovanni good luck with your career and I hope that one day you like to eat mushrooms again!

Book, you deserve a special place in these acknowledgments. We met when you started your PhD and I was a student at FBR. It was really nice to start my own PhD project and to share the ups and downs of a PhD life and personal life with you. I want to thank you for everything. See you soon in Thailand!

The PhD students from FBR thank you for the nice lunches and dinners! Marit, Lonneke, Yongfu, Noon, Pang, Vincent, Edwin, Lenny, Stefan and Geraldine good luck with your own PhD! Marit bedankt dat jij mijn paranimf wilt zijn!

Tijdens mijn project heb ik samen gewerkt met de mensen van de champignongroep van PRI. Anton and Johan, thank you for your expertise about mushroom growth, genetics, quality and much more. Wei, I enjoyed working together with you in the project and I wish you good luck finishing your PhD thesis. Patrick, Brain, Marcel, Jose, Narges, and other students from PRI thank you for the help during the big growing experiments. Ed bedankt voor het kweken van al die champignons en ook de andere medewerkers van de Unifarm, die altijd in waren voor een grapje maar serieus bleven doorwerken tot dat de klus af was.

Altijd als ik bij levensmiddelenchemie (FCH) was werd ik hartelijk begroet door iedereen. Velen zullen mij maar een paar keer gezien hebben, maar het is een hele collegiale groep. Iedereen bedankt waarvan ik het bureau geleend heb als ze zelf afwezig waren. In het speciaal wil ik Mark, Tomas en Yannick bedanken voor de hulp bij het UHPLC werk. Harry Gruppen en Jean-Paul bedankt voor jullie expertise en interesse voor mijn onderzoek. Together with Martine, Ruud, Simone, Book, Anja and Anne we organised an excellent PhD trip in 2010 to Italy and Switzerland. Thank you for the nice time together!

Daarnaast wil ik ook alle partners van het TTi Groene Genetica-project bedanken voor de samenwerking de afgelopen jaren. Wim Arts van CNC, Caroline van de Horst van C4C Holding, Geert Weijers van Lutèce, Mark Wach, Theo Lauwers en Anne Rodier van Sylvan Inc, Karst van der Werf van WeBe Engineering, Jan Vink van Productschap Tuinbouw, Els Verhaegh van Banken Champignons, Annelotte Crena de Iongh van The Greenery en Ineke Wijkamp van TTi Groene Genetica. Hopelijk volgen er nog meer mooie projecten vanuit ons onderzoek.

Natuurlijk is naast het drukke leven van een PhD ontspanning op zijn tijd ook erg belangrijk. Jack, Arwin, Hans, Anton, John, Edwin, Helmus, Marcel en Sandra bedankt voor de leuke en gezellige avonden naar concerten en films. Thanks to you, Book gave me the nickname *concert girl*. Daarnaast zijn de gezellige familieverjaardagen en etentjes ook erg belangrijk. Met de eeuwige vraag van mijn vader “Weet je het al? Waarom worden die champignons nou bruin?” Pap, mam, Sharon en Jan Willem, Valentijn, Nel en Gerard bedankt voor jullie interesse in mijn werk. Hoewel het soms erg moeilijk uit te leggen is wat je nu precies doet op een lab en waarom het 4 jaar moet duren en je dan nog steeds niet alle antwoorden hebt gevonden bleven jullie een steun en toeverlaat. Hopelijk geeft dit boekje een idee van wat ik de afgelopen 4 jaar met die champignons gedaan heb. Ook alle andere familieleden en vrienden bedankt voor jullie interesse in mijn werk!

En natuurlijk als allerlaatste Jack, mijn schatje. Bijna tegelijkertijd zijn we begonnen aan ons promotie-onderzoek. Bij mij was de finishlijn iets eerder in zicht, maar jij bent er nu ook bijna, op naar je eigen finale! Bedankt voor alle tenniswedstrijden waar we af en toe even lekker de frustratie er uit konden slaan en de lekkere etentjes als er weer een nieuw doel was behaald. Voor de meeste mensen waarschijnlijk een beetje saai, maar als je met twee wetenschappers in één

huis woont dan gaan de gesprekken tijdens het eten meestal over onderzoek. Wat bij ons in een hele mooie samenwerking heeft geresulteerd in hoofdstuk 5. Op naar een mooie wetenschappelijke en liefdevolle toekomst samen! Heel veel succes met je volgende uitdaging in Duitsland!

Liefs, *Amrah*

About the author

Curriculum Vitae

Amrah Weijn was born on September 1st 1985 in Apeldoorn, The Netherlands. After graduating from high school (VWO, profile Nature and Health) in 2003, she started studying Chemistry at the University Utrecht. The Bachelor diploma was obtained in 2007 after an internship at the group Biochemistry of Membranes at Utrecht University about the mode of action of the antibiotic telavancin. She continued her studies at Utrecht University with a master in Biomolecular Sciences. This master was completed with two internships. The major master internship was performed at the Microbiology group of the University Utrecht about the capsule biogenesis of *Cryptocuccus neoformans.* The minor master internship was performed at Food & Biobased Research (FBR) in combination with the Cellular Biology and Immunology group, Wageningen University. The research included the isolation and purification of Fungal Immunomodulatory Proteins (FIPs) from *Ganoderma lucidum* and *Flammulina velutipes* and the bioactivity of FIPs on different immunity-related cell lines. Afterwards, she obtained the opportunity to continue working at FBR as a PhD student under the supervision of prof. dr. Harry Wichers and dr. Jurriaan Mes. The results of the PhD research are described in this thesis. Currently, Amrah is looking for a job as a post-doc.

Contact email address: amrahweijn@hotmail.com

List of publications

Weijn, A., Bastiaan-Net, S., Wichers, H.J., Mes, J.J., Melanin biosynthesis pathway in *Agaricus bisporus* mushrooms. *Fungal Genetics and Biology*, accepted October 2012, http://dx.doi.org/10.1016/j.fgb.2012.10.004.

van der Meer, I.M., Mes, J.J., **Weijn, A.**, Wichers, H.J., Immunomodulation by food. *Multidisciplinary Approaches to Allergies*, Gao, Z.S., Shen, H.H., Zheng, M., Frewer, L.J., Gilissen, L.J.W.J. (2012).

Weijn, A., Tomassen, M.M.M., Bastiaan-Net, S., Wigham, M.L.I., Boer, E.P.J., Hendrix, E.A.H.J., Baars, J.J.P., Sonnenberg, A.S.M., Wichers, H.J., Mes, J.J., A new method to apply and quantify bruising sensitivity of button mushrooms. *LWT – Food Science and Technology*, 47 (2012) 308-314.

Sonnenberg, A.S.M., Baars, J.J.P., Hendrickx, P.M., Lavrijssen, B., Gao, W., **Weijn, A.**, Mes, J.J., Breeding and strain protection in the button mushroom *Agaricus bisporus*. *Proceedings of the 7th International Conference of the World Society for Mushroom Biology and Mushroom Products*, INRA Bordeaux (2011) 7-15.

Weijn, A., Tomassen, M.M.M., Bastiaan-Net, S., Hendrix, E.A.H.J., Baars, J.J.P., Sonnenberg, A.S.M., Wichers, H.J., Mes, J.J., Browning sensitivity of button mushrooms. *Proceedings of the 7th International Conference of the World Society for Mushroom Biology and Mushroom Products*, INRA Bordeaux (2011) 208-216.

Ismaya, W.T., Rozeboom, H.J., **Weijn, A.**, Mes, J.J., Fusetti, F., Wichers, H.J., Dijkstra, B.W., Crystal structure of *Agaricus bisporus* mushroom tyrosinase: identity of the tetramer subunits and interaction with tropolone. *Biochemistry*, 50 (2011) 5477-5486.

Weijn, A., van den Berg-Somhorst, B.P.M., Slootweg, J.C., Vincken, J.-P., Gruppen, H., Wichers, H.J., Mes, J.J., Main phenolic compounds of the melanin biosynthesis pathway in bruising-tolerant and bruising-sensitive *Agaricus bisporus* strains. *Submitted*.

Weijn, A., Sinistki, D.P., Tomassen, M.M.M., Wichers, H.J., Mes, J.J., A molecular approach to identify key components involved in bruising-tolerance of the button mushroom *Agaricus bisporus*. *To be submitted*.

Overview of completed training activities

Discipline specific activities

Conferences

- ALW-Experimental Plant Sciences, EPS, Lunteren, The Netherlands (2009)
- TTI gg symposium, Nieuwegein, The Netherlands (2010)[#,†]
- TTI gg symposium, Nieuwegein, The Netherlands (2011)[#,†]
- 7th International Conference of Mushroom Biology and Mushroom Products, Arcachon, France (2011)[†]
- 18th International Congress of the International Society of Mushrooms, Beijing, China (2012)[†]
- TTI gg symposium, Nieuwegein, The Netherlands (2012)

Courses

- Bioinformatics, a user's approach, EPS, Wageningen, The Netherlands (2010)
- Systems Biology: statistical analysis of ~omics data, VLAG, Wageningen, The Netherlands (2010)
- 11th Summer Course Glycosciences, VLAG, Wageningen, The Netherlands (2010)

General courses

- Basic statistics, PE&RC, Wageningen, The Netherlands (2009)
- Information literacy: including introduction Endnote ,WGS, Wageningen, The Netherlands (2010)
- Teaching and supervising thesis students, WU, Wageningen, The Netherlands (2010)
- Scientific writing, WGS, Wageningen, The Netherlands (2010)

- Techniques for writing and presenting a scientific paper, WGS, Wageningen, The Netherlands (2011)
- Career Orientation, WGS, Wageningen, The Netherlands (2012)

Additional activities

- Preparing Research Proposal, Wageningen, The Netherlands (2009)
- Food Chemistry Study trip, Ghent University, Belgium (2009)
- Organizing PhD study trip to Switzerland and Italy (2010)
- Food Chemistry PhD study trip, Switzerland and Italy (2010)†
- Basic statistics MSc course (MAT) (2010)
- Food Chemistry PhD study trip, Singapore and Malaysia (2012)†
- Research group meetings at FBR(2009-2013)

Poster presentation

† Oral presentation

Abbreviations

FBR	Food & Biobased Research
EPS	Experimental Plant Sciences
MAT	Mathematical and Statistic Methods Group
PE&RC	Production Ecology and Resource Conservation
TTI gg	Technical Top Institute green genetics
VLAG	Graduate School for Nutrition, Food Technology, Agrobiotechnology and Health Sciences
WGS	Wageningen Graduate Schools
WU	Wageningen University

The research described in this thesis was financially supported by TTi Green Genetics.

The thesis was printed by Wöhrmann Print Service.
The cover was designed by J.C. Slootweg and A. Weijn.

Printing of this thesis was financially supported by:
Wageningen University (WUR)
Sylvan BV Europe
Screening Devices b.v.
C4C Holding B.V.