Alterations in gene expression and secondary metabolite production during development of *Aspergillus nidulans*

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Für meine Frau Diana & meine Tochter Sophia

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Abbreviations

A	asexual development
aaRNA	amino allyl RNA
AF	aflatoxin
AFO	asperfuranone
ALA	alanine
ASN	asparagine
ASP	aspartate
Cy3/Cy5	cyanine 3 / cyanine 5
DEPC	diethylpyrocarbonate
DRE	dynamic range enhancement
EAS	emericellamides
GC MS	gas chromatography mass spectrometer
GLN	glycine
GLU	glutamate
GST	glutathione S-transferase
HOD	hydroxyoctadecadienoic acid
HOE	hydroxyoctadecanoic acid
DiHOD	di-hydroxyoctadecadienoic acid
DiHOE	di-hydroxyoctadecanoic acid
HIS	histidine
HPLC	high performance liquid chromatography
LYS	lysine
MDP	monodictyphenone
MET	methionine
NOX	NADPH oxidase
ORN	ornithine
PHE	phenylalanine
PPO	*psi* producing oxygenase
PRO	proline

PSI	precocious sexual inducer
S	sexual development
SDS	sodium dodecylsulfate
ST	sterigmatocystin
THR	threonine
TQ	terrequinone
UPLC	ultra performance liquid chromatography
TOF-MS	time-of-flight mass spectrometer
UV	ultraviolet
VEG	vegetative
VAL	valine
VSN	variance normalization and stabilization
Wt	wild type

Summary

Many studies describing development and secondary metabolism of the filamentous fungus *Aspergillus nidulans* contributed to a better understanding of fungal secondary metabolism regulation at the molecular level. However, a comprehensive picture of the regulation remained to be shown. Therefore, in this work we undertake a global transcriptomic and metabolomic overview, which describes the light-dependent developmental responses of this soil-borne fungus. Light favours the development of asexual spores and inhibits the formation of sexual fruiting bodies (cleistothecia), which are preferentially formed in the absence of light. Overall 2.014 genes, which correspond to 20 % of the genome, are differentially expressed and influenced during different developmental stages in the light and in the dark. Light controls development by inducing gene expression significantly during 24-48 hours of development. Targeted repression of light sensing complexes in early sexual development might delay differentiation and gene expression. Increased numbers of delayed genes during sexual differentiation reveal temporal consistency with the secondarily induced, delayed conidiation at sexual development. Interestingly, transcriptomics of vegetative growth and early sexual development exhibit similar profiles, which is consistent with highly similar growth phenotypes. A characteristic feature during the late phase of asexual spore formation of light-induced asexual development is the expression of stress response genes, which might provide resistance to various abiotic stress conditions, including UV irradiation and related oxidative stress compounds for the air-borne conidia. Fungal development depends on *psi* (*p*recocious *s*exual *i*nducer) factors, which are oxylipin hormones related to prostaglandins. *PsiC1β* (5,8-DiHOE) appears specifically in darkness during early sexual development. During the sexual cycle *A. nidulans* initiates the expression of many genes required for cell wall degradation, including genes for plant and bacterial cell wall and polysaccharides hydrolysis, which probably mobilize the energy and building blocks for the completion of sexual fruiting bodies during nutrient limitations. During the late sexual stage, protective secondary metabolites are present, which might be crucial to protect the fruiting bodies against fungivors and therefore, helping ascospores to germinate in the presence of a decreased number of competitors. The emericellamide C metabolite is secreted before the cleistothecia maturation and completion of sexual development. Many downregulated amino acid biosynthetic genes and cellular amino acids levels at late sexual development indicate a period of dormancy where translation stops due to lacking amino acids. Fungus initiates programmed cell death at late sexual development by inducing apoptotic gene expression, which corresponds to an aging process. Our results revealed that during light-dependent fungal development, a significant proportion of the genome (20 %) is affected by the light signal, which leads to various responses, including production of secondary metabolites and other adaptive responses, collectively contributing the fungus to adapt and survive through the current environmental conditions.

Zusammenfassung

Viele Studien beschreiben Entwicklung und Sekundärmetabolismus des filamentösen Pilzes *Aspergillus nidulans*, was zu einem besseren Verständnis der Regulation des Sekundärmetabolismus von Pilzen auf molekularer Ebene beisteuert. Dennoch muss ein umfassendes Bild dieser Regulation noch gezeigt werden. Aus diesem Grund geben wir in dieser Arbeit einen ausführlichen Überblick von Transkriptom und Metabolom, welcher die Antworten der lichtabhängigen Entwicklung dieses bodenbürtigen Pilzes aufzeigt. Licht begünstigt die Entwicklung asexueller Sporen und hemmt die Entstehung sexueller Fruchtkörper (Kleistothetien), die bevorzugt im Dunkeln gebildet werden. Insgesamt werden 2.014 Gene, was etwa 20 % dessen Genom entspricht, während unterschiedlicher Entwicklungsphasen in Licht und Dunkelheit differenziell exprimiert und beeinflusst. Licht kontrolliert die Entwicklung, indem es die Genexpression während 24-48 Stunden der Entwicklung erheblich induziert. Die gezielte Repression von Lichtsensor-Komplexen in der frühen sexuellen Entwicklung könnte die Genexpression und schließlich die Differenzierung des Pilzes verzögern. Die erhöhte Anzahl verzögerter Gene während der sexuellen Differenzierung zeigt eine zeitliche Übereinstimmung mit der sekundär induzierten und verzögerten Bildung von Konidien bei sexueller Entwicklung. Interessanterweise zeigen die Transkriptome vom vegetativen Wachstum und der frühen sexuellen Entwicklung ähnliche Expressionsmuster auf, was sich in den äußerst ähnlichen Phänotypen beider Phasen widerspiegelt. Ein charakteristisches Merkmal während der späten Phase asexueller Sporenbildung im Licht stellt die Expression von Genen für die Antwort auf Stress dar. Dadurch könnten Resistenzen gegenüber verschiedensten abiotischen Stressbedingungen, einschließlich UV-Bestrahlung and daraus entstehende reaktive Sauerstoffspezies, in den luftverbreiteten Konidien gebildet werden. Die Entwicklung der Pilze ist von *psi* (*p*recocious *s*exual *i*nducer) Faktoren, welche Prostaglandin verwandte Oxylipin-Hormone sind, abhängig. *PsiC1β* (5,8-DiHOE) erscheint spezifisch während der frühen sexuellen Entwicklung in Dunkelheit. Im Laufe der sexuellen Entwicklung aktiviert *A. nidulans* viele Gene für den Zellwandabbau, einschließlich Gene für den Abbau von Pflanzen- und Bakterienzellwand sowie für die Hydrolyse von Polysacchariden. Dadurch könnten Energie und die Grundbausteine für die erfolgreiche Fertigstellung sexueller Fruchtkörper während Nährstoffmangelbedingungen mobilisiert wird. Während der späten sexuellen Entwicklung sind Sekundärmetaboliten vorhanden, welche den Schutz der Fruchtkörper und Askosporen z. B. gegen Fressfeinde gewährleisten und daher den Askosporen beim Keimen in Anwesenheit einer geringeren Anzahl von Mitstreitern unterstützt. Das Emericellamide C Metabolit wird ausgeschieden bevor die Reifung der Kleistothecien und die sexuelle Entwicklung abgeschlossen sind. Viele herunter regulierte Gene für die Synthese von Aminosäuren und die geringe Anreicherung zellulärer Aminosäuren in der späten sexuellen Entwicklung spiegeln einen Ruhezustand wider, bei welchem die Translation aufgrund des Mangels an Aminosäuren gestoppt wurde. Der Pilz initiiert den programmierten Zelltod in der späten sexuellen Entwicklung durch die Expression von Apoptose-Genen, welche mit einem Alterungsprozess einhergeht. Unsere Ergebnisse zeigen, dass während der lichtabhängigen Entwicklung des Pilzes ein beachtlicher Teil des Genoms (20 %) durch das Lichtsignal beeinflusst wird. Dieses führt zu verschiedenen Antworten einschließlich der Produktion von Sekundärmetaboliten und anderen Anpassungen, welche es zusammengenommen dem Pilz ermöglichen sich anzupassen und in den vorherrschenden Umweltbedingungen zu überleben.

1. Introduction

1.1 Fungi within the ecological system

1.1.1 Importance of fungal organisms on our planet

Fungi are very successful organisms in the adaptation to their environment for many hundred million years. They developed many different largely still unknown mechanisms to maintain the habitat they exist in. Fungi are potential sources for the discovery of secondary metabolites e.g. antibiotics, mycotoxins, phytotoxins etc. and for the understanding of such mechanisms (Bhetariya, *et al.*, 2011, Dickman & Figueiredo, 2011, Khlangwiset, *et al.*, 2011, Bayram & Braus, 2012, Scharf, *et al.*, 2012). Some secondary metabolites are either carcinogenic or anti-therapeutic (Wainright, 1992). On the other hand some fungal derived secondary metabolites have therapeutic relevance. For instance terrequinone A initially found in the filamentous ascomycete *Aspergillus terreus* (He, *et al.*, 2004) possesses anti-tumor properties. Fungi are crucial for the recycling of organic material within the terrestrial ecosystem.

Fungi also serve as basis for our food. Among them are prominent examples like the baker's yeast *Saccharomyces cerevisiae* for bread baking, beer and wine production, for which another (filamentous) ascomycete *Aspergillus oryzae* is also used. Food industry utilizes *Aspergillus niger* for citrate synthesis in huge industrial scales (Bomstein & Johnson, 1952, Papagianni, 2007, Dhillon, *et al.*, 2011, Acourene & Ammouche, 2012). For instance, citrate serves as cleaning and preservative agent as well as dietary supplement.

Fungi are part of our daily life and have enormous biotechnological potentials. Filamentous fungi serve as excellent platforms to analyze and understand the regulation of biology, physiology, genetics and biochemistry of a eukaryotic cell. This allows us to understand the mechanism of eukaryotic cells and also provide information about the human pathogenic fungi.

1.1.2 Pathogenic fungi

Pathogenesis describes the competence of an organism to cause deceases in another organism. Among countless pro- and eukaryotes the fungal kingdom contains numerous pathogenic representatives possessing harming effects on organisms of the two animal and plant kingdoms (Hof, 2003). Damages caused by fungi result in massive financial losses in our agriculture and health system every year (Agrios, 1997, Latge, 1999, Agrios, 2005).

Phytophthora infestans is a plant pathogenic oomycote causing the serious potato

disease known as late blight (Nowicki, *et al.*, 2012). The effects of *Phytophthora infestans* infection of potatoes in Ireland from 1845 to 1857 caused over one million to starve to death and forced another two million to emigrate from affected countries. Another plant pathogenic fungus is *Verticillium dahliae* belonging to the class of sordariomycetes. This fungus causes verticillium wilt in many plant species showing symptoms like leaves to curl and discolour (Douglas, 2011).

In contrast, *Candida albicans* is a human pathogen that belongs to the Saccharomycetales, the real yeasts. This pathogen causes candidasis in immunocompromized patients suffer in AIDS, cancer and diabetis mellitus (Nielsen & Heitman, 2007). Invasive mycoses cause high morbidity and mortality rates in severely ill patients. *Candida*, *Cryptococcus*, *Pneumocystis* and *Aspergillus* are most prevalent agents for mycoses (Peman & Salavert, 2012).

Aspergilli are heterogeneous according to their benefits and disadvantages they bring to mankind. Among the *Aspergillus* genus that comprises about 190 species most *Aspergilli* are non-pathogenic saprophytic soil organisms. Despite of this, inhaling their spores can result in different types of respiratory hypersensitive disorder. Mainly three *Aspergillus* species were classified as human pathogens in immunocompromized patients. These are *A. flavus*, *A. terreus* and *A. fumigatus*. *A. flavus* favors hot climate, whereas *A. fumigatus* is mainly present in temperate climates. Both species cause invasive pulmonary aspergillosis leading to death in more than 90% of the patients (Sethi, *et al.*, 2012, Pabst, *et al.*, 2013). *A. parasiticus* and *A. flavus* produce the carcinogenic aflatoxin and are often found in crops representing a permanent problem in food industry (De Lucca, 2007).

1.1.3 Symbiotic fungi

During symbiosis a community between fungus and another organism, mostly plants and trees, are formed. In contrast to parasites, this relationship is in mutual advantage and is called mycorrhiza. Fungi often have important mycorrhizal symbiosis with other organisms. Particularly 90% of plants have some kind of mycorrhizal relationship with various fungi and are dependent upon this relationship for their survival (Smith & Read, 1997). For instance, mycorrhiza is specifically employed to give roses an excellent start for their growth. In this symbiosis fungi enhance the uptake of water and minerals for the plant and get sugar compounds in return. A successful growth and development of many orchids also requires symbiosis with specific fungi.

The Agaricomycetes *Amanita muscaria* is capable to undergo symbiosis with different

trees, whereas *Leccinum scabrum* and *Suillus viscidus* form their symbiosis with a specific tree (Reis, *et al.*, 2011). Fungi form another symbiosis together with algae result in lichens. As for the symbiosis between fungus and plant, algae deliver the fungus with carbohydrates synthesized during photosynthesis and get water and minerals by the fungus (Perrine-Walker, *et al.*, 2011, Ba, *et al.*, 2012, Zambare & Christopher, 2012).

1.1.4 Saprophytic fungi

Fungal growth and differentiation are very energy consuming processes and require exploitation of external energy sources. Along with fungi numerous saprophytic pro- and eukaryotic organism genomes contain and express several genes encoding enzymes secreted for the hydrolysis of cell wall material derived from dead animals, plants, fungi and bacteria. Cell wall composition of the different organisms is specific for the kingdoms. Plant cell wall degraded by saprophytic organisms like *A. nidulans* is primarily composed of a primary, secondary layer and middle lamella (Buchanan, *et al.*, 2000). The primary layer consists of pectins, cellulose, hemicellulose and glycoproteins. Xylan belongs to hemicelluloses and is also part of the primary plant cell wall. The epidermis an outer part of the primary plant cell wall consists of cutin and wax generating the plant cuticle a permeability barrier. Waxes protect the plant from drying-out. Suberin or cutin two epidermal polyester-like polymers protect the cell from herbivores (Moire, *et al.*, 1999). The secondary plant cell wall named cuticula consists of microfibrilcellulose and hemicellulose which strengthen and waterproof the wall additionally. Plant cell wall hydrolysis requires specific enzymes. Among them are xylanases, pectinases, cutinases, polygalacturonase etc. In contrast, bacterial cell walls are mainly composed of peptidoglycan which is also called murein (van Heijenoort, 2001). Muramidases are able to hydrolyse murein.

Polysaccharides like starch and lichenin which assure energy storage in plants are also at fungal disposal. Thereby lichenin is mainly synthesized in moos and lichen for long-term energy storage. Saprophytic fungi contain and secret enzymes like amylases and licheninase for the utilization of such external polysaccharides. Hydrolysis of starch requires amylases. The *A. nidulans* genome comprises seven known amylase genes (*amyA – amyF, glaA, glaB*) (Nakamura, *et al.*, 2006). Beside starch, lichenin presents a further polysaccharide with an immense meaning for the survival of countless organisms since licheninases are conserved from prokaryotes to eukaryotes. For instance, the *eng2* orthologue *xgeA* (AN2385) from *A. nidulans* is also thought to be a putative GPI anchored endo-1,3(4)-beta-glucanase (Bauer, *et al.*, 2006, de Groot, *et al.*, 2009). XgeA possesses also licheninase activity. Licheninases are

also present in other *Aspergilli* like *A. japonicus* and were tested extensively (Grishutin, *et al.*, 2006).

1.2 Secondary metabolism

Secondary metabolites are characteristic for plant and fungi and are low-molecular-weight chemicals that have potent physiological effects on living organisms. Morphine, atropine, cocaine, tannin and resin are typical plant secondary metabolites whereas penicillin, aflatoxin and its precursor sterigmatocystin, the aflatrem paxilline, asterriquinones, emericellin etc. are classified as fungal specific secondary metabolites. Secondary metabolites often play an important role in defense against herbivores in plants and fungivores in fungi (Stamp, 2003, Yin, *et al.*, 2012).

Their synthesis has to be adapted to different environmental conditions that might harm and damage fungal mycelia and developmental structures. Thus, secondary metabolites escort and protect the fungus. *Aspergillus nidulans* serves as an amenable model system to study fungal development and secondary metabolite production. The importance of the closely related species either in medicine (*A. fumigatus, A. flavus*) or biotechnology (*A. oryzae*, *A. niger*) makes *A. nidulans* more important as a model system. Various protective secondary metabolites namely antibiotics, pigments etc. are synthesized and present in *A. nidulans*. While some secondary metabolites are designed to attack pro- and eukaryotic organisms in their neighborhood, others protect the fungus from UV radiation, or serve as chemical signals that enable fungal response to environmental stimulus.

Compared to primary metabolites that are primarily important for organisms to maintain their cellular physiology, secondary metabolites are not essential for the growth of the fungus (Fraenkel, 1959). Unlike primary metabolites absence of secondary metabolites does not result in immediate death, but rather in long-term impairment of the organism's survival, fertility, or phenotype. Secondary metabolites are often restricted to few species within a phylogenetic group.

Secondary metabolites can be grouped into four major categories: alkaloids, peptides, polyketides and terpenes, depending on their structure and synthesis. Alkaloids: Along with the dimethylallyl pyrophosphate and tryptophan derived gibberellin GA3, trichothecene T2 toxin and aristolochene (Keller, *et al.*, 2005) the indolocarbazole alkaloid staurosporine is also a specific fungal product with antitumor properties (Sanchez, *et al.*, 2005). Many are extremely poisonous to humans. Peptide secondary metabolites are divided into non-ribosomal (NRP) and ribosomal peptides. The first fungal NRP to be found was the

immunosuppressant cyclosporine. Phytopathogenic fungi produce NRP-like HC-toxin (*Helminthosporium carbonum*) (Walton, 2006), AM-toxin (*Alternaria alternata* pv. *Mali*) (Johnson, *et al.*, 2000) and the *Cochliobolus victoriae* specific victorin. Penicillin G and gliotoxin belong to the NRP group as well. Microcin J-25 is a ribosomal peptide. Polyketides are a large group of fungal secondary metabolites exhibiting heterogeneity regarding their chemical structure and pharmacological properties. Zearalenon, alternariol, aflatoxin, sterigmatocystin, erythromycin or the polyen-antimycoticum amphotericin B are prominent fungal polyketides. Terpenes are a prevalent and huge group of natural compounds already applied in cancer- and malaria-therapies. Over 22,000 terpenes have been described to date. They include steroids, which, like alkaloids, are eminently useful in medicine. Steroids are complex compounds that all have the same basic structure. Little structural modification results in different compounds with different properties, such as male and female sex hormones. Alpha-, beta- and gamma-carotenes, retinol, lanosterol, a precursor of steroids, resin etc. are among the important terpenes. The medically relevant tremorgenic paxilline is an indole diterpene produced by *Penicillium paxilli* and also present in *Aspergilli* sp. (Nicholson, *et al.*, 2009).

1.2.1 Antibiotics in *A. nidulans*

Since many hundred million years fungal kingdom members have developed different strategies to protect themselves from various changing abiotic and biotic environmental threats. Along with abiotic threats like UV-radiation, reactive oxygen species, aridity, heavy metals, pH, heat and cold, biotic threats like amoeba, insects, nematodes, and bacteria pose potential threat to fungi. Numerous secondary metabolites such as antibiotics secreted by the fungi or soil bacteria serve as growth inhibitor for competing organisms in soil. Apoptosis inducing compounds like farnesol or staurosporine are further biotic derived chemo-tactic weapons (Berkova, *et al.*, 2006, Semighini, *et al.*, 2006). Like farnesol in *A. nidulans*, staurosporine is connected to the induction of apoptosis in *A. fumigatus*. Thereby, secondary metabolites have a central position for fungal protection. Various protective secondary metabolites are located inside the fungus serving as passive protection such as pigments or repellents. In contrast, several secondary metabolites are known to be active against specific pro- or eukaryotic organisms and are secreted actively.

An *Emericella* sp. specific antibiotic is emericellamide that was first found in two different conformations/forms namely emericellamide A and B, respectively (Oh, *et al.*, 2007). Emericellamides are antibiotic compounds of mixed origins with polyketide and amino

acid building blocks. Induction of emericellamide A and B production, by the marine-derived fungus *Emericella* sp., was observed during co-cultivation with the marine actinomycete *Salinispora arenicola*. Five emericellamide derivatives are already identified in *A. nidulans* and called emericellamide A - F. Beside isolation from co-cultivation, emericellamides could also be isolated from four days vegetatively and five days asexually induced *A. nidulans* cultures grown in or on YAG complex medium, respectively (Szewczyk, *et al.*, 2008, Chiang, *et al.*, 2009).

Orsellinic acid belongs to the secondary metabolites like emericellamides whose expression and production was found to be induced in co-cultivation with prokaryotic organisms (Schroeckh, *et al.*, 2009). Thereby, orsellinic acid was claimed to be another antibiotic that is produced by *A. nidulans* in co-cultivation with *Streptomyces hygroscopicus*. Expression of *ors* genes is activated at bacterial co-cultivation. In contrast, *ors* gene expression is beneath microarray detection threshold and orsellinic acid could not be detected at standard laboratory conditions in *A. nidulans* wild type without *Streptomyces*. As for orsellinic acid, it was shown that production of bacterial induced secondary metabolites like penicillin or sterigmatocystin depends on the activation of histone acetylases (Nutzmann, *et al.*, 2011). These histone acetylases encoded by *ngn* genes belong to the GNAT-type acetyltransferase family.

Though several secondary metabolites are already identified and characterized, overall profile of secondary metabolites in *A. nidulans* has not been studied by analyzing various developmental stages.

1.2.2 Toxins for protection in *A. nidulans*

One of the best characterized secondary metabolites in *A. nidulans* is sterigmatocystin (ST) (Brown, *et al.*, 1996, Butchko, *et al.*, 1999, Bok, *et al.*, 2006). As a precursor of aflatoxin, which is a known mycotoxin causing Turkey X disease in humans, ST in *A. nidulans* is used to analyze the regulation of the pathway of this carcinogenic, human-pathogenic relevant compound. We could show that ST concentration is highest in sexually differentiated *A. nidulans* grown for 3 d at 30°C in darkness (Bayram, *et al.*, 2010). Genes important for the synthesis of ST are clustered within a huge gene cluster. It was shown that the expression of the essential transcription factor *aflR* which regulates the proper expression of all *stc* genes required for ST production in *A. nidulans* is *laeA*-dependent. Therefore, *laeA*-dependent gene regulation of secondary metabolites is chromosome location dependent. This might be most likely mediated by local histone methylation since LaeA was reported to

control the methylation of histones like H3K9 (Histone 3, Lysine 9) (Keller, *et al.*, 2005, Bok, *et al.*, 2009, Reyes-Dominguez, *et al.*, 2010).

An asterriquinone synthesized by *A. nidulans* is terrequinone A. Most isolated asterriquinones are cytotoxic compounds that have been shown to intercalate genomic DNA, thus predisposing tumor cells to apoptosis (Kaji, *et al.*, 1997). Terrequinone A is a fungal benzoquinone with anti-tumor properties (He, *et al.*, 2004). Like many other identified secondary metabolite genes, terrequinone *tdi* synthesis genes are clustered. The *tdi* gene cluster was also shown to be *laeA*-dependent regulated (Bok, *et al.*, 2006). *tdi* genes are upregulated in *A. nidulans* wild type at 48 h and 60 h asexual development. Furthermore *tdiA* and *tdiB* expression is decreased in strains deleted for the mitogen-activated protein kinase encoded by *mpkB* (Atoui, *et al.*, 2008)

1.2.2.1 Repellents in *A. nidulans*

Rohlfs and co-workers (2007) showed that fungal mutants with impaired production of various secondary metabolites, which might serve as a chemical shield, are favorite targets for fungivores. Even fungivore insects prefer fungi with lacking secondary metabolites (Rohlfs, *et al.*, 2007, Rohlfs & Churchill, 2011).

Repellents are produced by many organisms protecting them against predators. Numerous plants developed physical defenses to deter herbivores but also invest in chemical defense mechanisms as feed protection (Moore, *et al.*, 2007). For instance, methylated anthranilate is synthesized and accumulated in concord grapes and other plants and serves as repellant against birds (Wang & De Luca, 2005). Feed protection is not restricted to eukaryotic organisms but is also present in prokaryotes. For protection, bacteria incorporate endotoxins like Lipooligo- (LOS) and lipopolysaccharides (LPS) within their membrane (Rivest, *et al.*, 2000). These compounds are localized in the outer membrane of gram-negative bacteria and are essential for their structural integrity as well as for protection of the membrane from certain kinds of chemical attacks (Rietschel, *et al.*, 1994, Rietschel, *et al.*, 1996, Raetz & Whitfield, 2002, Wang & Quinn, 2010). LOS and LPS increase the negative charge of the bacterial cell membrane and help to stabilize the overall membrane structure. They are endotoxins that cause several diseases in humans and animals. It has also been implicated in non-pathogenic aspects like surface adhesion and interactions with predators such as amoebae. Since a rough LPS is more hydrophobic, Gram negative bacteria have more penetrable cell membranes to hydrophobic antibiotics. LOS and LPS synthesis is not described in *Aspergilli* ssp. However, two proteins encoded by *breA* (AN7123) and AN1663

are thought to be implicated in a possible lipooligosaccharide synthetic process. The *Streptomyces*, synthesizing antifungal *b*afilomycin, *re*presses *breA* in *A. nidulans* (Melin, *et al.*, 1999). Bafilomycin is targeted to a lipopolysaccharide synthetic pathway that might be directed against passive diffusion/infiltration of several antifungal compounds since gram-negative and -positive bacteria exhibit a different LPS dependent cell membrane permeability.

Isoquinole alkaloids appear to be protection from grazing animals and herbivorous insects in plants as well. Among all compounds found in plants, alkaloids are the most powerful and very effective. The strength or effectiveness of the alkaloids commonly includes all substances that are poisonous in plants. Prominent isoquinole alkaloids are divided into several sub classes and comprise isoquinolines, benzylisoquinolines, phthalideisoquinolines, protopines, morphine alkaloids and protoberberines. Beside its function as repellent berberine exhibits mild antibiotic properties. Berberine possesses antifungal activity against *Candida albicans*, yeast, parasites, and also activity against bacterial or viral infections (Birdsall & Kelly, 1997, Gibbs & Seddon, 2000, Yu, *et al.*, 2005, Ozbalci, *et al.*, 2010). Berberine seems to exert synergistic effects with fluconazole even in drug-resistant *Candida albicans* (Xu, *et al.*, 2009). Berberine synthesis has not been shown in *Aspergilli* yet.

1.2.2.2 Polyamines in *A. nidulans*

Spermine, spermidine and putrescine are polyamines, which are known to have signaling properties in many organisms (Dudley, *et al.*, 1926, Tabor & Tabor, 1985, Ruiz-Herrera, 1994, Lopez, *et al.*, 1997). Like for the plant hormone ethylene also polyamines were shown to force wilting in plants that would also enhance the supply with material for a lifestyle of a saprophytic organism like *A. nidulans* (Pandey, *et al.*, 2000). Polyamine synthesis starts from the non-proteinogenic amino acid ornithine. Spermine is converted from spermidine and is an important growth factor in bacteria. In plants, spermidine is a growth regulating hormone promoting somatic embryogenesis (Bouche, 1981, Tabor & Tabor, 1984, Traynelis & Cull-Candy, 1990). It was also shown to retard aging in yeast, worms, flies and human tissue mediated through induction of autophagy (Eisenberg, *et al.*, 2009). Putrescine is synthesized in small quantities by healthy cells through ornithine decarboxylase activity. The polyamines, of which putrescine is one of the simplest, appear to be growth factors required for cell division. It is toxic in large doses and an apoptosis-inducing agent in the highly competitive habitat (Til, *et al.*, 1997).

Polyamine synthesis is present in *A. nidulans* (Jin, *et al.*, 2002). Spermidine synthesis is mediated through the spermidine synthase encoded by *spdA* (AN0687). Deletion of *spdA*

results in a phenotype affected in germination, increased asexual sporulation but decreased in sterigmatocystin (ST) production. ST production could be restored by addition of exogenous spermidine. The impact of polyamines on aflatoxin production in *A. parasiticus* could also be shown through inhibition of ornithine decarboxylase required for polyamine synthesis (Guzman-de-Pena, *et al.*, 1998). Respective strains reveal decreased aflatoxin production. Consequently, polyamines are also involved in toxin production and possess a connection to fungal defense. Beside all important cell differentiation processes including sporulation and secondary metabolite production polyamines are involved in, it remains an open question whether they also have toxic properties when they are secreted and might induce apoptosis with other competitors in soil as it was reported by Til and co-workers (Til, *et al.*, 1997).

1.2.3 Fungal oxylipins and development

Oxylipins are a class of hormones present in filamentous fungi (Brodhun & Feussner, 2011). Oxylipins represent oxygenated natural compounds converted from fatty acids through at least one step of dioxygen-dependent oxidation (Gerwick, *et al.*, 1991). Numerous oxylipins have physiological relevance. They are prevalent in all three kingdoms, including plants, animals and fungi (Gobel & Feussner, 2009). Animal oxylipins (eicosanoids) often have opposite effects in cell tissue. Some eicosanoids are pro-inflammatory whereas others are anti-inflammatory and mediate decay processes resulting in tissue injuries. Oxylipins in plants mainly control reproduction, ontogenesis and resistance to various pathogenic microorganisms or other plant vermin. Plant oxylipins include various fatty acid hydroperoxides, hydroxy fatty acids, aldehydes and the hormones 12-oxo phytodienoic acid (OPDA) and jasmonic acid (JA) amongst others (Grechkin, 1998). Along with other phytohormones like auxin, abscisic acid, and gibberellic acid, jasmonate regulates plant growth, development and aging. Phytohormones like jasmonate have an additional role as signal in defense to abiotic and biotic stress.

Studies on oxylipins and fungal enzymes crucial for their formation have been initiated 25 years ago (Matsuda, *et al.*, 1978, Hamberg, 1986). However, the exact physiological function of oxylipins is still rudimentarily. In contrast, an impact of oxylipins on the development of *Aspergillus nidulans* could be shown (Calvo, *et al.*, 2001, Tsitsigiannis & Keller, 2007). Oxylipin-forming enzymes can be predicted in a number of fungal species since the genomes of numerous fungi have been sequenced completely and published (Galagan, *et al.*, 2003, Galagan, *et al.*, 2005). Among them are several dioxygenases mediating synthesis of oleic- and linoleic acid derived oxylipins. Defined ratio of oleic- and

linoleic acid derived oxylipins are required as trigger for proper developmental regulation in *Aspergilli* (Champe, *et al.*, 1987, Champe & el-Zayat, 1989). For instance, these oxylipins stimulate early sexual development through inhibition of asexual sporulation in *A. nidulans*. Thus, these oxylipins are called *psi* (*p*recocious *s*exual *i*nducer) factors. They are related to prostaglandin hormones secreted by several mammalian tissues. *psi*-factor receptors have not yet been identified in *Aspergilli*. Three *psi*-factors *p*roducing *o*xygenases (*ppo*) have been described in *A. nidulans* (Tsitsigiannis, *et al.*, 2005, Tsitsigiannis & Keller, 2006). PpoA - C have direct influence on development. Deletion of *ppoA* results in increased conidiation concluding that the dioxygenase PpoA inhibits asexual development (Tsitsigiannis, *et al.*, 2004). Expression of *brlA* encoding for a central regulator of asexual development is increased in Δ*ppoA* strains. Thus, inhibitory effect of PpoA on asexual differentiation is mediated through inhibition of *brlA* expression. *PsiB1*χ (10-HOD, 10-hydroxy-9,12 octadeca dienoic acid) and *psiB1*α (8-HOD, 8-hydroxy-9,12-octadecadienoic acid) biosynthesis depend on PpoA presence and are prerequisites for sexual development levels. In addition to the asexual inhibitory effect PpoB also stimulates ascospore formation. PpoB is required for *psiB1*β (8-HOE, 8-hydroxy-9-octadecanoic acid) synthesis. However, specific 8-HOE:8-HOD ratios are characteristic for defined differentiation cycles in *A. nidulans*. This ratio shifts from 1:8 in dark (sexual) to 1:3 in light (asexual). The third dioxygenase encoded by *ppoC*, is essential for proper *psiB1*β (8-HOE) and *psiC1*α (5,8-DiHOD) levels and effects the ratio of asexual to sexual spores. In contrast to the other dioxygenases, PpoC stimulates asexual development. A delayed *brlA* and an increased *nsdD* expression appear in the Δ*ppoC* strain consequently (Tsitsigiannis, *et al.*, 2004). Stimulation of asexual development is mediated by PpoC through exact induction of *brlA* accompanied by the inhibition of *nsdD* expression.

In contrast to hydroxylated derivatives of linoleic acid, the impact of hydroxylated oleic acid *psi*-factors (*psiA1*β (*psiC1*β with lactone ring at C-5) and *psiC1*β (5,8-DiHOE)) on *A. nidulans* development is mostly unknown. Calvo and co-workers (Calvo, *et al.*, 2001) analyzed a desaturase *odeA* deletion strain and could show that this strain is depleted of polyunsaturated fatty acids (18:2 and 18:3) but increased in oleic acid (18:1). The total amount of linoleic acid derived *psi*-factors exhibit massive decrease whereas oleic acid derived *psi*-factors concentration is increased. Development of the deletion strain shifted to sexual direction since ascospore formation was elevated. They concluded that oleic acid derivatives have an effect on the asexual to sexual spore ratio in *A. nidulans*.

Beside the already characterized desaturases *odeA* (AN1037), another monofunctional oleoyl-Delta12 desaturase encoded by *an2* (AN7204) reveals the same expression pattern as

odeA. An2 was biochemically and structurally characterized but the effect of an *an2* deletion on *A. nidulans* development has not been analyzed yet (Hoffmann, *et al.*, 2007).

psi-factors in *A. nidulans* are prominent examples for the tight connection between development and secondary metabolite production since they have impact on fungal development and secondary metabolite production simultaneously. Δ*ppoA* strains possess an increased sterigmatocystin production whereas *ppoB* deletion results in an increased penicillin concentration additionally. Alterations in the sterigmatocystin level are detectable in *ppoC* mutants. *ppoA*/*ppoC* double mutants cannot produce sterigmatocystin, whereas lack of *ppoB* increases sterigmatocystin levels (Tsitsigiannis, *et al.*, 2005).

Deletion in *csnE* encoding a fungal COP9 signalosome deneddylase in *A. nidulans* results not only in an impaired sexual development but also in changes in the secondary metabolism (Nahlik, *et al.*, 2010, Gerke, *et al.*, 2012). Expression pattern of *ppoA* and *ppoC* is altered in this mutant. Increased *ppoA* expression might explain why the mutant is unable to repress sexual differentiation in light.

1.3 Development of fungi

1.3.1 *Aspergilli* and development

Most filamentous fungi have to develop vegetative hyphae before they can induce other developmental programs (Axelrod, *et al.*, 1973). Vegetative growth starts with the germination of a spore that could be either a mitotically derived conidiospore or a meiotically formed ascospore. The germinated spores establish tubular hyphae growing in a polar manner through apical extension of the *Spitzenkörper*. Branched hyphae form a network of interconnected cells named as mycelia. Although mycelia seems to be similar and homogenic, in fact it contains diverse cells possessing special functions such as in the uptake of nutrients from the environment and in determining the precise time to synchronize the formation of reproductive structures (Adams, *et al.*, 1998). Among filamentous fungi two different ways of reproduction have developed. During asexual development asexual spore-bearing conidiophores are established generating conidia. Sexual life-cycle of ascomycetes can be either homothallic (self-fertile) or heterothallic. In contrast to homothallic, heterothallic ascomycetes require the presence of an opposite mating type to develop sexually. Sexual development is completed with the maturation of the sexual fruit body cleistothecia. Numerous *Aspergilli* do not possess any known sexual cycle as for instance *A. niger* or *A. flavus*.

The ascomycete *A. nidulans* is a homothallic (self-fertile) filamentous fungus capable

to undergo both differentiation pathways. Developmental competence in *A. nidulans* is achieved between 14 – 20 hours after spore germination. Environmental signals do result in the initiation of differentiation of the vegetative hyphae to asexual or sexual structures depending on illumination and aeration conditions (Braus, *et al.*, 2010, Helmstaedt, *et al.*, 2011). Any impairment on developmental program is often accompanied by the loss of certain secondary metabolites that serve for nutrient supply, fungal defense or as communication signal with the environment (Bayram, *et al.*, 2010, Rodriguez-Romero, *et al.*, 2010).

1.3.2 Asexual development of *A. nidulans*

After asexual induction of *A. nidulans* in light an approximately 70 µm long stalk is formed. The stalk swells and forms a vesicle at its tip. Metulae are formed from these multinuclear sterigmata through budding. From the metulae, a second row of mononuclear sterigmata, the phialides, originate through budding. Finally, multiple conidia arise from the phialides through asymmetric cell division. Conidial laccase (p-diphenol oxidase, *yA*, AN6635) activity ensures proper conidiospore formation and production of the dark green pigment in the conidium cell wall (Clutterbuck, 1972, Aramayo & Timberlake, 1990). Pigments most likely absorb UV-radiation and protect asexual spores.

Asexual differentiation in *A. nidulans* is a light-induced process regulated by various well-described and analyzed genetic elements. Important factors for the light induced asexual cycle are the transcription factors: BrlA, WetA, MedA, and VosA (Clutterbuck, 1969, Boylan, *et al.*, 1987, Adams, *et al.*, 1988, Ni & Yu, 2007). BrlA is a C_2H_2 zinc finger type transcription factor responsible for the light induced activation of conidiophore development. Overexpression of *brlA* results in an increased expression of downstream asexual development regulators like *wetA*, *abaA*. Reduced *brlA* expression is accompanied by a decreased sterigmatocystin production (Wieser, *et al.*, 1997) demonstrating the co-regulation of secondary metabolism with asexual development of *A. nidulans*.

Light controls development in *A. nidulans*. Therefore, the fungus comprises several light sensing complexes for the detection of red, blue and green light. In *A. nidulans,* red light triggers asexual conidiation while inhibiting sexual fruit body formation through the red light receptor and phytochrome FphA. *fphA* mutants are blind to red light resulting in the disappearance of the red light inhibitory effect on sexual development (Blumenstein, *et al.*, 2005).

Table 1: Asexual regulators of the filamentous fungus *A. nidulans*

Gene	Function / Deletion	References
brlA	Light induced regulator of conidiophore development	(Adams, *et al.*, 1988)
wetA	Mutant lacks conidia pigment and autolyze before maturation	(Boylan, *et al.*, 1987)
medA	Multiple tiers of sterigmata in the mutant strain	(Clutterbuck, 1969)
abaA	Required for phialide differentiation	(Boylan, *et al.*, 1987)
vosA	Nuclear protein for spore formation and trehalose accumulation. Mutants suffer from low spore viability	(Ni & Yu, 2007, Bayram & Braus, 2012)

Selected regulators of asexual development in *A. nidulans*. The table shows additional information about their exact function and/or effect of a deletion on fungal development. The corresponding references are listed in the third column on the right side.

In contrast, the blue light receptor and cryptochrome CryA is a nuclear localized protein that senses UVA and blue light and represses sexual development by regulating regulators such as VeA, NsdD and RosA (Bayram, *et al.*, 2008). Deletion of *cryA* results in a strain defective in light response leading to abnormal formation of sexual structures. Hülle cells are formed in submerged cultures and cleistothecia formation is absent.

lreA encodes a putative zinc-finger transcription factor involved in blue-light responsive differentiation (Purschwitz, *et al.*, 2008). It is a homologue to *Neurospora crassa* blue-light-sensing component White Collar 1 (WC-1). *A. nidulans* LreA is a positive regulator of sexual development and interacts with the zinc-finger transcription factor LreB. It is homologue to the blue-light-sensing component WC-2 in *N. crassa* and is involved in blue-light response. Beside its interaction with LreA, LreB also interacts with the sexual regulators VeA and FphA. As described for LreA also LreB is a positive regulator of sexual development in *A. nidulans*.

The still uncharacterized green light receptor NopA is a homologue to the bacterial rhodopsin family G-protein coupled receptor-like proteins (Lafon, *et al.*, 2006). An impact of NopA on *A. nidulans* development has not been shown yet.

1.3.3 Sexual development of *A. nidulans*

A. nidulans is capable to undergo sexual differentiation. Its sexual form has been named *Emericella nidulans*. In darkness and at anoxia with low O_2/CO_2 partial pressure sexual differentiation is induced whereas it is inhibited at light and hypoxia. Specialized vegetative hyphae start budding, producing Hülle cells. Hülle cells in *A. nidulans* have strong

phenol oxidase activity due to the accumulation of laccase type II enzyme (*cpeA*, AN7388) (Scherer, *et al.*, 2002). The consequence of phenolic compound oxidation is reactive oxygen species (ROS) formation. Therefore, Hülle cells as oxidative active structures employ enzymes like CpeA and NoxA that trigger and control ROS levels (Lara-Ortiz, *et al.*, 2003). Deletion of the sexually expressed *noxA* results in a diminished superoxide concentration and accumulation of Hülle cells and primordia as cleistothecia precursors blocked in development. The Hülle cells surround the fruit body during the complete development (Zonneveld, 1975). They are thought to nurse developing cleistothecia. Two days after sexual induction primordia become visible in a nest-like structure, which matures to a micro-cleistothecium. Specialized ascogenous hyphae fuse forming multinuclear hyphae. The resulting zygote undergoes meiosis and consecutive mitosis. Finally, within the sac like structure, the ascus, sexually formed ascospores are formed. The mature closed sexual fruit body of *A. nidulans* is called cleistothecium and contains numerous asci with sexual proliferation units, the ascospores.

Sexual development in *A. nidulans* employs several genetic elements (Tab. 2). For instance, it is regulated by the velvet family proteins VelB, VeA, methyltransferase LaeA, Ime2 like kinase ImeB, transcription factors NosA, NsdD and SteA (Mooney, *et al.*, 1990, Han, *et al.*, 2001, Bok & Keller, 2004, Vienken & Fischer, 2006, Stinnett, *et al.*, 2007, Bayram, *et al.*, 2008, Purschwitz, *et al.*, 2008, Bayram, *et al.*, 2009). The putative histone methyltransferase LaeA (loss of aflR expression A) is a part of the VelB/VeA/LaeA velvet complex and is required for a proper sexual development and also controls secondary metabolite production. Deletion of *laeA* results in smaller cleistothecia, less Hülle cells and a delay in the formation of sexual ascospores. Furthermore, secondary metabolite production is decreased in the *laeA* deletion strain (Bok, *et al.*, 2006). Bayram and co-worker (2010) could show that *laeA* null mutants exhibit constitutive sexual differentiation revealing LaeA as an essential inhibitor of sexual differentiation in light (Sarikaya Bayram, *et al.*, 2010). LaeA is an example revealing that development and secondary metabolism in *A. nidulans* are closely connected to each other. Deletion of *veA* or *velB* also results in a decreased secondary metabolite production and in a misregulation of sexual development (Kato, *et al.*, 2003, Bayram, *et al.*, 2008). ImeB is a serine/threonine protein kinase involved in light-mediated regulation of sexual development in *A. nidulans*. The *imeB* deletion strain reveals slower growth. Constitutive sexual induction is detectable in this strain. Hülle cells are formed in submerged cultures as it was seen for the Δ*cryA* strain, but light could not inhibit cleistothecia formation. Sterigmatocystin production is impaired in the Δ*imeB* strain (Bayram, *et al.*, 2009).

Table 2: Sexual regulators of the filamentous fungus *A. nidulans*

Gene	Function / Deletion	References
veA	Involved in light-sensitive control of differentiation and secondary metabolism	(Mooney, *et al.*, 1990, Bayram, *et al.*, 2008)
velB	Along with VeA and LaeA coordination of development and secondary metabolism	(Bayram, *et al.*, 2008)
laeA	See *velB*	(Bayram, *et al.*, 2008)
imeB	Constitutive sexual structure formation	(Bayram, *et al.*, 2009)
nosA	Mutants with immature cleistothecia and reduced numbers of ascospores	(Vienken & Fischer, 2006)
nsdD	Cleistothecia are absent from mutants	(Han, *et al.*, 2001)
steA	Mutant are blocked in sexual cycle, forms Hülle cells but no ascogenous tissue nor cleistothecia	(Vallim, *et al.*, 2000)

Selected regulators of sexual development in *A. nidulans*. The table shows additional information about their exact function and/or effect of a deletion on fungal development. The corresponding references are listed in the third column on the right side.

Another Zinc(II)2Cys6 transcription factor involved in the regulation of sexual development is NosA (Number of sexual spores). The corresponding deletion strain produces immature cleistothecia and reduced numbers of ascospores (Vienken & Fischer, 2006). NsdD (Never in sexual development) is a further zinc-finger transcription factor of GATA-type required for sexual development. The absence of NsdD causes a defective strain not able to be induced for sexual development. Mutants of STE-like transcription factor with homeobox and zinc finger domains containing SteA (Sterile12 like) are blocked in sexual differentiation. Asexual development remains unaffected by this deletion. Δ*steA* forms Hülle cells but no ascogenous tissue or cleistothecia (Vallim, *et al.*, 2000).

1.3.4 Amino acids and sexual development

Generally, amino acids are crucial for the decision of whether development in fungi takes place or not. Limitation results in the induction of a genetic network, which affects genes for enzymes of several amino acid synthetic pathways and for aminoacyl-tRNA synthases. In filamentous fungi this genetic system is called *c*ross*p*athway-*c*ontrol (*cpc*) (Braus, *et al.*, 2004). The *A. nidulans* genome comprises two *cpc* genes, *cpcA* and *cpcB*. Both are involved in cross-pathway control in response to amino acid starvation and are required for sexual development. Deletion of the Gcn4p c-Jun-like transcriptional activator *cpcA* causes a block in sexual development at the stage of microcleistothecia development (Hoffmann, *et al.*, 2000). A similar phenotype is observed for the *cpcB* mutant revealing a

block after microcleistothecia formation. Generally, fruit body formation in *A. nidulans* is not induced at amino acid starvation conditions. A corresponding system is also present in the baker's yeast *Saccharomyces cerevisiae* and is named general control (Braus, 1991, Hinnebusch, 2005). Gcn4p (General Control Nonderepressible) is a basic leucine zipper (bZIP) transcriptional activator of amino acid biosynthetic genes that mediates response to amino acid starvation in yeast. *GCN4* mutants reveal a general growth defect. They are decreased in vegetative growth and increased in filamentous/pseudohyphal growth.

In the ascomycete *Sordaria macrospora* arginine controls *cyn1* encoding a cyanase transcriptionally resulting in a defective ascospore germination at the lack of arginine. (Elleuche & Poggeler, 2008). Expression of this cyanase, important for ascospore formation, is markedly decreased by the addition of arginine.

1.3.5 Influence of the environmental factors on fungal growth and development

In permanent contact with their environment fungi have to face several external abiotic parameters like temperature, pH, aeration, light and nutrient supply for growth, differentiation and propagation (Takaya, 2009, Bayram & Braus, 2012, Dyer & O'Gorman, 2012). Temperature affects enzymatic activity and growth. A balanced homeostasis depends on the pH value, which is optimal for fungal growth at pH < 7, which means a preference for more acidic pH. Solar radiation possesses a broad wavelength spectrum. Defined wavelengths are environmental signals that trigger fungal differentiation. Depending on the type of the signal, a fungal organism can develop from vegetative into asexual and sexual growth phase. The nutrition factor is most important for fungal development. Acquisition of the carbons (mono-, di- and polysaccharides, glycerol, acetate) and nitrogen sources (nitrate, ammonium, glutamate, amino acids and peptides) is crucial for the synthesis of macromolecules. Nucleic acids, lipids and proteins are required for the formation of complex differentiated structures like fruit bodies. As biotic factors competing organisms influence fungal growth and development, being a soil inhabitant brings many challenges including competition for nutrients and being eaten by stronger organisms, which makes a range of defense mechanisms emerge against potential danger. Competitors might secret factors like chitin hydrolyzing enzymes or anti-fungal compounds, which impair fungal growth. Antibiotics like penicillin produced by *Penicillium* and other filamentous fungi possess a broad spectrum of activity against Gram-positive *Streptococcus* ssp. and *Staphylococcus* ssp. (Garrod, 1960, Brakhage, *et al.*, 2004, Houbraken, *et al.*, 2011). Penicillin includes procaine penicillin, benzathine penicillin or penicillin G. However the exact function and impact on physiology, growth and

development of other antibiotics is often less understood.

1.4 Aim of the work

In this work, new insights into changes in the transcriptome during *A. nidulans* wild type asexual and sexual development were of main interest. Developmental decisions require drastic reprogramming of gene expression of developmental and metabolic genes. In order to assess which genes are stage specific, delayed activated or inactivated during the transition of vegetative growth to asexual and sexual development as well as in the course of both development cycles, gene expression of both developmental cycles was compared to vegetative stage. Therefore, strains growing vegetatively had to be induced for asexual (under light) and sexual (in the dark) development. By using two different, independent transcriptome platforms quantity and quality of differentially expressed genes was analyzed. Numerous genes comprised and differentially expressed genes in the genome of *A. nidulans* are still uncharacterized. Therefore, manual sequence analyses required for functional categorization had to be executed. In order to identify novel expressional clusters transcriptomes had to be manually analyzed for expressional hot spots.

Second major goal of this work was to establish an intra- and extracellular metabolic database of *A. nidulans* wild type cultures induced for the same conditions as for the transcriptome analyses. We asked the question whether the expressional changes on the transcriptome level also reflects in metabolite production. During these metabolic analyses, important developmental molecules including oxylipin hormones, amino acids and secondary metabolites were investigated with the aim of getting insights into their regulation during development.

2. Materials and Methods

2.1 Material

2.1.1 Growth media, solutions and buffers

Chemicals used for media, solutions and buffers were obtained from BRAND GMBH & CO.KG (WERTHEIM, GERMANY), INVITROGEN GMBH (KARLSRUHE, GERMANY), SARTORIUS (GÖTTINGEN, GERMANY), MERCK (DARMSTADT, GERMANY), ROCHE GMBH (MANNHEIM, GERMANY), CARL ROTH GMBH & CO.KG (KARLSRUHE, GERMANY), SIGMA-ALDRICH CHEMIE GMBH (STEINHEIM, GERMANY) or FLUKA (NEU-ULM, GERMANY).

2.1.2 *Aspergillus nidulans* strains

A. nidulans FGSC A4 Glasgow wild type strain (FUNGAL GENETICS STOCK CENTER (UNIVERSITY OF MISSOURI, KANSAS CITY, MO, USA) was used as single strain for all performed experiments in this work. A4 strain contains no auxotrophic markers and bear the wild type *veA* gene required for the development and secondary metabolite production.

2.1.3 Material for transcriptome and metabolome analyses

Chemicals, material and devices for transcriptome and metabolome analyses were obtained from AGILENT TECHNOLOGIES (SANTA CLARA, CA, USA), TIGR GRAIG VENTER INSTITUTE (ROCKVILLE, MA, USA), AMBION (AUSTIN, TX, USA), AMERSHAM BIOSCIENCE (PISCATAWAY, NJ, USA), QIAGEN (HILDEN, GERMANY), SARTORIUS (GÖTTINGEN, GERMANY) or MACHEREY-NAGEL (DÜREN, GERMANY), WATERS CORPORATION (MILFOR, CT, USA), J&W SCIENTIFIC (FOLSOM, CA, USA) or PEQLAB BIOTECHNOLOGIE GMBH (ERLANGEN, GERMANY)

2.2 Methods

2.2.1 Cultivation of *Aspergillus nidulans* FGSC A4 for transcriptome and metabolome analyzes

Aspergillus nidulans wild type strain FGSC A4 was grown in or on minimal medium (0.52 $g \cdot l^{-1}$ KCl, 0.52 $g \cdot l^{-1}$ $MgSO_4$, 1.52 $g \cdot l^{-1}$ KH_2PO_4, 0.1 % trace element solution, pH 6.5) + 1% glucose. Vegetative mycelia was obtained from submerged liquid cultures inoculated with 10^6 spores/ml and grown on rotary shaker for 20 h. They were washed with NaCl/Tween20

solution (0,96% NaCl, 0,02% Tween20) and synchronized mycelia were shifted to 2% agar plates containing minimal medium. Cultures were induced 24 h (A24) and 48 h (A48) for asexual development under white fluorescence light (90 μWm^2) containing wavelengths ranging from blue to far red light (400 – 700 nm). Cultures induced for sexual development were cultivated for 24 h (S24), 48 h (S48), 72 h (S72) and 96 h (S96) in the darkness. Thereby plates with sexually induced cultures were wrapped with Parafilm M (BRAND GMBH & CO.KG, WERTHEIM, GERMANY) to avoid further oxygen entering cultures (Clutterbuck, 1974). Conidiospore quantification was modified from Bussink and Osmani (1998) as described by (Bussink & Osmani, 1998, Busch, *et al.*, 2003).

2.2.2 Microscopic analysis

A. nidulans colonies, hyphae and structure picture were taken with a KAPPA PS30 digital camera (KAPPA OPTO-ELECTRONICS GMBH, GLEICHEN, GERMANY) used in combination with a ZEISS AXIOLAB (ZEISS AG, OBERKOCHEN, GERMANY) light microscope or an Olympus SZX12 binocular (Olympus, Hamburg, Germany). KAPPA IMAGEBASE SOFTWARE (KAPPA OPTO-ELECTRONICS GMBH, GLEICHEN, GERMANY) was used for editing pictures and the calibration of magnification.

2.2.3 Transcriptome analysis

2.2.3.1 Sequence analysis

Sequences from *A. nidulans* were retrieved from the NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION (NCBI, gi: 40747330), from BROAD INSTITUTE ASPERGILLUS COMPARATIVE DATABASE and ASPERGILLUS GENOME DATABASE (ASPGD) (Galagan, *et al.*, 2005). Homologue sequences from other organisms also retrieved from the NCBI Entrez Protein, BROAD and ASPGD database (Machida, *et al.*, 2005, Nierman, *et al.*, 2005, Wei, *et al.*, 2007). Sequence comparisons were performed for different criteria e-value, score, conserved domains and thus predicted functions.

2.2.3.2 RNA extraction and quality control

Harvested mycelia from *A. nidulans* cultures induced for different developmental stages were frozen in liquid nitrogen and grounded immediately. Total RNA was extracted from 2 mg grounded culture using TRIZOL™ reagent (INVITROGEN GMBH, KARLSRUHE, GERMANY) as recommended by the manufacturer (Chomczynski, 1993). Crude RNA preparations were dissolved in 250 µl diethyl pyrocarbonate (DEPC)-treated water at 65°C for

12 min. Phenolic precipitation of the RNA probes were performed twice. RNA probes were mixed with one volume phenol/chloroform/isoamylalcohol (25 volume/24 volume/1 volume) and centrifuged (13,000 g, 30 min; 4°C). Aqueous phase was mixed with one volume isopropanol and 20 µl sodium acetate (3M) and placed for 30 min at -20°C. After centrifugation (13,000 g, 30 min, 4°C) pellet was washed twice with 75% ethanol. The dried pellets were dissolved in 200 µl DEPC-treated water at 65°C for 15 min. RNA concentration was measured by Nanodrop ND-1000 (PEQLAB BIOTECHNOLOGIE GMBH, ERLANGEN, GERMANY). Absorbance ratios A_{260}/A_{280} and A_{230}/A_{260} indicating the purity/quality of the samples were determined to be higher than 2.0 for both ratios. RNA was stored at -80°C in 20 µl aliquots. Integrity and composition of the isolated RNA was checked using Bio-analyzer 2100 (AGILENT TECHNOLOGIES, SANTA CLARA, CA, USA).

2.2.3.3 Selected Microarray platforms and processing

2.2.3.3.1 TIGR microarray platform

The TIGR *A. nidulans* version 1 microarrays employed throughout this work contained two replicates with 23,962 oligomers in total (TIGR, J. CRAIG VENTER INSTITUTE, ROCKVILLE, MA, USA). *A. nidulans* version 1 microarrays were used for transcriptome analysis on the first biological replicate of FGSC A4 grown for different periods of sexual and asexual development as well as for the 20 h vegetatively grown culture.

DNA microarray hybridization was performed according to the Ambion (Austin, TX, USA) provided Amino Allyl MessageAmp™II aRNA Kit (AMBION, AUSTIN, TX, USA, Cat. N° 1753). 1 µg purified RNA was used for first and second strand cDNA synthesis. aaRNA was labeled with AMERSHAM BIOSCIENCE (PISCATAWAY, NJ, USA) provided Cy3 and Cy5 dyes respectively. Washed microarray slides were dried through acetonitrile. AGILENT TECHNOLOGIES G2505B Microarray Scanner and program analyzed slides.

2.2.3.3.2 AGILENT microarray platform

For the confirmation of expression data on the first biological replicate with TIGR *Aspergillus nidulans* microarray version 1, sensitive AGILENT TECHNOLOGY custom microarray system was used. AGILENT TECHNOLOGIES custom microarrays were used for the second biological replicate. The ordered custom arrays comprised 10,560 gene transcripts with 35,353 gene exons derived from CADRE (MANCHESTER, UK) based on the third annotation. However three to four independent oligomers per gene provide the opportunity to calculate mean values inside this system. A scoring system for the quality of the spotted 60-

mer oligomers revealed high specificity to the gene exons. 2 µg total RNA from a second biological replicate derived from *A. nidulans* wild type strain FGSC A4 was applied to first strand cDNA (copy DNA) synthesis by Low RNA Input Linear Amplification Kit, PLUS, two color (AGILENT TECHNOLOGIES SANTA CLARA, CA, USA, Cat. N° 5188-5340). Procedure was performed as recommended by the corresponding AGILENT protocol. After first strand cDNA synthesis, cDNA was directly transcribed to cRNA and cRNA immediately cyanine 3 or cyanine 5 labeled, respectively. Labeled cRNA was purified using RNeasy RNA purification Kit (QIAGEN, HILDEN, GERMANY). Yield and concentration of purified labeled cRNA was determined through NanoDrop ND-1000. 0.825 µg of each Cy3-labelled cRNA derived from a defined developmental point of time was mixed with 0.825 µg Cy5-labeled pool cRNA for hybridization with AGILENT *A. nidulans* whole genome custom arrays. Hybridized arrays were washed and analyzed through G2505B Microarray Scanning Unit (AGILENT TECHNOLOGIES SANTA CLARA, CA, USA).

2.2.3.4 Microarray experimental design and statistical analysis

For the TIGR based transcriptional profiling of *A. nidulans* FGSC A4 wild type strain during different developmental stages B-swap design with four microarrays was used for comparisons. Microarray intensity data were extracted using the „Automatic Image Processing for Microarrays“ software (personal communication TRANSCRIPTOME ANALYSIS LABORATORY, HUMBOLDT.ALLEE 23, UNIVERSITY OF GÖTTINGEN, GERMANY). Normalization of the raw intensity data was performed with a non-linear Loess regression method (Yang, *et al.*, 2002). Differentially expressed genes were identified by an ANOVA fixed effects model (Landgrebe, *et al.*, 2004) and adjusted p-values were obtained by the Benjamini-Hochberg method to control the False Discovery Rate (Benjamini & Hochberg, 1995, Bretz, *et al.*, 2005). Normalization and statistical computation was done for two independent datasets derived from a high gain and a low gain scan, allowing replacement of saturated features in the high gain scan with data from the low gain measurement. Contrasts refer to log2 normalized intensity ratios between the samples of the different developmental stages. Genes with log2 ratios $\geq \pm 1.5$ and adjusted p values ≤ 0.01 in both biological replicates and platforms were regarded as differentially expressed.

In order to analyze the AGILENT based transcriptional profile of the second biological replicate on *A. nidulans* FGSC A4 wild type strain during different developmental stages, each stage was compared and hybridized with a RNA pool generated by RNA of each point of

time (Fig. 1). Three technical replicates per comparison rather hybridization were prepared.

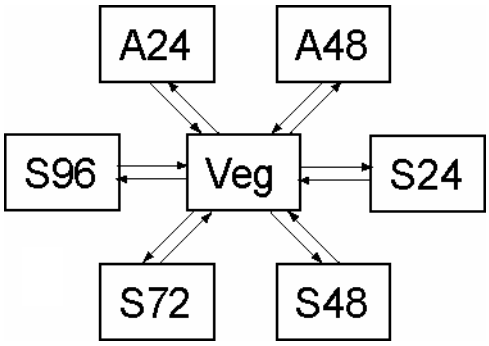


Figure 1: Experimental design of the performed transcriptome analyses on *A. nidulans* development
Each stage of asexual (A) and sexual (S) development was compared to vegetative (Veg) stage giving us insights into gene regulation in transition of vegetative growth and both development cycles and into changes in the time course of each gene during asexual and sexual development in *A. nidulans*. Numbers behind A and S show hours after induction of asexual and sexual development respectively after achieving developmental competence.

AGILENT Feature Extraction 9.1 was used for microarray intensity extraction. Single microarrays were normalized with a non-linear Loess regression method. Normalization among the microarrays was performed with Variance Stabilized Normalization (VSN) (Huber, *et al.*, 2002).

For determination of differentially expressed genes, statistical tests were accomplished employing Limma. p-values were adjusted as previously described (Smyth, 2004). All computations were performed with the statistical software 'R' (http://cran.r-project.org/).

2.2.3.5 Data mining for transcriptome analysis

In order to sort data by their functionality, gene probes were annotated manually by homology searches via translated nucleotide BLAST algorithm (blastx) at NCBI (http://www.ncbi.nlm.nih.gov/BLAST). A functional category was assigned to the gene probe only if the best scoring hit with a known or predicted function showed an alignment score of 100 in total or higher and expect value e^{-8} or lower. The exception was the 'unknown' category, to which no gene probes were assigned due to the low score values in homology searches. These probes had certain homology to other proteins. Genes being involved in processes apart from the shown categories were added to the ´other´ category. Genes without indicated domain was sorted into the ´no domain´ category. Each gene probe was assigned to one functional category only.

Raw expression data derived from TIGR and AGILENT transcriptomes were stored at www.dropbox.com/sh/yj8r6dx5z2n5zqp/PVpPVIV0mN. Thereby, one expression value for

each gene was generated from TIGR microarrays and four expression values were generated from AGILENT customized microarrays.

A simplified list for all differentially expressed genes with -1, 0 and 1 description was also added to www.dropbox.com/sh/yj8r6dx5z2n5zqp/PVpPVIV0mN. Analyses data from manual annotation and categorization for differentially expressed genes concerning the whole transcriptome, the development specific and delayed genes are also stored at www.dropbox.com/sh/yj8r6dx5z2n5zqp/PVpPVIV0mN. These lists are subdivided into data showing the different analyses points of time, compared and overlapping as well as a regarding enclosed summary revealing an overview on the numbers of all differentially expressed genes for each category.

2.2.4 Metabolome analysis

2.2.4.1 Metabolite fingerprinting by UPLC TOF-MS

For non-targeted metabolite fingerprinting of the intracellular and extracellular metabolome, *A. nidulans* wild type A4 was cultivated (see 2.2.3.2.). For intracellular metabolome 20 h vegetative grown cultures were shifted to solid minimal medium plates and induced for asexual or sexual development respectively. Thereby, cultures were harvested regarding the asexual (A24, A48) and sexual (S24, S48, S72, S96) points of time chosen for transcriptome analysis. Endpoint extracellular metabolomes were analyzed additionally. Therefore $8x10^6$ conidiospores were inoculated into horizontal flasks filled with 1.5 l liquid minimal medium as it was used for transcriptome analysis. Flasks with sexually induced *A. nidulans* cultures were closed with parafilm and covered with a double layer aluminum foil. After 48 h asexual and 96 h sexual induction cultures were washed twice with 1 liter 0.96% NaCl, immediately frozen with liquid nitrogen, grounded and processed immediately or stored at -80°C. Three extractions of mycelium from vegetative, asexual and sexual cultures or extracellular material using a two-phase extraction with methyl-tert-butylether according to Matyash and co-workers were performed for each condition on two independent biological replicates (Matyash, *et al.*, 2008). The metabolite analysis was done twice for each extract by Ultra Performance Liquid Chromatography (UPLC, ACQUITY UPLC™ System, WATERS CORPORATION, MILFORD, CT, USA) coupled with an orthogonal time-of-flight mass spectrometer (TOF-MS, LCT Premier™, WATERS CORPORATION, MILFORD, CT, USA). For LC an ACQUITY UPLC™ BEH SHIELD RP18 column (1 x 100 mm, 1.7 µm particle size, WATERS CORPORATION, MILFORD, CT, USA) was used at a temperature of 40°C, a flow rate of 0.2 ml/min and with the following gradient for the analysis of the polar phase: 0 - 0.5 min 0%

B, 0.5 - 3 min from 0% B to 20% B, 3 - 6 min from 20% B to 99% B, 6 - 9.5 min 99% B and 9.5 - 13 min 40% B (solvent system A: water/methanol/acetonitrile/formic acid (90:5:5:0.1, v/v/v/v); B: acetonitrile/formic acid (100:0.1, v/v). The TOF-MS was operated in negative as well as positive electrospray ionization (ESI) mode in W optics and with a mass resolution larger than 10,000. Data were acquired by MassLynx™ software (WATERS CORPORATION, MILFORD, CT, USA) in centroided format over a mass range of m/z 50 – 1,200 with scan duration of 0.5 sec and an interscan delay of 0.1 s. The capillary and the cone voltage were maintained at 2,700 V and 30 V and the desolvation and source temperature at 250°C and 80°C, respectively. Nitrogen was used as cone (30 l/h) and desolvation gas (600 l/h). For accurate mass measurement the Dynamic Range Enhancement (DRE) mode was used for data recording. All analysis were monitored by using Leucine-enkephaline ($[M+H]^+$ 556.2771 as well as its ^{13}C isotopomer $[M+H]^+$ 557.2799, Sigma-Aldrich, Deisenheim, Germany) as lock spray reference compound at a concentration of 0.5 µg/ml in acetonitrile/water (50:50, v/v) and a flow rate of 30 µl/min.

The raw mass spectrometry data of all samples were processed using the MarkerLynx™ Application Manager for MassLynx™ software (WATERS CORPORATION, MILFORD, CT, USA) resulting in four data sets. For further data processing, like filtering, adduct correction, data base search as well as data visualization and clustering the MarVis toolbox (MarkerVisualization, http://marvis.gobics.de) has been used. A Kruskal-Wallis test was performed to extract a subset of 3,162 high-quality markers of a confidence threshold < 10^{-6} for the marker candidates of the intracellular (Meinicke, *et al.*, 2008). Metabolome and 3,234 marker candidates with pVal<$5x10^{-4}$ for the extracellular metabolome. The exact mass information of the marker candidates were used for searching public and internal data bases. Finally a cluster analysis was performed by training a one-dimensional self-organizing-map (1D-SOM) model. (Kaever, *et al.*, 2009).

The putative identity of all metabolites of interest has been confirmed by coelution with identical standards or by MS/MS analysis. For that a LC 1290 Infinity (Agilent Technologies, Santa Clara, USA) coupled with a 6540 UHD Accurate-Mass Q-TOF LC MS instrument with Agilent Jet Stream Technology as ESI source (Agilent Technologies, Santa Clara, USA) has been used. For LC an ACQUITY UPLC BEH SHIELD RP18 column (2.1 x 100 mm, 1.7 µm particle size, Waters Corporation, Milford, USA) was used at 40 °C, a flow rate of 0.5 ml/min, and a solvent system and gradient comparable with that for UPLC analysis. The Q-TOF MS instrument was operated for positive and negative ionization in the targeted MS/MS mode with a detection frequency of 2 GHz. The following source conditions

has been used: gas temperature: 250 °C; drying gas flow: 8 L min^{-1}; nebulizer pressure: 35 psi; sheat gas temperature: 300 °C; sheat gas flow: 8 L min^{-1}; VCap voltage: 3000 V; nozzle voltage: 200 V; fragmentor voltage: 100 V. For exact mass measurement the reference mass correction with trifluoroacetic acid ($[M-H]^-$ 112.98559) and HP-921 ($[M+CH_2O_2-H]^-$ 966.00073) were used. The MassHunter Workstation Acquisition software B.04.00 (Agilent Technologies, Santa Clara, USA) as well as the MassHunter Qualitative Analysis software B.05.00 (Agilent Technologies, Santa Clara, USA) was used for data acquisition and analysis, respectively.

2.2.4.2 *psi*-factor extraction and analysis by GC MS

For comprehensive metabolite analyzes at Veg, A24, A48, S24, S48, S72 and S96 *A. nidulans* wild type strain was cultured with two independent biological replicates as for transcriptome analyzes. In order to analyze *psi*-factors, 0.6 g of frozen and ground *A. nidulans* cells were extracted by adding 8 ml of extraction medium (n-hexane: 2-propanol, 3:2 [v/v] with 0.0025% [w/v] butylated hydroxytoluene) and $^{13}C_{18}$-8-HOD as internal standard. The extract was shaken for 10 min and centrifuged at 3,200 x *g* at 4°C for 10 min. After collecting the clear upper phase, a 6.7% (w/v) solution of potassium sulfate was added up to a volume of 13 ml. Drying of the upper hexane-rich layer under streaming nitrogen was subsequently followed by the vigorous shaking and centrifugation at 3,200 x *g* at 4°C for 10 min. The remaining lipids were redissolved in 0.4 ml methanol and methylated after addition of 380 µl methanol and 6.5 µl trimethylsilyldiazomethane (2 M in hexane; SIGMA, TAUFKIRCHEN, GERMANY). After shaking for 30 min, 0.2 µl of glacial acetic acid was added. The corresponding methyl esters were dried under streaming nitrogen and redissolved in 80 µl methanol:water:acetic acid (75:25:0.1 [v/v/v]). At first, *psi*-factors were purified on reversed phase HPLC on an ET250/2 Nucleosil 120-5 C18 column (2.1 x 250 mm, 5 µm particle size; MACHEREY-NAGEL, DÜREN, GERMANY) assembled in an Agilent 1100 HPLC system coupled to a diode array detector (AGILENT TECHNOLOGIES, SANTA CLARA, CA, USA). The solvent systems were as follows: solvent system A, methanol: water: acetic acid (75:25:0.1 [v/v/v]); and solvent system B, methanol: acetic acid (100:0.1 [v/v]). The gradient elution profile was as follows: flow rate of 0.18 ml/min, 0 – 5 min, 100% A; 5 – 10 min from 100% A to 100% B and flow rate increase to 0.36 ml/min; 10 – 20 min, 100% B; 20 – 25 min from 100% B to 100 % A; and 25 – 30 min, 100% A and flow rate decreased to 0.18 ml/min. For purification of *psi*-factors, eluate fraction was collected between 8 and 13.5 min and dried under streaming nitrogen. The residue of this fraction was redissolved in 3 µl acetonitrile. After addition of 1

µl pure N,O-bis(trimethylsilyl)trifluoroacetamide (SIGMA, TAUFKIRCHEN, GERMANY), analysis of trimethylsilyl ethers/methyl esters of *psi*-factors was carried out using an AGILENT 5973 network mass selective detector connected to an AGILENT 6890 gas chromatograph (AGILENT TECHNOLOGIES, SANTA CLARA, CA, USA) equipped with a capillary DB-23 column (30 mm x 0.25 mm; 0.25 µm coating thickness; J&W SCIENTIFIC, FOLSOM, CA, USA and AGILENT TECHNOLOGIES, SANTA CLARA, CA, USA). Helium was used as a carrier gas at a flow rate of 1 ml/min. The temperature gradient was 150°C for 1 min, 150 - 200°C at 4 K min^{-1}, 200–250°C at 5 K min^{-1}, and 250°C for 6 min. Electron energy of 70 eV, an ion source temperature of 230°C and a temperature of 260°C for the transfer line was used. For quantification, the ions *m/z* 241 (8-HOE; R_f = 11.41 min), *m/z* 250 ($^{13}C_{18}$-8-HOD; R_f = 11.75 min), *m/z* 239 (8-HOD; R_f = 11.76 min) and *m/z* 271 (10-HOD; R_f = 12.18 min), were used. Standard curves were constructed by analyzing known mixtures of unlabeled *psi*-factors and $^{13}C_{18}$-8-HOD and plotting the ratios of ion intensities (ion of unlabeled/ion of ^{13}C-labeled) *vs*. the molar amount of unlabeled.

2.2.4.3 Data mining for metabolome analysis

A table of all identified metabolites is attached to this work (see attachment at the very end of this thesis) and stored at www.dropbox.com/sh/yj8r6dx5z2n5zqp/PVpPVIV0mN. Uploaded raw data base on two biological and three technical replicates. A simplified list for all verified primary and secondary metabolites was also uploaded.

3. Results

3.1 One fifth of *A. nidulans* genes are differentially expressed during developmental stage

In order to determine the differentially expressed genes during developmental stages, a developmental transcriptome analysis of *A. nidulans* wild type grown vegetatively, asexually and sexually was performed (Fig. 2A). For this purpose, 20 h vegetatively (Veg) grown mycelia were shifted on plates and induced for asexual development under light conditions for 24 and 48 h (A24, A48) and for sexual development in the darkness for 24, 48, 72 and 96 h (S24, S48, S72, S96). Therefore, 20 h vegetative cultures served as standard for the fluctuations in gene expression during asexual and sexual development. These transcriptome analyses data based on two independent biological replicates and two independent transcriptomic platforms. Gene expression data were generated from a comparison of each developmental stage to the reference stage Veg 20 h. This setup gave us inside into the expressional time course of each gene. During sexual development *A. nidulans* even performs conidiation secondarily (Fig. 2B). Despite of this the numbers of conidiospores are around 20-fold lower in comparison of A48 and S96.

In total, 19.1% (2,014) of the genes (10,560) in the genome of *A. nidulans* were differentially expressed during development. Cultures induced for sexual development for 24 h did not show drastic changes in gene expression. Merely expression of the 9.6% (194 genes) of the total differentially expressed 2,014 genes changed during S24 (Fig. 2C). In contrast, the number (711 genes) of differentially expressed genes after 24 h asexual development was 3.7-fold higher than at S24. For 24 h stages, light induced the expression of genes while in darkness activation of those genes is repressed.

Gene expression pattern was very similar in early sexual (S24) cultures in comparison to the cultures grown vegetatively, which was also supported by the almost identical hyphal morphologies of the 20 h Veg and S24 cultures.

Darkness delays differential gene expression in comparison to light. This effect was compensated at late sexual development and the number of differentially expressed genes was similar or even higher compared to those of the asexually expressed genes. This corresponds with the phenotypical delay in conidiospore formation during sexual differentiation in darkness. Results concerning delayed genes in sexual development of *A. nidulans* most likely linked to asexual development are described more intensive in chapter 3.1.3 (page 46).

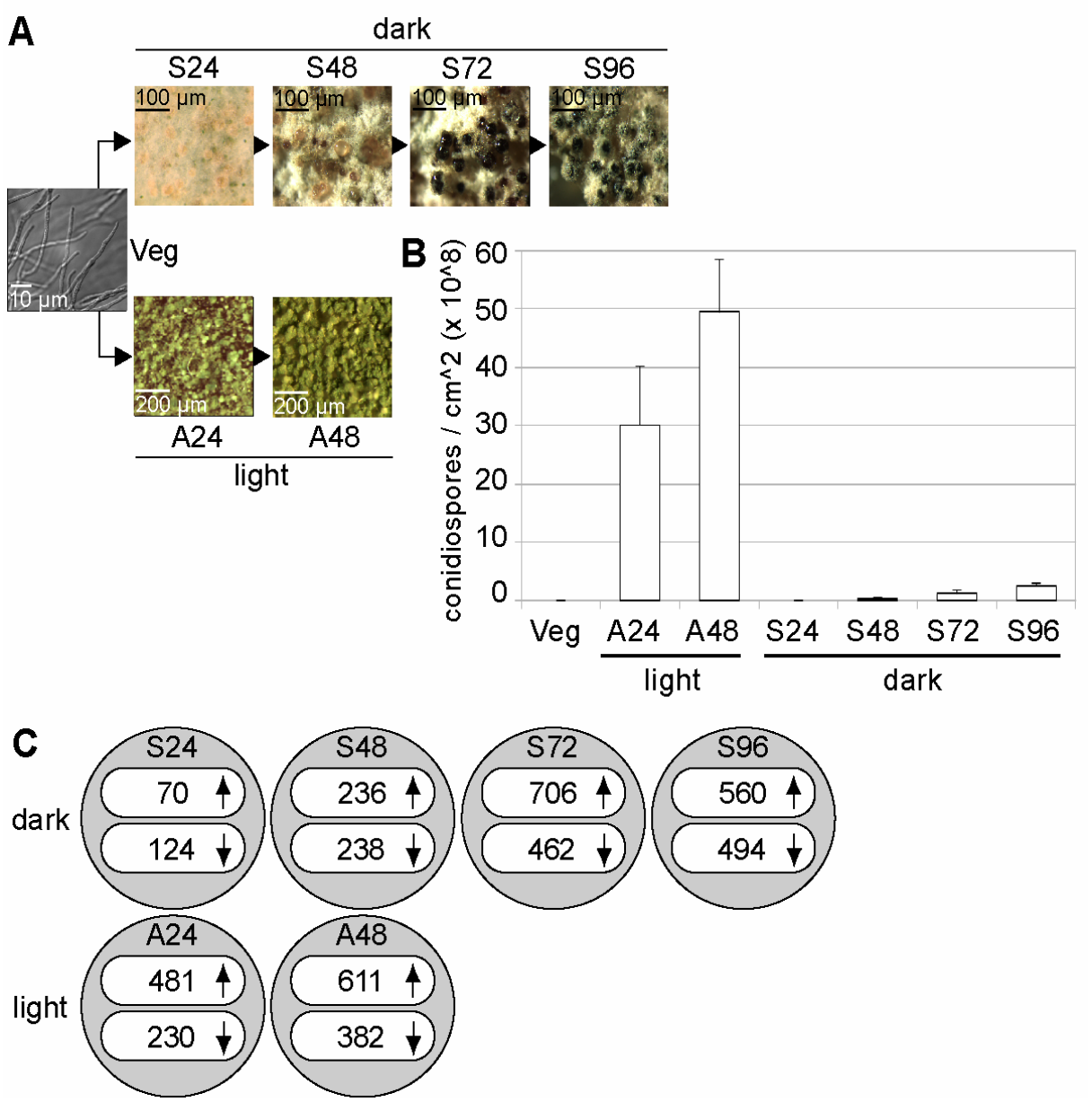


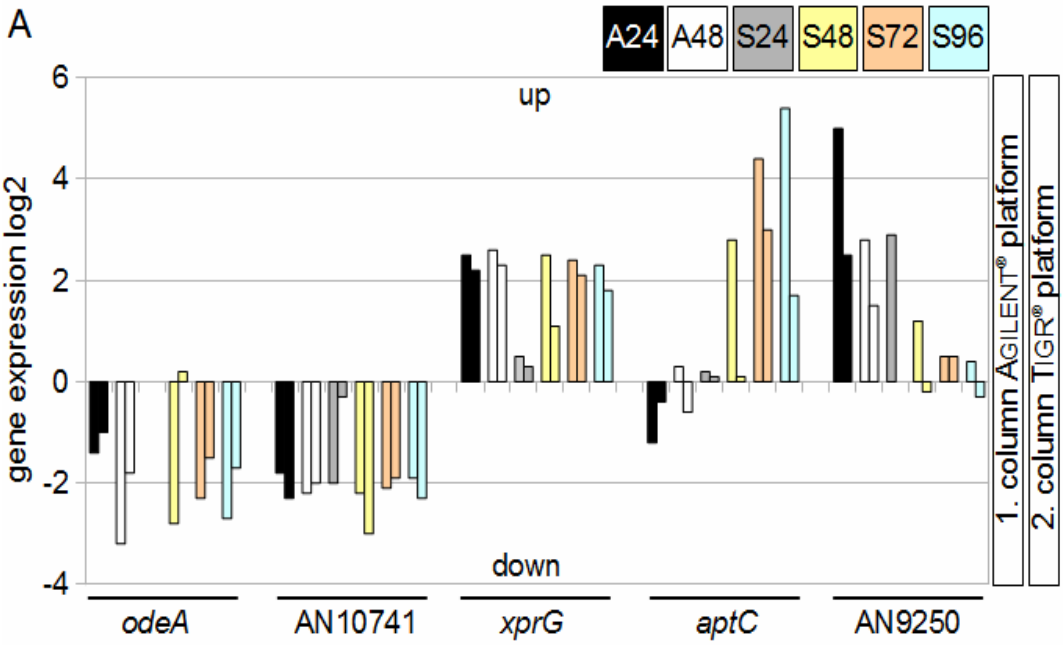
Figure 2: Analyzed *A. nidulans* cultures processed for transcriptomics and metabolomics.
(A) *A. nidulans* vegetative (Veg) cultures were induced for asexual (A) and sexual (S) development and harvested at the indicated stages. Numbers behind A and S exhibit hours after induction of the corresponding differentiation. (**B**) *A. nidulans* conidiospore formation is delayed in darkness. Despite of this after primarily performed sexual cycle the number of formed conidiospores is less compared to growth in light. (**C**) Summary of differentially expressed genes. Light (full spectrum) induces or derepresses the expression of genes. Induction of gene expression is delayed in darkness. Comparison of gene expression between Veg 20 and S24 shows lowest number of differentially expressed genes. Highest number of upregulated genes at S72. Highest number of downregulated genes at S96 for the preparation of programmed cell death and aging. Arrows directing upside indicate the upregulated genes and downward arrows represent downregulated genes. Data derived from transcriptomes of two independent biological replicates and two microarray platforms (TIGR and AGILENT). Primary data are stored at www.dropbox.com/sh/yj8r6dx5z2n5zqp/PVpPVIV0mN. Cut off was set to log2 ≥ ±1.5.

Most drastic changes in gene expression were observed at S72 (Fig. 2C). At this stage of development 58% (1,168 genes) of the differentially expressed genes were up- or downregulated. In other word, more than 11% of the genes in the genome of *A. nidulans* were differentially expressed at S72. Presence of drastic upregulation (706) of the gene expression at S72 suggests that the most comprehensive transcriptional activation took place during this stage when the ascospores are formed. This effect could also be observed for the specific genes that were merely up- or downregulated at one stage which will be shown in detail in chapter 3.1.2 (page 41).

Expression values of five differentially expressed candidates derived from both transcriptome platforms were shown in figure 3 to demonstrate high reproducibility among both microarray systems of TIGR® and AGILENT®. Single genes from figure 3 are not described closer in this context since they are going to be part of following chapters.

Generally, expression values in figure 3 and all shown tables in this work possess (+) and (-) values or symbols respectively. Based on the experimental setup of the transcriptomes that compares each asexual and sexual to vegetative stage, (+) means an upregulation and (-) a downregulation at asexual and/or sexual stage. Consequently, genes with (-) exhibit an upregulation already at vegetative stage.

A



B

	A24	A48	S24	S48	S72	S96	arrays
odeA	-1.4	-3.2	0	-2.8	-2.3	-2.7	AGILENT®
	-1	-1.8	0	0.2	-1.5	-1.7	TIGR®
AN10741	-1.8	-2.2	-2	-2.2	-2.1	-1.9	AGILENT®
	-2.3	-2	-0.3	-3	-1.9	-2.3	TIGR®
xprG	2.5	2.6	0.5	2.5	2.4	2.3	AGILENT®
	2.2	2.3	0.3	1.1	2.1	1.8	TIGR®
aptC	-1.2	0.3	0.2	2.8	4.4	5.4	AGILENT®
	-0.4	-0.6	0.1	0.1	3	1.7	TIGR®
AN9250	5	2.8	2.9	1.2	0.5	0.4	AGILENT®
	2.5	1.5	0	-0.2	0.5	-0.3	TIGR®

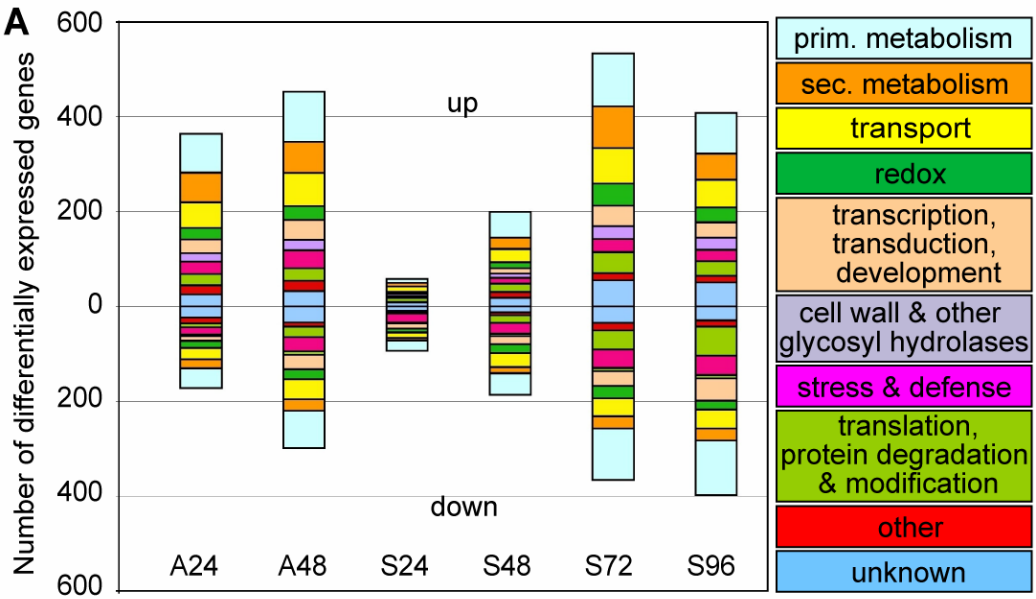
Figure 3: Demonstration of reproducible gene expression values derived from both independent microarray platforms AGILENT® and TIGR®. (**A**) Five differentially expressed *A. nidulans* genes were taken for this demonstration randomly. Among them are already validated genes like *odeA* (AN1037) coding for an oleate desaturase which has impact on development processes of *A. nidulans*. Our transcriptome analyses on different platforms were performed using two biological replicates separately. Expression data derived from these analyses were not used to generate mean values and standard deviation since both platforms possess different detection sensitivities. Thus expression values were shown in separated columns for each analyzed stage of development. (**B**) Table containing expression values of chosen genes. A direct confrontation with the expression values in comparison of both microarray platforms mediates a high reproducibility being a solid basis for reliable results. TIGR microarrays use one 70 bases oligomers for binding corresponding RNA to the slide whereas AGILENT generates four independent 60 bases oligomers binding four different sites of one RNA. Raw data are stored at www.dropbox.com/sh/yj8r6dx5z2n5zqp/PVpPVIV0mN. Folder: Transcriptome → AGILENT or TIGR → check corresponding excel files. AGILENT lists contain four expression values for one differentially expressed gene since this system uses four oligomers.

3.1.1 Functional categorization exhibits massive expressional changes in a wide range of pathways during *A. nidulans* development

In order to characterize the changes in transcriptome during the development of *A. nidulans*, a functional categorization with eleven defined categories was performed (Fig. 4). For this aim, we took the categories already used in one of our publications (Nahlik, *et al.*, 2010).

Despite the expressional delay at 24 h sexual development, *A. nidulans* exhibits a similar number of differentially expressed genes at late sexual (S72) compared to asexual (A48) development. Differences in the composition of differentially expressed genes are present and characteristic for the different stages of development. Though the number of primary metabolism genes covering many different pathways e.g. carbohydrate-, lipid- and amino acid- metabolism were similar at A48 and S72, the number of genes for the specific pathways were different. While the number of upregulated amino acid metabolism genes were similar at A48 and S72 (25 vs. 23 genes), the number of downregulated was much higher at S72 (34 vs. 19 genes) which reveals a massive amino acid downregulation at late sexual development in darkness. Most secondary metabolite genes (88) were expressed at S72, which might hint for the chemical response of the fungus against most competitors and other harming conditions within soil. Furthermore, the fungus exhibits massive downregulation of transcription and translation genes at late sexual stage S72 and S96. Decreased expression of these genes was most likely the consequence of an induced programmed cell-death that is going to be discussed.

In agreement with the downregulation of primary metabolism genes at late sexual development S72 and S96 in the transcriptome analyses, most genes are particularly downregulated at S72 and S96. Especially genes for amino acid metabolism were drastically affected. Downregulation in amino acid biosynthesis might have presumably direct impact on translation. Downregulation of amino acid metabolism genes was accompanied by a massive downregulation of stage specific genes for transcription and translation (20 genes) at S96 which is going to be described more intensive in chapter 3.4.1.4 (page 76). The broad downregulation for genes involved in amino acid metabolism and translation was consistent with the observation in the complete transcriptome.



B

upregulated genes	A24	A48	S24	S48	S72	S96
prim. metabolism	78	100	8	52	101	78
sec. metabolism	62	65	7	24	88	54
transport	54	70	12	28	75	59
redox enzymes	24	29	2	13	46	31
transcription, signal transduction ...	30	43	1	11	43	32
cell wall & glycosyl hydrolases	22	28	6	11	37	34
stress and defense	26	38	4	12	29	25
translation, protein degradation ...	24	26	9	18	44	31
other	19	22	1	12	15	14
unknown	25	32	9	18	55	50
no domain (not shown in **A**)	117	158	11	37	173	152
total	481	611	70	236	704	560

downregulated genes	A24	A48	S24	S48	S72	S96
prim. metabolism	37	74	21	44	103	109
sec. metabolism	19	24	6	13	26	25
transport	24	42	12	29	38	40
redox enzymes	14	20	7	19	25	18
transcription, signal transduction ...	10	31	11	17	31	47
cell wall & glycosyl hydrolases	9	12	3	7	12	13
stress and defense	15	30	18	23	39	41
translation, protein degradation ...	8	22	3	16	40	61
other	12	10	3	6	16	13
unknown	24	35	9	13	35	30
no domain (not shown in **A**)	58	82	31	51	97	97
total	230	382	124	238	462	494

Figure 4: Transcriptomics based global functional categorization of genes differentially expressed during *A. nidulans* development. (A) Overview of differentially expressed and functionally categorized genes at different stages of development. Ten categories are shown except for the ´no domain´ category for transcripts without known domain. Redox genes known to be involved in secondary metabolism are sorted into secondary metabolism category (orange). Redox genes without defined function were sorted into the dark-green category. **(B)** Numbers of differentially expressed genes sorted in eleven categories. Gene expression was either stimulated by light or inhibited in darkness. In light much more genes are expressed within a shorter time. In darkness expression is delayed. Since each stage of asexual and sexual development was compared with vegetative growth, transcriptionally, S24 can be considered to be very similar to the vegetative growth. After 72 h of sexual development the number of differentially expressed genes is at least as high as at A48. At late sexual development expression of genes encoding ribosomal proteins are decreased. In contrast ubiquitin-dependent proteolysis genes are activated at late sexual development. The skin-colored = transcription, signal transduction and development. The pink = stress & defense. The bright-green = translation, protein degradation and modification. Within the ´unknown´ category (blue) proteins with domains without connection to fungal physiology and not described to be present in fungi are collected. Proteins without a conserved domain are contained in the ´no domain´ category (white). Data derived from transcriptomes of two independent biological replicates and two microarray platforms. Cut off was set to log2 ≥ ±1.5. Primary data are stored at www.dropbox.com/sh/yj8r6dx5z2n5zqp/PVpPVIV0mN.

Since *A. nidulans* development is a light-dependent process, genes regulated by light were of special interest. Therefore, direct comparison between 24 h and 48 h stages of both differentiation cycles was performed (Fig. 5). 32 up- and 50 downregulated overlapping genes expressed at both 24 h stages are present (Fig. 5A). While more downregulated, overlapping genes are at this stage, more upregulated, 101 up- and only 19 downregulated genes specific for asexual stage are detectable. This observation is restricted to asexual development and absent at S24 with 26 up- and 44 downregulated genes. Light initiates expression of specific genes most likely closely connected to asexual development in *A. nidulans.* Even at 48 h stages light stimulates expression to a higher extend compared to sexual stage. There, 171 asexually and 44 sexually upregulated genes were identified, demonstrating impact of light on *A. nidulans* gene expression and a possible preference for its asexual propagation driven by light. Especially genes involved in transcription, signal transduction and development are upregulated at both asexual stages. Beside the already known essential asexual regulator genes like *brlA* (AN0973) or *wetA* (AN1937) further yet uncharacterized transcription factors are specifically upregulated at asexual development. Among them are C2H2 finger domain protein (AN4586), C2H2 finger + Zn(2)-Cys(6) DNA binding domain protein (AN6747), Zn(2)-Cys(6) DNA binding domain protein (AN7072).

Even the putative cytosolic Cu/Zn superoxide dismutase (SOD) (AN1131) is already present at early asexual development and might trigger asexual development through hydrogen peroxide generation specifically at asexual stage. Ruger-Herreros and co-workers (2011) found SOD (AN1131) repressed by light at shorter light exposure and an earlier stage than A24. We found this SOD as the only asexual specific expressed ROS generating enzyme present at both asexual stages. This point at SOD possesses an important role in asexual development of *A. nidulans*.

Furthermore, 20 genes for asexually specific secondary metabolites like terrequinone A are already expressed at A24. Some genes for asperfuranone *afoB* (AN1031), *afoF* (AN1035) and sterigmatocystin *stcF* (AN7818) synthesis are upregulated at A24. *ausA* (AN8383) encodes a polyketide synthase (PKS) expressed at A24 exclusively. Thereby, *ausA* is the only PKS expressionally restricted to the early asexual stage. It was described to be required for 3,5- dimethyl orsellinic acid synthesis, the first intermediate in the biosynthesis of austinol and dehydroaustinol (von Dohren, 2009, Nielsen, *et al.*, 2011). Since austinol could not be detected at asexual stages this PKS might also be linked to synthesis of further secondary metabolites.

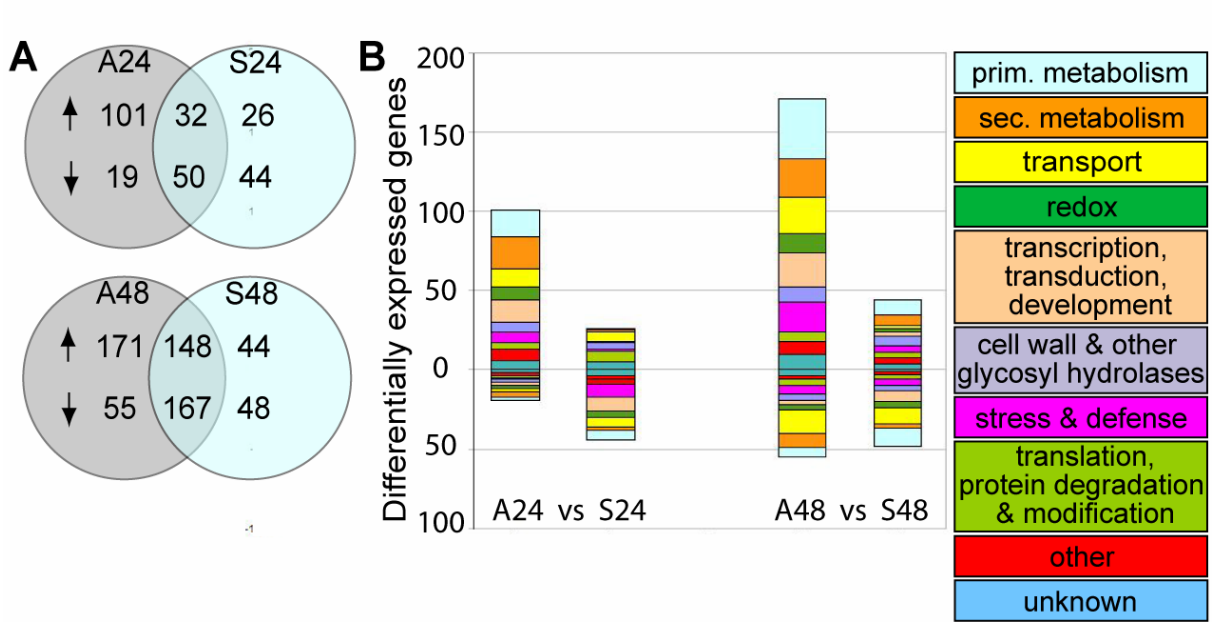


Figure 5: Differentially expressed genes specific for asexual and sexual development in *A. nidulans* (A) Identification of specific light dependent up- and downregulated genes after 24 h and 48 h asexual and sexual induction. Only genes specific for asexual (171 up & 55 downregulated) and sexual (44 up- & 48 downregulated) development generally were taken for the comparison. Light stimulates or derepresses expression of genes. Asexual and sexual specific genes were identified through Venn analysis. Genes independent from light equally expressed or repressed at both conditions are shown in the overlapping area of the ellipses. Redox genes known to be involved in secondary metabolism are sorted into secondary metabolism category (orange). Redox genes without connection to one of the used categories were sorted into the dark-green category. (**B**) Broad upregulation of genes for all functional categories in light. For the comparison between asexual and sexual cultures to identify light specific genes light-inducing or darkness-repressing effect can be asserted for the global transcriptomic analysis. Correspondingly, number of genes from the different categories is much bigger than at analogous sexual stages. Skin-colored category contains genes for transcription, signal transduction and development. Pink-colored category contains genes for stress & defense. The bright-green category consists of genes for translation, protein degradation and modification. Within the ´unknown ´ category (blue) proteins with domains without connection to fungal physiology and not described to be present in fungi are collected.

In contrast to A24, no secondary metabolite gene is upregulated at S24. Among the S24 specific upregulated genes are four putative histone acetylases (AN0969; AN5330; AN6411; AN7944) comprising GCN5-related N-acetyltransferases (GNAT). Since histone acetylases are crucial for transcriptional regulation and are most likely involved in *A. nidulans* developmental control at early sexual stage and the control of secondary metabolism, this topic will be described in more detail in chapter 3.4.1.1 (page 70).

Expression of several known asexual regulators like *brlA*, *wetA*, *cetA* (AN3079) is detectable at A48. A neighbour downstream of *wetA* (AN1937) is also upregulated at this stage and codes for a putative developmentally regulated MAPK interacting protein with GPI-anchor (AN1941). This family of proteins appear to be involved in both fruit body formation and also in host attack. One member is named Hesp-379 (haustorially expressed secreted protein). Haustoria are structures required for nutrient uptake from hosts and are, for instance,

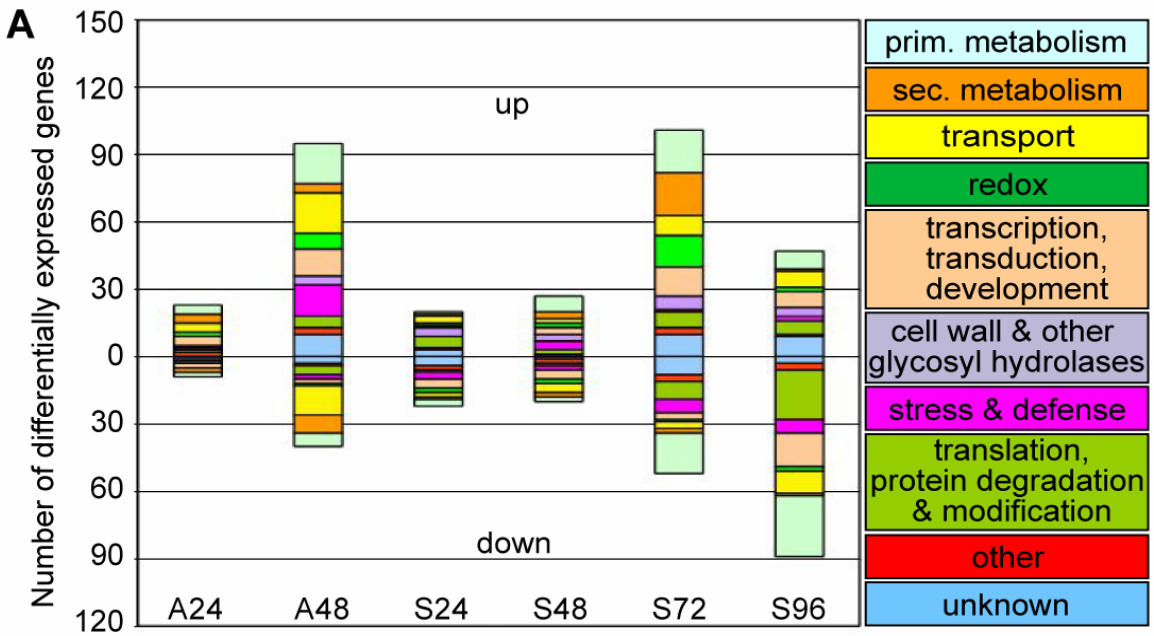
formed by parasitic fungi. Its sequence was analyzed manually at NCBI platform since BROAD exhibit GPI-anchor merely.

Beside the already known and intensively analyzed regulators like *brlA* and *wetA*, we see many additional regulators upregulated in this work. Amongst 232 comprised Zn(2)-Cys(6) and 15 comprised bZIP transcription factors (source: BROAD) further transcription factors could be identified as asexually specific expressed. Five uncharacterized transcription factors are expressed at A48 specifically: four Zn(2)-Cys(6) DNA binding domain containing transcription factors (AN2677; AN3433; AN5924, AN6747) and one bZIP transcription factor (AN8643). In contrast, only one S48 specific Zn(2)-Cys(6) transcription factor (AN2650) could be identified, which shows a higher transcriptional activity of development specific transcription factors at 48 h asexual development in light.

Expression of 24 secondary metabolite genes is increased at A48, whereas only seven genes are upregulated at S48. Even more interesting is the increased number of upregulated transporter genes in comparison of both 48 h stages. 23 asexually and two sexually upregulated transporter are detectable. *A. nidulans* initiates a massive secretion at asexual stages generally. Beside the exchange of primary metabolites the fungus also translocates secondary metabolites for different reasons. Later this issue is going to be analyzed and described extensively. S96 expresses only one specific secondary metabolism gene, suggesting that preparation for synthesis of protective secondary metabolites actively takes place at S72 and is completed by the S96 stage. Most specific secondary metabolism genes (8) are downregulated at A48. Among them are three genes AN5846, AN7081 and AN7274 encode uncharacterized proteins with berberine bridge-like domain that is found in enzymes involved in the biosynthesis of numerous isoquinoline alkaloids. Many alkaloids are toxic to other organisms. Their toxic properties made them a potential feed protectant against animals (chapter 3.3.1.4, page 60).

3.1.2 Stage specific genes in *A. nidulans* development

Transcriptome data were used to identify genes which are only expressed at one specific point of time (Fig. 6). Thereby, vegetative, asexual and sexual specific genes are depicted. As for the general transcriptome also most of the differentially expressed stage specific genes are present at middle or late stages of asexual and sexual development. Thereby, 58 genes are downregulated at S48 and 122 genes at S96.



B

upregulated genes	A24	A48	S24	S48	S72	S96
prim. metabolism	4	18	1	7	19	8
sec. metabolism	4	4	1	3	19	1
transport	4	18	3	2	9	7
redox enzymes	2	7	1	2	14	2
transcription, signal transduction ...	4	12	1	3	13	7
cell wall & glycosyl hydrolases	1	4	1	3	6	4
stress and defense	1	14	0	4	1	2
translation, protein degradation ...	1	5	5	2	7	6
other	2	3	1	0	3	1
unknown	0	10	3	1	10	9
no domain (not shown in **A**)	11	40	2	6	41	27
total	34	135	22	33	142	74

downregulated genes	A24	A48	S24	S48	S72	S96
prim. metabolism	2	6	3	2	18	27
sec. metabolism	2	8	1	2	2	1
transport	0	13	2	4	3	10
redox enzymes	0	1	2	2	1	2
transcription, signal transduction ...	2	2	4	4	3	15
cell wall & glycosyl hydrolases	1	0	0	0	0	0
stress and defense	0	2	3	2	6	6
translation, protein degradation ...	1	4	1	1	8	22
other	0	1	2	2	3	3
unknown	1	3	4	1	8	3
no domain (not shown in **A**)	6	18	9	9	20	33
total	15	58	31	29	72	122

Figure 6: Functional categorization of stage specific genes in *A. nidulans* development.
(A) Differentially expressed genes in only one stage of development extracted from the global transcriptome. Redox genes known to be involved in secondary metabolism are sorted into secondary metabolism category (orange). Redox genes without defined function were sorted into the dark-green category. (**B**) Detailed description of the total number of genes sorted into various functional categories. It demonstrates that the fungus expresses much more sexual (S) specific genes (see S72) compared to asexual (A) specific secondary metabolite genes. Light represses or darkness induces their expression. The number of specifically expressed genes for stress response and defense is higher in light than dark (A48). Genes for translation and transcription were downregulated at latest stages as we could already observe and identify in the global transcriptomic view. Corresponding genes are restricted to the analyzed late sexual stages. Previously stated parameters were used for this transcriptomic extraction. Skin-colored category contains genes for transcription, signal transduction and development. Pink-colored category contains genes for stress & defense. The bright-green category consists of genes for translation, protein degradation and modification. Within the ´unknown´ category (blue) proteins with domains without connection to fungal physiology and not described to be present in fungi are collected. Proteins without a conserved domain are contained in the ´no domain´ category (white).

Most specifically upregulated genes in sexual development were present at S72, when cleistothecia maturation is not yet finished. At this stage 142 specific genes were upregulated. In contrast, most specifically upregulated genes (135) in asexual development were at A48 when asexual differentiation was nearly completed. These data suggest that even after very late asexual development, fungal cells or spores prepare for the next environment by accumulating or expressing transcripts of some genes.

Most stage specific secondary metabolism genes (19) were expressed at S72. For instance, four genes *mdpC* (AN0146), *mdpL* (AN10023), *mdpI* (AN10035) and *mdpK* (AN10044) of the already characterized monodictyphenone (*mdp*) synthesis gene cluster were found to be expressed after 72 h induction in darkness. The *mdp* gene cluster is also required for emericellin and shamixanthone synthesis and described in chapter 3.4.2.2.2 (page 95).

One of four additional genes, *xptA*, coding for a prenyltransferase (AN6784) involved in emericellin and shamixanthone synthesis was also found to be expressed at S72. Another S72 specific gene encodes a DMATS aromatic prenyltransferase (AN11080). Members of this protein family are mostly fungal enzymes involved secondary metabolite and toxin biosynthesis. Two clavaminic acid synthetase (CAS)-like encoding genes are specifically expressed at S72. CAS is a trifunctional Fe(II)/ 2-oxoglutarate (2OG) oxygenase that is described to produce clavaminic acid which is an inhibitor of beta-lactamases in bacteria. The biological function of both genes is still unknown in *A. nidulans* but implies a possible role in enhancing the effect of penicillin, directed to beta-lactamase producing bacteria. Additionally, expression of the gene *aptA* that encodes the polyketide synthase (AN6000) within the asperthecin (*apt*) gene cluster was restricted to this stage in sexual development. This polyketide synthase is crucial for the synthesis of asperthecin. Deletion of AN6000 results in the lack of asperthecin production (Szewczyk, *et al.*, 2008) as it was described for deletion of *aptB* (AN6001) and *aptC* (AN6002). Both genes belong to the *apt* gene cluster and are upregulated at S96 additionally.

Beside the previously given examples from the secondary metabolism category Top 20 of stage specific expressed genes were depicted and listed in table 3. Among A24 specifically expressed genes are two genes coding for a putative chromosome segregation ATPase (AN10385) and a heterokaryon incompatibility protein (HET, AN3554). The AN3079 encoded thaumatin-like protein CetA was shown to have a role in early conidiation and is expressed at A48 specifically. Protein degradation mediated by ubiquitin ligases is specifically initiated at A48 since the ubiquitin ligase encoded by AN7294 is upregulated at this point of time.

Table 3: Top 20 of stage specific genes in asexual or sexual development of *A. nidulans*.

Gene ID	Function
A24 specifically expressed	
AN10385	Chromosome segregation ATPase, put.
AN3554	Heterokaryon incompatibility protein (HET), put.
A48 specifically expressed	
AN3079	***cetA***, secreted thaumatin-like protein with role in early conidiation *
AN3382	***salA***, salicylate 1-monooxygenase confers resistance to antifungal terbinafine *
AN7294	PHD and RING finger domain protein → HRD ubiquitin ligase complex, put.
S24 specifically expressed	
AN0460	F-box protein + WD40 domain, put.
AN0969	***ngn17***, uncharact., GNAT family N-acetyltransferase → histone modification
AN5330	GNAT family N-acetyltransferase, put.
AN6411	***ngn28***, uncharact., GNAT family N-acetyltransferase → histone modification
AN7010	Phenazine biosynthesis-like protein → putative antibiotic synthesis, put.
S48 specifically expressed	
AN5397	***lccC***, extracellular laccase *
AN7418	Phenol 2-monooxygenase, put. → detoxification
S72 specifically expressed	
AN0307	F-box protein, put.
AN10558	cAMP-mediated signalling protein, put. → cell cycle progress
AN2755	***matB***, α-domain mating-type protein regulating sexual development, verified
AN7553	***devR***, HLH transcription factor required for conidiophore development
AN8683	NADPH oxidase (NOX), put.
S96 specifically expressed	
AN0482	E2 ubiquitin conjugating enzyme, put.
AN10141	Interferone-related developmental regulator (IFRD), put.
AN5453	Arrestin, N-terminal domain protein → ubiquitin ligase Rsp5 associated, put.

Among numerous differentially expressed stage specific genes, interesting candidates involved in development, protein degradation- and modification, secondary metabolism and defense of *A. nidulans* are listed in this table. (+) = upregulated and (-) downregulated at asexual (A) and sexual (S) development in relation to vegetative stage. Consequently (-) = already upregulated at vegetative stage. Numbers behind A and S show hours after induction start for asexual and sexual development respectively Data derived from transcriptome data of two independent biological replicates and two microarray platforms. put. = putative, uncharact. = uncharacterized. Cut off was set to $\log2 \geq \pm 1.5$. (*) verified gene.

Protein degradation might affect proteins from several categories like development, secondary metabolism, cell cycle control, signal transduction and many other pathways. A salicylate 1-monooxygenase (*salA*, AN3382) is involved in the resistance to the antifungal terbinafine and is only expressed at A48. Among less differentially expressed genes, early sexual stage S24 genes for protein degradation and modification are the most interesting candidates. Expression of three histone N-acetyltransferases *ngn17*, AN5330 and *ngn28* reveals the requirement for transcriptional control at S24. Developmental processes might also be affected by the transcriptional activation controlled by acetylation of histones. The AN0460 encoded putative F-box protein, which contains a WD40 protein-protein interaction domain, is involved in protein degradation and already expressed at S24. It might be crucial for degradation of proteins which represses sexual development and fruiting. Again a stage specific putative phenazine biosynthesis-like protein required for the synthesis of the antibiotic phenazine, encoded by AN7010, is even activated at S24 when sexual induced *A. nidulans* cultures still possess high phenotypical similarity to vegetative cultures.

lccC (AN5397) codes for an extracellular laccase, which oxidizes phenolic compounds. Together with the also listed phenol 2-monooxygenase (AN7418) *A. nidulans* seems to prepare for intra- and extracellular detoxification and establishes defense towards toxic phenolic compounds at S48 when fruiting is sensible and at a critical stage.

Another putative F-box protein (AN0307) is upregulated at S72. The S72 activated cAMP-mediated signalling protein (AN10558) is crucial for cell cycle progress and might also be a specific protein required for cleistothecia composition and maturation. *matB* (AN2755) is expressed at S72 and comprises an alpha-domain mating-type protein that regulates sexual development. In contrast, at S72 the fungus even activates *devR* (AN7553), which codes for an HLH transcription factor that is required for conidiophore formation. This observation implies that *A. nidulans* induces conidiation at sexual stage systematically. With the background of NoxA being crucial for fruit body formation in *A. nidulans* specific expression of an additional putative NADPH oxidase (AN8683) at S72 let us presume a similar function for this Nox in sexual cycle.

Genes involved in protein degradation are also upregulated at S96. Protein degradation evolves the E2 ubiquitin conjugating enzyme (AN0482), which associates ubiquitin to proteins to be degraded. Arrestin, the N-terminal domain protein (AN5453) is associated with the ubiquitin ligase Rsp5. A putative interferone-related developmental regulator (IFRD) encoded by AN10141 is also activated at late sexual stage. It might have hormone-like properties and regulate several physiologic processes at the completed sexual

development of *A. nidulans*. A protein involved in cell aging is the retrograde regulation protein 2 (*thiA*, AN10492). Thereby, its expression at S96 confirms the fungus being at the end of development, lysing cell material for another round of differentiation requiring nutrients and energy.

3.1.3 Delayed genes in sexual development of *A. nidulans*

In order to demonstrate the delay in genetic expression genes of both analyzed asexual stages where compared to the corresponding sexual stage genes as well as to the pool of differentially expressed genes of later stages of sexual development (Fig. 7). Thereby, single genes not being differentially expressed to an earlier point of time than the one used for delay analysis.

Especially the comparison of the early asexual stage A24 reveals increasing numbers of upregulated delayed genes from S24 to S72 in *A. nidulans*. While A24 and S24 share 32 up- and 50 downregulated genes, A24 and S72 exhibit 268 up- and 132 downregulated overlapping genes. In contrast, the number of upregulated overlapping genes in comparison of A48 to S48/S72/S96 remains similar. The comparison of A48 to S48 reveals 148 up- and 167 downregulated genes, whereas the A48 and S96 overlay contains 142 up- and 98 downregulated genes. The number of downregulated genes is decreasing in the course of this comparison from S48 to S96.

Categorization of delayed *A. nidulans* genes reveals increased numbers of delayed genes in comparison of A24 vs. S48 to A24 vs. S72 affecting each category (www.dropbox.com/sh/yj8r6dx5z2n5zqp/PVpPVIV0mN → Delayed genes detailed.xls). For instance, the number of upregulated secondary metabolism genes increases from five genes (A24 vs. S48) to 38 genes (A24 vs. S72). For cell wall remodelling and degradation an increase from one gene (A24 vs. S48) to 15 genes (A24 vs. S72) is detectable, demonstrating a delay of genes from this category of about 48 h. In contrast, genes from primary metabolism, transport, transcription, signal transduction and development categories denotes highest increases in the comparison of A24 to S48, suggesting the requirement and importance for the expression of genes from this categories with only 24 h delay as preparation for another round of development in case of a secondary induced asexual cycle during sexual development. An activation of genes from primary metabolism assures a stable energy supply. Upregulation of transport genes stimulates uptake of essential nutrients from extracellular environment and integration of these nutrients into primary metabolism or for direct usage.

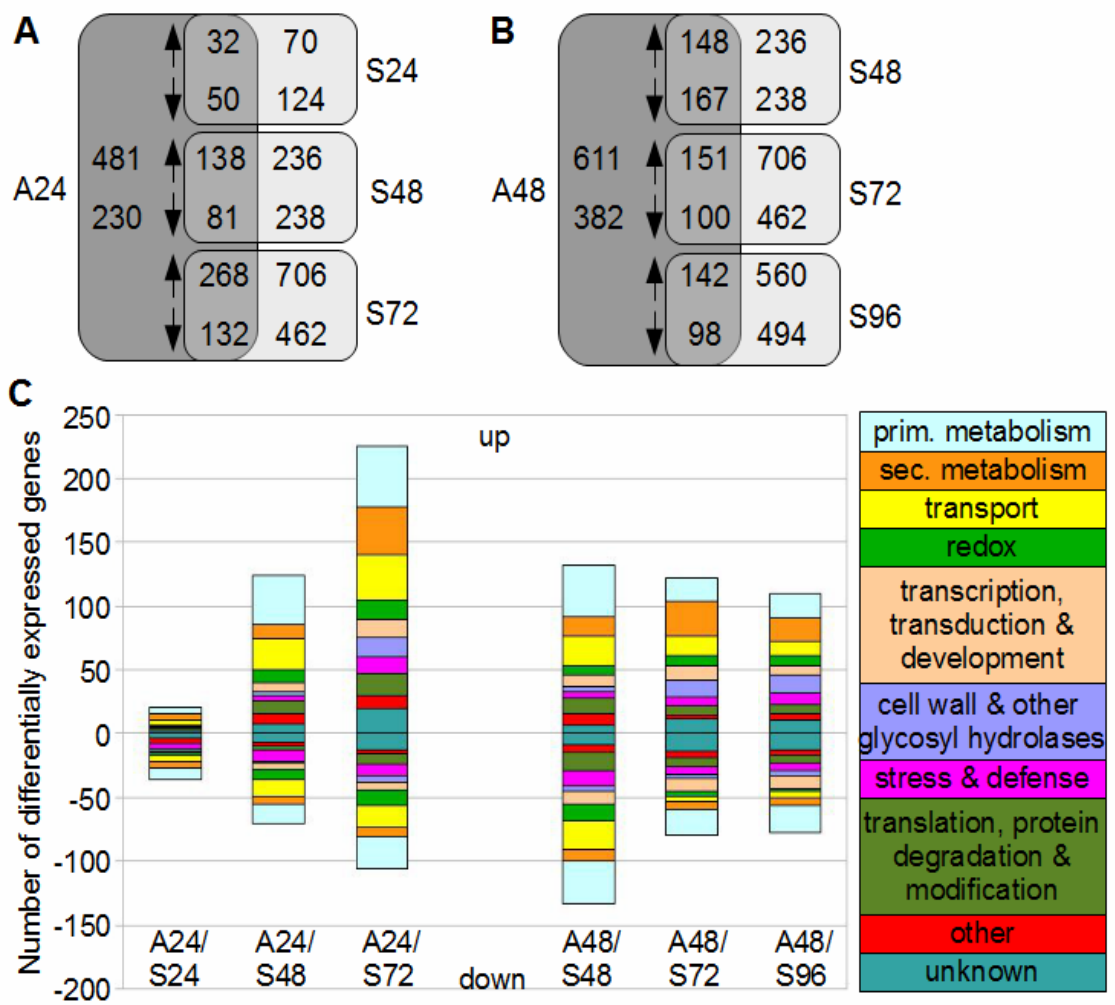


Figure 7: Early asexual genes reveal delayed up- and downregulation at late sexual stage in *A. nidulans* **(A)** The number of overlapping up- and downregulated genes in comparison of A24 to later sexual stages (S48 and S72) is already increased at S48 (138 genes up and 81 genes down) and achieves its highest numbers at S72 (268 genes up and 132 genes down). Light induces and accelerates gene expression required for asexual development in *A. nidulans* which is delayed in darkness. This observation is confirmed by *A. nidulans* forming asexual structure in darkness at a later point of time compared to its growth in light. Total number of differentially expressed genes at the single stages of asexual (dark grey boxes) and sexual (bright grey boxes) stages are also shown **(B)** In contrast, the number of upregulated genes in comparison of A48 and sexual stages S48, S72, S96 is constant whereas the number of A48 overlapping, downregulated genes decreases in the course of sexual differentiation S48 (167 genes), S72 (100 genes) and S96 (98 genes) revealing a decreasing derepression resulting in an activation of genes for asexual development at late sexual stage. Criteria for delayed genes shown in (A) was that S48 and S72 overlapping, delayed genes are not up- or downregulated at S24. Respectively, selected overlapping genes in (B) S72 and S96 are not up- or downregulated at A24 and A48. **(C)** Categorization of overlapping delayed genes. Genes encode for proteins without domain are not shown. The direct comparison between A24 and later sexual stages reveals that genes from all categories are among the overlapping delayed genes. For instance, highest increase in the number of upregulated genes from A24 vs. S24 to A24 vs. S72 is detectable for secondary metabolism (5 to 38) genes and cell wall hydrolysing (1 to 15) genes. Data derived from two independent biological and technical replicates performed on two different microarray platforms (TIGR and Agilent). Cut off is at log2 $\geq \pm 1.5$.

Contrastingly, in comparison of A24 vs. S48 and A24 vs. S72 to A48 vs. S72 and A48 vs. S96 the numbers of delayed genes remain the same or decrease. For instance, 18 (A48 vs. S72) and 19 (A48 vs. S96) primary metabolism genes are upregulated respectively. The numbers of upregulated secondary metabolism genes are even decreased with 28 (A48 vs.

S72) to 19 (A48 vs. S96) upregulated genes revealing high consistency/similarity regarding both asexual stages to S72.

Among the delayed genes derived from different categories Top20 genes of secondary metabolite, transcription-signal transduction-development, cell wall remodelling and glycosyl hydrolyzation and protein degradation and modification were depicted and listed in table 4. Within the secondary metabolite category are four interesting delayed candidates. A putative bioluminescence fatty acid reduction in *A. nidulans* might be catalyzed by a LuxE-like acyl-protein synthetase encoded by AN4201. A putative tyrosinase (AN8435) is expressed at A48 and with 24 h delay at S72. AN4806 codes for a putative dopa 4,5-dioxygenase. It converts 3,4-dihydroxyphenylalanine to betalamic acid (betalain), a yellow chromophore also present in basidiomycetes. Betalain belongs to alkaloids and possesses antioxidative properties that might also protect *A. nidulans* from harming ROS and photooxidation. Betalain synthesis gene expression is already initiated at vegetative stage and still detectable at A24, whereas its expression is extended for 24 h. Furthermore, *A. nidulans* activates AN8134, coding for an enzyme of a transferase family, homologue to an anthranilate N-hydroxycinnamoyl/ benzoyltransferase that catalyzes the first committed reaction of phytoalexine biosynthesis in plants. Phytoalexines are first direct reactions of the plant on bacterial or fungal infections to avoid their proliferation and growth. Its expression starts at vegetative stage and is still present at S48. *A. nidulans* might also possess a similar mechanism to react on attacking and competing bacteria or fungi directly.

Most listed candidates belong to transcription-signal transduction-development category since a main focus in this thesis is on the search for possible genes involved in the regulation and induction of asexual development also initiated at sexual development. AN10600 codes for a fungal-specific Zn(II)2Cys6 transcription factor with potential for initiation of asexual differentiation and conidiophore formation. It is present at both asexual stages and expressed at S72 merely. RNA silencing is another opportunity to control translation posttranscriptionally. Thereby, argonaute proteins are involved in inverted repeat transgene (IRT)-induced RNA silencing. With a delay of 24 h a putative argonaute protein encoded by AN1519 is expressed during asexual and sexual development. It is already activated at early asexual stages whereas it appears at S72 merely. Either it is passively involved in the activation of sexual development or it derepresses asexual differentiation factors. The AN1553 encoded aegerolysin-like protein is expressed at early asexual stage and expressed at sexual development with 24 h delay.

Table 4: Top 20 of delayed genes expressed comparing asexual and sexual development of *A. nidulans.*

Gene ID	Function	A24	A48	S24	S48	S72	S96
Secondary metabolism							
AN4201	Acyl-protein synthetase, LuxE	+			+	+	
AN8435	Tyrosinase, put. → pigment formation		+			+	+
AN4806	Dopa 4,5-dioxygenase, put. → betalain		-			-	-
AN8134	Transferase family put. → phytoalexine	-	-			-	-
Transcription, signal transduction, development							
AN10600	Zn(II)2Cys6 transcription factor, fungal, put	+	+			+	
AN1519	Argonaute protein, put. → RNA silencing	+	+			+	+
AN1553	Aegerolysin-like protein, put. initial fruiting	+	+		+	+	
AN5408	RNAse III, put. → RNAi → silencing	+			+		
AN6382	Phospholipase C, put. → signal cascade		+			+	+
AN7388	***cpeA***, catalase-peroxidase*	+	+		+	+	
AN8923	Heterokaryon incompatibilty protein, put.	+	+			+	+
AN0055	***tmpA***, transmembrane protein involved in regulation of conidium formation*		-				-
AN1037	***odeA***, oleate delta-12 desaturase*		-			-	-
AN10741	Cyclin box fold for transcriptional control	-	-		-	-	-
AN10893	NADPH oxidase, put.		-			-	-
AN3310	Extracellular serine-rich protein, put.	-	-		-	-	-
AN5844	Conidiation protein 6		-			-	-
AN8132	Zinc-dependent metalloprotease	-	-		-	-	-
Cell wall remodelling & glycosyl hydrolysis							
AN4871	***chiB***, Chitinase class V* → autolysis,aging	+	+			+	+
Protein degradation and modification							
AN6625	F-box protein, put.	+			+	+	+

Among numerous differentially expressed delayed genes earlier expressed during light and delayed in darkness interesting candidates involved in secondary metabolism, development, signal transduction, cell wall and protein degradation are listed in this table. For instance, genes from secondary metabolism category like the tyrosinase (AN8435) that might be involved in pigment formation or the putative dopa 4,5-dioxygenase (AN4806) that might be linked to betalamic acid, a yellow chromophore, synthesis. Thereby AN8435 expression is detectable in light at A48 pointing at a possible function in conidiospore pigmentation, which takes place delayed in darkness since asexual (A) differentiation is induced secondarily when sexual (S) development is at an advanced stage and almost finished. AN4806 expression is already present at vegetative stage. It remains upregulated at both 24 h stages whereas expression is prolonged and delayed for 24 h in darkness. Numbers behind A and S show hours after induction start for asexual and sexual development respectively. Data derived from transcriptome data of two independent biological replicates and two microarray platforms. put. = putative. Cut off was set to log2 ≥ ±1.5. (*) validated genes.

Aegerolysin and ostreolysin were found to be expressed during formation of primordia and fruit bodies in fungi. It has been suggested that these haemolysins play an important role in initial phase of fungal fruiting. Since this gene is involved in fungal development generally and is also expressed at asexual stage it might also participate in the initiation and control of asexual differentiation. A putative RNAse III is upregulated at A24 and S48. Eukaryotic RNase III participates in rRNA processing, in processing of small nucleolar RNAs (snoRNAs) and snRNA components of the spliceosome. In eukaryotes RNase III or RNaseIII like enzymes such as Dicer are involved in RNAi and miRNA gene silencing. As for the differentially expressed argonaute gene (AN1519), RNAase III is an additional factor for post-transcriptional control and might have an important impact on *A. nidulans* development generally or even control delayed asexual differentiation. Phospholipases are part of signal cascades in pro- and eukaryotic organisms. Thereby, phospolipase C catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) to D-myo-inositol-1,4,5-trisphosphate (1,4,5-IP3) and sn-1,2-diacylglycerol (DAG). Both products function as second messengers in eukaryotic signal transduction cascades and could also have impact on development of *A. nidulans*. The already verified catalase-peroxidase (*cpeA*, AN7388) was also identified as laccase II expressed during sexual development (Scherer & Fischer, 1998, Scherer, *et al.*, 2002). We find *cpeA* also expressed at both asexual stages and with 24 h delay at sexual stages S48 and S72. Since CpeA was localized to cleistothecia and nursing Hülle cells with a potential role in ROS triggering, it might trigger initiation of asexual development even at sexual conditions through defined ROS levels. AN8923 encodes a heterokaryon incompatibility protein (HET) restricted to ascomycetes. Genetic differences in HET genes prevent a viable heterokaryotic fungal cell from being formed by the fusion of filaments from two different wild types. This HET gene is already activated at early asexual stage and present at S72 merely suggesting an asexual specific expression accompanying the secondarily induced asexual cycle during sexual development. A direct hint for the formation of asexual structures at sexual stage in *A. nidulans* is based on the expression of *tmpA* (AN0055), coding for a transmembrane flavoprotein involved in conidiophore formation (Soid-Raggi, *et al.*, 2006). *tmpA* expression starts at vegetative stage and is present until A24 and S72 with 48 delay. The oleate delta-12 desaturase encoded by *odeA* (AN1037) is also expressed at vegetative stage. Additionally, *odeA* expression is still present at A24 and S48. Since *odeA* participates in conidiation and is also expressed with 24 h delay it might also control conidiation at sexual differentiation in *A. nidulans*. Cyclin box fold encoded by AN10741 contains a protein binding domain functioning in cell-cycle and transcriptional control. Its

expression already starts at vegetative growth and is still present at early sexual stage S24. Either it might be involved in the initiation of sexual differentiation directly or in delayed initiation of asexual development at sexual conditions. A homologue of NoxA (NADPH oxidase), a positive regulator of cleistothecia formation, is the putative NADPH oxidase (AN10893). Beside its initial upregulation at vegetative growth, expression remains stable until A24 and S48. Like NoxA, this uncharacterized Nox might also control and regulate *A. nidulans* development through triggered ROS generation. AN3310 codes for a putative extracellular serine-rich protein, a developmentally regulated MAPK interacting protein. Its expression is restricted to vegetative stage and S24 and, therefore, is most likely linked to initiation of sexual development and fruiting. Conidiation proteins accompany asexual differentiation and trigger successful initiation and performance of asexual development. Conidiation protein 6 encoded by AN5844 is also activated at S24 and S48 with 24 h delay representing strong evidence for a delayed initiation of asexual differentiation at sexual development. Beside its expression at vegetative stage the zinc-dependent metalloprotease (AN8132) expression is still detectable at S24. Although it is basically classified in protein degradation category, this metalloprotease possesses developmental connection. It has wide phylogenetic distribution and contains sub-families involved in vertebrate development and might also be required in initiation of sexual development.

At the end of asexual and sexual development *A. nidulans* performs autolysis of differentiated tissue not needed anymore. *chiB* (AN4871) encodes a chitinase class V crucial for autolysing and aging in *A. nidulans*. *chiB* is already expressed at A24, whereas it is activated at S72 merely when sexual development is at an advanced stage. Autolysis assures fungal nutrient supply and finally survival even in a nutrient exhausted environment. Autolysis of differentiated structures symbolise an aging process of *A. nidulans*.

The putative F-box protein (AN6625) is expressed at A24 and S48 reveals a 24 h delay. It might control asexual development through targeted degradation of factors probably responsible for the initiation of asexual differentiation.

3.2 Development-specific gene and metabolite pattern in *A. nidulans*

Within this study we give an overall picture of gene expression and metabolite content for a time course during vegetative, asexual and sexual growth, which might be relevant for biotechnological issues and usage in order to identify novel clusters, products and understand their kinetics.

The transcriptome as well as the intra- and extracellular metabolome data of the developmental stages of *A. nidulans* were clustered by training an one-dimensional self-organizing map (1D-SOM) model to obtain a global overview of these comprehensive data sets (Fig. 8). The data mining by 1D-SOMs allows arranging intensity profiles of microarray and metabolome data according to similarities within the expression/accumulation pattern during development. The metabolite fingerprinting analysis and data interpretation was performed by Dr. Kirstin Feussner, Department of Plant Biochemistry, Göttingen.

For the transcriptome (Fig. 8A) and the intracellular metabolome analysis (Fig. 8B) seven stages of development were compared (Veg, A24, A48, S24, S48, S72, S96), while only three stages (Veg, A48, S96) were analyzed for the extracellular metabolome (Fig. 8C).

Therefore, a subset of high quality marker candidates was generated by filtering with Kruskal-Wallis test. For the intracellular and the extracellular metabolome analysis a subset of 3,162 and 3,234 marker candidates with pVal<1x10-6 and 5x10-4, respectively, were used for further analysis. For the analysis of the transcriptome data expression values of 2,014 differentially expressed genes were normalized, calculated regarding their absolute expression and used for 1D SOM.

After intensity-based clustering by 1D-SOMs, all three data set show blocks of comparable intensity pattern (colored frames in Fig. 8). The framed blocks represent marker candidates specific for the vegetative (blue labelled), the asexual (yellow labelled) or the sexual developmental stages (red labelled).

The transcriptome data show additionally a large block of markers (cluster 13-15), which are up regulated in the asexual and the sexual stage. A comparable pattern is detectable on metabolite level by the prototypes 16-18, but with one essential distinction. While transcripts are already upregulated in the early sexual stage (S24), the intracellular metabolites are still not enhanced. The levels of these metabolites marker are not enriched in the vegetative and in the early sexual stage. The strong analogy between vegetative and early sexual stage on metabolite level becomes also apparent for cluster 2. Metabolite markers, specific for the vegetative growth are still present in the S24 stage.

A remarkable profile is also shown by cluster 7 of the intracellular metabolome data. This prototype represents marker candidates, which exclusively occur during the early sexual stage. These could be metabolite candidates, which may operate as signals/compounds essential for successful reprogramming of the fungal metabolism.

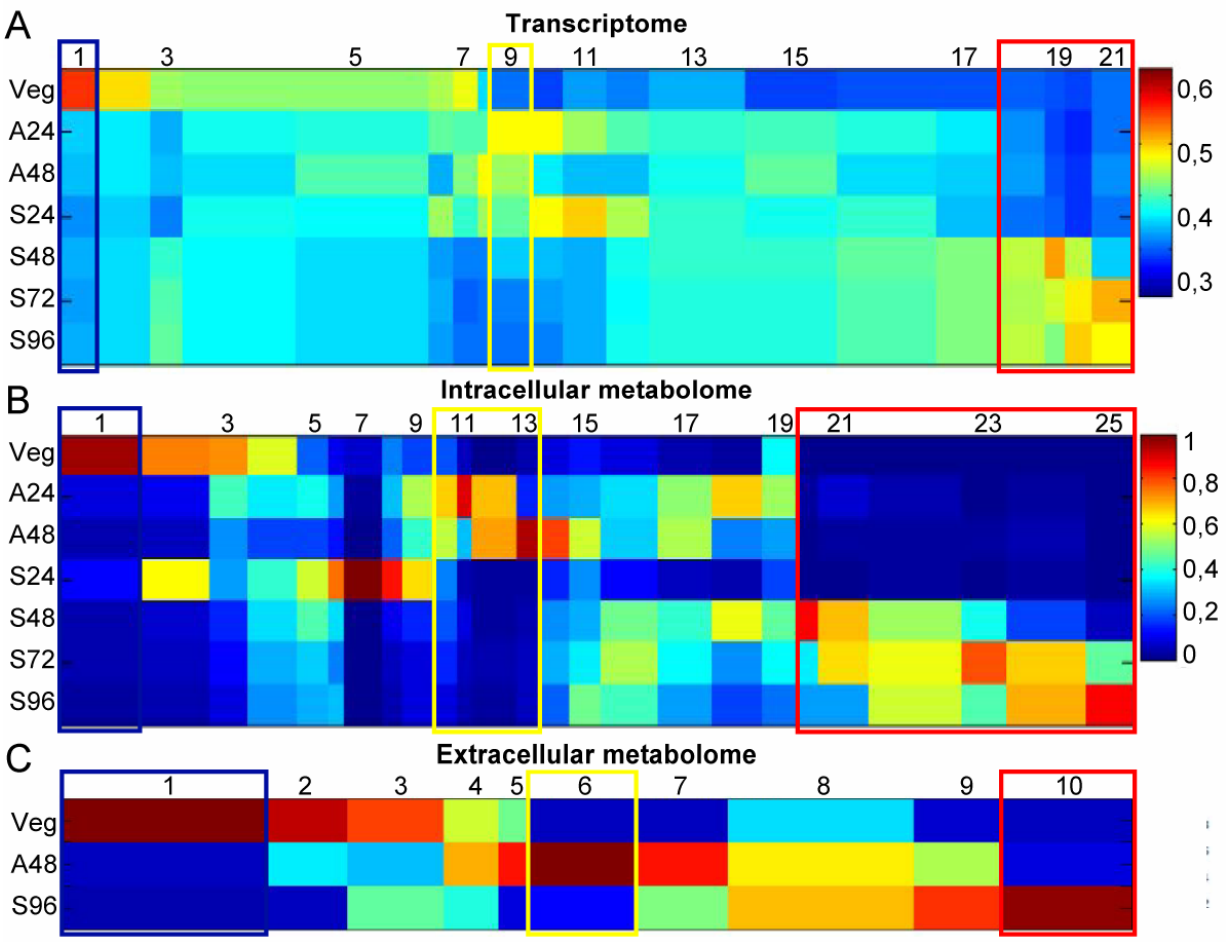


Figure 8: Clustering and visualization of the transcriptome, intra- and extracellular metabolome data by 1D-SOMs. Transcriptome (A), intracellular (B) and extracellular metabolome (C) data of different stages of vegetative (Veg), asexual (A24, A48) and sexual (S24, S48, S72, S96) development were arranged according to similarities within the intensity pattern by training a 1D-SOMs model. For that a subset of high quality marker candidates were generated by filtering by Kruskal-Wallis. The horizontal and vertical dimensions correspond to prototypes and developmental stages, respectively. The color of matrix elements represents (average) intensity values according to the color map on the right hand side. The width of the matrix column for each prototype is proportional to the number of marker candidates assigned to this prototype. The metabolite fingerprinting analyses was performed by Dr. Kirstin Feussner, Department of Plant Biochemistry, Göttingen.

Metabolites, which are intra- and extracellular enriched during the asexual stage are emericellamide C and anthranilate. High amounts of emericellamide A and E and terrequinone A are only intracellular enriched at A24 and A48.

Amongst asperthecin, emodin, and emericellin, other light-dependent secondary metabolites could be identified, which are specifically enriched during the asexual and sexual phase. A more detailed consideration of the omics data on the level of selected gene clusters, its gene products as well as the corresponding biosynthetic compounds, respectively will be given in the next chapters.

3.3 Light-dependent asexual development in *A. nidulans*

3.3.1 Transcriptome

3.3.1.1 Asexual regulators and development specific genes in *A. nidulans*

Development in *A. nidulans* is examined since many years. Numerous developmental regulators and genes specific for development are described. We present an expressional overview of these genes during the course of *A. nidulans* development for the analyzed stages (Tab. 5).

Table 5: Asexual regulators & development specific genes expressed in *A. nidulans* as control for the proper induction of asexual development.

Gene ID	Function	A24	A48	S24	S48	S72	S96
Asexual development regulators							
AN0973	***brlA***, regulator of asex development*	+	+				
AN1937	***wetA***, regulatory protein involved in conidial development *	+	+				
AN1959	***vosA***, involved in conidia formation*		+				
Asexual specific genes							
AN0231	***ivoB***, Conidiophore-specific phenol oxidase*		+				
AN10148	***chpA***, sporocarp development involved in asex reproduction*				-		-
AN10628	***spoC1***, MAC/Perforin domain, conidia specific RNA of unknown function	+	+				
AN3079	***cetA***, Secreted thaumatin-like protein, role in early conidial germination		+				
AN6635	***yA***, conidial laccase*	+	+				
AN8006	***dewA***, conidium wall hydrophobin*	+	+				
AN8021	***vmaA***, vacuolar ATPase (V-ATPase), subunit A → growth and asexual sporulation						-
AN8209	***wA***, conidial polyketide synthase*	+	+				
AN8638	***cetJ***, Conidia enriched Transcript	+	+				
AN8640	Conidiation prot 6 Con-6	+	+				
AN8803	***rodA***, Conidium wall hydrophobin	+	+				

A. nidulans contains numerous developmental regulators specifically expressed at and required for the development the fungus is able to undergo. The listed differentially expressed genes are already described to be specific for asexual (A) and sexual (S) development and are triggers one can use as control. Beside developmental regulators numerous genes specifically expressed at defined development stages were found. Among them are asexually, conidial specific genes like *ivoB* (AN0231, phenol oxidase), *cetA* (AN3079, thaumatin-like protein), *yA* (AN6635, conidial laccase) and many more candidates. Numbers behind A and S show hours after induction start for asexual and sexual development respectively. Data derived from transcriptome data of two biological replicates and two microarray platforms. Cut off was set to log2 > ±1.5

Typical regulators of asexual development like *brlA* (AN0973), *wetA* (AN1973) and *vosA* (AN1959) appear differentially expressed in this study. *brlA* and *wetA* are expressed at

both asexual stages, whereas *vosA* transcripts increase at A48. VosA (viability of spores) might assure protection of conidiospores from environmental influences important for their germination in soil finally.

Proteins specific for asexual development and serving as control are *ivoB* (AN0231) encoding a conidiophore specific phenol oxidase, the conidial laccase *yA* (AN6635), two conidium wall hydrophobins *dewA* (AN8006) and *rodA* (AN8803) and the conidial specific polyketide synthase *wA* (AN8209). These genes are expressed at A24 and A48 except for *ivoB* and are among other differentially expressed asexual genes.

3.3.1.2 Light-dependent saprophytic genes provide energy supply during asexual development of *A. nidulans*

Beside mobilization of own carbohydrates from hydrolysis of its cell wall, *A. nidulans* expresses genes required for its saprophytic life. For the usage of plant and bacterial cell wall glucans/peptidoglycans and stored polysaccharides, e.g. starch and lichenins, the fungus expresses numerous hydrolases on specific stages in its development (Tab. 6). Plant cell wall consists of a primary and secondary layer. The primary layer is a composition of pectins, cellulose, hemicellulose (like xylan) and glycoproteins. Covering and protecting secondary plant cell wall named cuticula consists of microfibrilcellulose and hemicellulose and contains cutin and waxes that protect plants from drying-out.

Most of these genes are already expressed at vegetative growth. It seems to be important for the fungus to get additional energy from the extracellular environment since it has to store a lot of carbohydrates for the composition of novel cell wall for asexual and sexual structure formation. It is important to differ between vegetative specific genes and genes whose expression is initiated at vegetative stage and is continued at asexual and/or sexual differentiation. Overall 12 saprophytic genes are already expressed at vegetative stage. *pmeA* (AN3390), which was already shown to be a pectinesterase (Bauer, *et al.*, 2006, Coutinho, *et al.*, 2009), is expressed at vegetative stage exclusively (Tab. 6). Expression of 11 out of the 12 vegetatively expressed genes is continued during differentiation. Two of these genes are muramidases (AN6470; AN8969), which are able to hydrolyze bacterial cell walls. Nine genes, which are expressed at vegetative stage, encode hydrolyzing enzymes for plant cell wall. The rest of the genes belong to following categories: five xylan hydrolyzing enzymes (*xgcA* AN1542; AN2359; AN7158; *xlnB* AN9365), two cutinases (AN10346; AN7541), *xgcA* an end-specific cellobiohydrolase (AN1542), three pectin hydrolyzing enzymes (*abfB* AN1571; *pgaB* AN4372) and a glycosyl hydrolase family 61 with cellulose

binding domain (AN7891). Since vegetative growth takes place on the surface of soil and/or within soil (invasive growth), *A. nidulans* expresses early genes required for a broad range of carbohydrates that are possibly present and can be hydrolyzed during its saprophytic life.

Table 6: Selected genes mediating plant and bacterial cell wall hydrolysis assure energy supply for *A. nidulans* development in light.

Gene ID	Function	A24	A48	S24	S48	S72	S96
Cell wall degradation of plant & bacteria							
Vegetative specific							
AN3390	***pmeA***, Pectinesterase *	-	-	-	-	-	-
Already at vegetative growth							
AN10346	Cutinase	-					
AN1542	***xgcA***, end-specific cellobiohydrolase*	-	-		-	-	-
AN1571	***abfB***, alpha-arabinofuranosidase*	-	-				-
AN2359	xylan 1,4-beta-xylosidase		-				
AN4372	***pgaB***, Polygalacturonase *				-	-	-
AN6470	N,O-diacetyl muramidase, put. *						-
AN7158	Xylanase, put				-	-	-
AN7541	Cutinase, put	-	-			-	-
AN7891	GH family 61, extracellular, put		-		-	-	-
AN8969	Muramidase > peptidoglycan hydrolysis		-			-	-
AN9365	***xlnB***, endo-1,4-beta-xylanase *	-	-				
Asexual specific							
AN3883	Extracellular endo-1,3(4)-beta-glucanase, put.	+	+				
AN5267	***faeC***, ferulic acid esterase *	+	+				
AN7180	Cutinase*		+				

Identified differentially expressed glycosyl hydrolysing genes were checked for the function of their conserved domains and additionally for homology to already characterized plant and bacterial cell wall and polysaccharide hydrolysing genes in other organisms. Selected genes were sorted for their specific expression during vegetative growth, asexual (A) and sexual (S) differentiation respectively. For instance, *pmeA* (AN3390) encodes a pectinase catalyzing hydrolysis of pectins contained in the plant primary cell wall layer. It is upregulated at Veg specifically and secures energy supply from external sources like plants at vegetative growth. AN8969 encodes a muramidase that gives the fungus opportunity to hydrolyse bacterial cell wall. AN8969 expression is already increased at Veg and is continued at early stage of asexual A24 and sexual development S24, S48. Numbers behind A and S show hours after induction start for asexual and sexual development respectively. Data derived from transcriptome data of two independent biological replicates and two microarray platforms. put. = putative. Cut off was set to log2 > ±1.5. (*) validated genes.

In contrast, the number of differentially expressed asexual and sexual specific, saprophytic genes is less. At asexual development three additional hydrolases for the hydrolysis of extracellular glucans/xylans/cutins are expressed specifically. Interestingly, no additional bacterial cell wall hydrolases are expressed. AN3883 encodes a secreted, extracellular endo-1,3(4)-beta-glucanase, which might be required for hydrolysis of the cell wall of other fungi or plants since its activity is not specified yet. 1,3-beta glucans are for instance lichenin, laminarine and zymosane, whereas cellulose and chitin are 1,4-beta glucans. Finally, this enzyme is extracellular and ensures energy mobilization for the fungus. AN3883 is expressed at both asexual stages A24 and A48. A ferulic acid esterase encoded by *faeC* (AN5267) is described to hydrolyze plant xylans (Bauer, *et al.*, 2006, Coutinho, *et al.*, 2009). AN7180 encodes a verified cutinase and is expressed at A48. Obviously, *A. nidulans* does not need to express additional bacterial cell wall hydrolases on the surface of soil when it develops asexually.

3.3.1.3 *A. nidulans* undergoes an aging process through controlled cell death mediated by regulators of apoptosis and reactive oxygen generating factors in light

Sessile organisms like filamentous saprophytic soil-born fungi exhaust nutrients within their habitat and suffer in nutrient starvation after a certain period. Therefore, the fungus initiates programmed cell death after maturation and release of asexual and sexual spores. Apoptosis is apparently initiated already after 24 h at asexual growth in light. An *A. nidulans* orthologue protein of the apoptosis initiation factor 1 (*AIF1*) in yeast encoded by AN9315 is upregulated at A24 (Tab. 7). *AIF1* is a mitochondrial cell death effector that enters nucleus in response to apoptotic induction/stimuli (Wissing, *et al.*, 2004). Overexpression of *AIF1* increases induction of programmed cell death. Generally, a consequence of mitochondrial alterations during apoptosis is disruption of the electron transport chain and incidental production of ROS (Bras, *et al.*, 2005). For instance, hydrogen peroxide (H_2O_2), which induces oxidative stress, triggers apoptosis in *S. cerevisiae* (Madeo, *et al.*, 1999). Indeed maturation of conidiospores is not completed at this stage of asexual development but programmed cell death is clearly induced before this developmental cycle is finished. AN4874, which encodes another initiator of apoptosis signalling with caspase activity, is upregulated at A48 and might be crucial for aging at late asexual stage. Its ornithology in *S. cerevisiae* Rny1p has endoribonuclease activity and has a role in cell morphogenesis and apoptosis (MacIntosh, *et al.*, 2001, Thompson & Parker, 2009).

Table 7: Light-specific genes important for development, programmed cell death and oxidative stress response trigger reactive oxygen levels in *A. nidulans.*

Gene ID	Function	A24	A48	S24	S48	S72	S96
Apoptosis							
Asexual specific							
AN9315	Yeast orthologue apoptosis initiation factor 1 (*AIF1*)	+					
AN4874	Yeast orthologue *RNY1* promotes apoptosis		+				
NADPH oxidases (Nox)							
Already at vegetative growth							
AN0773	Nox, uncharacterized			-			-
AN10893	Nox, uncharacterized		-			-	-
Superoxide dismutases (Sod)							
Already at vegetative growth							
AN5148	Sod, Fe, uncharacterized						-
Asexual specific							
AN1131	Sod, Cu/Zn, uncharacterized	+	+				
Catalases & Catalase-peroxidase							
AN7388	***cpeA***, catalase-peroxidase *	+	+		+	+	
AN8637	***catA***, conidia specific catalase *	+	+				
AN9339	***catB***, hyphal catalase *		-				
AN8553	Catalase, uncharacterized	+	+				

A. nidulans expresses two genes AN9315 and AN 4874 with connection to apoptosis during light-dependent asexual development in *A. nidulans.* AN4874 encodes an orthologue of RNY1 that is involved in apoptosis in *S. cerevisiae* and is expressed at late asexual (A) development. AN9315, already activated at A24, is a serious hint for *A. nidulans* to induce programmed cell death already before asexual development and conidiophore formation is finished. Additionally, *A. nidulans* contains several enzymes required for reactive oxygen species (ROS) triggering development and induction of programmed cell death as reaction to oxidative stress. Within this work novel, not yet characterized and/or identified and development specific expressed genes coding for NADPH-oxidases (Nox), superoxide-dismutase (Sod), catalases were found. AN0773 and AN10893 encode two Nox already expressed at vegetative growth. Both still remain activated at A24 when conidiophore formation is not yet finished. In contrast to sexual cycle in darkness *A. nidulans* did not express an asexual specific Nox. Beside a Sod already upregulated at vegetative growth, another still uncharacterized Cu/Zn Sod is specifically activated at both asexual stages A24/A48. *catA* encodes a well described conidia specific catalase. AN8553 codes for another still uncharacterized catalase that reveals a similar expression pattern and thus belongs to asexual specific catalases. A sexual specific catalase could not be detected. Numbers behind A and S show hours after induction start for asexual and sexual development, respectively. Data derived from transcriptome data of two independent biological replicates and two microarray platforms. Cut off was set to $\log2 \geq \pm 1.5$. (*) validated gene.

Here, expression of several genes, coding for putative and still not identified ROS-connected enzymes, was detected (Tab. 7). There are four putative Nox (AN0773, AN10893, AN4906, AN8683) with ferric reductase FAD-binding domain (BROAD) and ferric-chelate domain (NCBI/PFAM) found in the genome, respectively. AN10893 contains a ferric reductase-like transmembrane component, additionally. AN0773 and AN10893 encode Nox, which are already expressed at vegetative growth and might be crucial for initiation of differentiation through H_2O_2 and/or O_2^- formation. Subsequent expression of AN0773 is still detectable at early stage of asexual development, whereas AN10893 expression is still present at both early stage of asexual and sexual development.

Interestingly, an asexually activated Nox could not be detected showing either vegetative Nox possessing an important role for asexual differentiation already at vegetative stage or asexual development requires inactivated Nox generally. In contrast, AN4906 and AN8683 encode sexual specifically expressed Nox and are described in more detail in sexual development chapters (page 81, chapter 3.4.1.5, Tab. 15).

Additionally, superoxide-dismutases (Sod) catalyzing conversion of superoxide to hydrogen peroxide reveal development specific expression as well. AN1131 encodes an asexual specifically expressed (A24 + A48) putative cytosolic Cu/Zn Sod not identified and characterized yet. An uncharacterized ferric Sod (AN5148) is expressed already at vegetative stage but the exact molecular function is still unknown like many other gene products identified and expressionally described in this work. This ferric Sod is merely downregulated at late sexual development S96.

Since Nox generates superoxides from hydrogen peroxides and Sod catalyzes the reverse reaction, we can assign development specific, antagonistic Nox and Sod based on our transcriptome data. Thus, vegetative Nox (AN0773; AN10893) are counterparts of the Fe Sod (AN5148), the sexual Nox (AN4906; AN8683) of the Mn Sod *sodM* (AN0785), whereas the asexual specific Cu/Zn Sod (AN1131) has no opposite Nox detected in this study. Probably asexual stage requires another specific H_2O_2 / O_2^- ratio.

Catalases catalyze the decomposition of hydrogen peroxide into water and oxygen (Chelikani, *et al.*, 2004). Beside the already identified and described catalases A – C (AN8637; AN9339; AN5918) (Navarro, *et al.*, 1996, Kawasaki, *et al.*, 1997, Kawasaki & Aguirre, 2001) another asexual specifically expressed catalase (AN8553) was identified in this work. The conidia-specific catalase CatA exhibits expression at A24 and A48 as described. Expression of *catB*, which encodes a hyphal catalase, was indeed detectable at vegetative stage but it was also found at asexual and sexual development. Its expression

merely decreases at late asexual development A48 and is present at each analyzed stage of sexual differentiation. *catC* expression is constitutive during development. Finally, AN8553 presents in conjunction with *catA* a second asexual specific catalase.

These results suggest that apparently, *A. nidulans* mobilizes numerous enzymes to get rid of toxic ROS produced predominantly during asexual growth at high oxygen and UV radiation. The lack of asexual specific Nox is an important way to avoid the formation of further toxic ROS like superoxide in an environment with high oxygen partial pressure and high potential for the formation of UV-generated ROS. Thus, *A. nidulans* at asexual stage possesses characteristic ROS ratio to keep cellular damages as low as possible and thereby assure proper regulation to complete developmental cycle successfully.

3.3.1.4 Light-dependent production of lipopolysaccharide, isoquinole alkaloide and anthranilate by the fungus as chemical repellents against predators

Numerous organisms produce repellents to protect themselves against competitors and predator/herbivore. For instance, many plants rely on physical defense to deter herbivores but also invest in active chemical defense mechanisms. They produce numerous repellents like anthranilate derivatives as feed protection. Even bacteria incorporate endotoxins within their membrane for protection. Lipooligo- (LOS) and lipopolysaccharides (LPS) are compounds localized in the outer membrane of gram-negative bacteria. They are endotoxins causing several diseases in humans and animals (Rivest, *et al.*, 2000). *A. nidulans* expresses two genes (AN10601; AN6460) encoding glycosyl transferase family 25 proteins involved in LPS or LOS biosynthesis, respectively (Tab. 8).

Table 8: Lipopoly- (LPS) and Lipooligosaccharide (LOS) synthesis genes expressed in *A. nidulans*

Gene ID	Function	A24	A48	S24	S48	S72	S96
Already at vegetative growth							
AN6460	Glycosyl transferase family 25, lipooligosaccharide (LOS) synthesis	-	-			-	-
Asexual specific expressed							
AN10601	Glycosyl transferase family 25, lipopolysaccharide (LPS) synthesis	+					

Identified differentially expressed Lipopoly- (LPS) and Lipooligosaccharide (LOS) synthesis genes. Both contain glycosyl transferase family 25 domains involved in LPS- and LOS-synthesis. AN6460 is already expressed at vegetative growth, whereas AN10601 is specific for asexual (A) development. LPS and LOS synthesis genes are not specifically expressed at sexual (S) differentiation. Numbers behind A and S show hours after induction start for asexual and sexual development respectively. Data derived from transcriptome data of two independent biological replicates and two microarray platforms. Cut off was set to log2 > ±1.5.

Both genes are uncharacterized and their exact molecular function is still unknown. AN10601 is described at BROAD to be a LPS glycosyl transferase. AN10601 is specifically expressed during asexual sporulation. Since feeding through animals is more likely on the surface of soil, fruits or leaves, the fungus protects itself with expression of light dependent LPS biosynthesis genes and might integrate LPS in its outer membrane as feed protectant. The LOS biosynthesis glycosyl transferase encoded by AN6460 is already expressed at vegetative growth during both, growth on surfaces in light and invasive in soil. Expression is still present at early and middle sexual phase.

Additionally, isoquinole alkaloids also serve as repellent in plants. At A48 we found three downregulated genes AN5846, AN7081, AN7274 coding for uncharacterized proteins with berberine bridge-like domain found in enzymes involved in the biosynthesis of numerous isoquinoline alkaloids. Many alkaloids are toxic to other organisms. For instance, plants synthesize numerous toxic isoquinoline alkaloids as feed protectant. It is most likely that a similar synthesis and protection also resides in *A. nidulans*. Since the three genes are already activated at vegetative stage, isoquinole alkaloid synthesis is presumably of high importance for the fungus to protect the mycelia as basis for its proliferation. *A. nidulans* seems to protect conidiophore formation through their expression until it is decreased at A48 in light when conidiophore formation is finished. Conidiospores are spread and the conidiophore requires no further protection.

Additionally, we could identify asexual specific accumulated anthranilate in the extra- and intracellular metabolome. Basically, anthranilate is an amino acid used as an intermediate for production of dyes, pigments, and saccharin as well. It serves as UV-absorber and mould inhibitor in Soya as well. Furthermore, methylated anthranilate is another feed protection, which is synthesized and accumulated through concord grapes and other plants as repellent against e.g. birds (Wang & De Luca, 2005). Anthranilate might also serve as repellent since this compound owns a wide range of chemical activities. The metabolomic appearance is going to be described in more detail in the corresponding metabolome chapters 3.3.2.1 (page 65) and 3.4.2.2.2 (page 95).

3.3.1.5 Activation of several secondary metabolite clusters without product during asexual development of *A. nidulans*

Expression of several already known secondary metabolite clusters in *A. nidulans* is closely connected with the induction of the two light-dependent developmental pathways. It was proposed that expression of several secondary metabolite gene clusters are low at

standard laboratory conditions and some clusters are not expressed generally and their production is to low to detect in these conditions. One prominent example is the asperfuranone gene cluster whose gene product is not detectable under normal lab conditions (Chiang, *et al.*, 2009).

Our transcriptome revealed an increased expression of *afoB* (AN1031) encoding an MFS efflux pump, a salicylate hydroxylase (AN1033) and a FAD/FMN-dependent oxygenase (AN1035) at asexual development (Tab. 9). Neither the important transcription factor gene (AN1029) nor the two polyketide synthases encoded by AN1034 and AN1036 respectively are upregulated within the examined stages of development. There is no hint for the signal activating the asperfuranone gene cluster. Its gene product is also absent in the metabolome. Either it is not synthesized or the amount is beneath the detectable concentration.

A directed production of the secondary metabolite orsellinic acid in *A. nidulans* could be linked to co-cultivation with the bacterium *Streptomyces hygroscopicus* (Schroeckh, *et al.*, 2009). Expression of each gene (AN7909 – AN7914) participated in orsellinic acid synthesis was highly upregulated after *S. hygroscopicus* treatment but its production by the fungus is at below detection limits in normal laboratory conditions. In contrast, three genes (*orsB* AN7911, *orsC* AN7912, *orsE* AN7914) belonging to that gene cluster are upregulated at early asexual A24 and late sexual development S72 + S96. *orsB* encodes a putative amidohydrolase which is upregulated at A48 additionally. The putative alcohol dehydrogenase encoded by *orsE* is also upregulated at S24. The polyketide synthase (AN7909) is constitutively expressed at the analyzed stages of development. Orsellinic acid could not be detected by the metabolome analysis.

Penicillins are β-lactam containing antibiotics, which are produced by several fungi as well as in *A. nidulans*, respectively (Tobin, *et al.*, 1990, MacCabe, *et al.*, 1991, Martin, 1992, Brakhage, *et al.*, 1994, Martin, *et al.*, 1994, Sprote, *et al.*, 2008). Although *acvA* (AN2621) and *aatA* (AN2623) crucial for penicillin production in *A. nidulans* are upregulated at late asexual (A48) and sexual (S48 - S96) development, no penicillin production could be detected. Fermentation experiments of *A. nidulans* for penicillin production by Sprote et al. (2008) were carried out in special fermentation media with phenoxyacetic acid as substrate for IAT/AatB essentially according to Litzka, *et al*. (1996). Cultures were induced for 72 h. Penicillin synthesis in *A. nidulans* wild type strain FGSC A4 grown on minimal medium used in this study was never observed.

Table 9: Secondary metabolite gene clusters without detectable product in *A. nidulans* asexual development

Gene ID	Function	A24	A48	S24	S48	S72	S96
Asperfuranone (Afo) biosynthesis							
AN1031	***afoB***, Efflux pump in Afo synthesis	+	+				
AN1033	***afoD***, Salicylate hydroxylase		+				
AN1035	***afoF***, FAD/FMN-dependent oxygenase	+	+				
Orsellinic acid (Ors) biosynthesis							
AN7911	***orsB***, Amidohydrolase, put.	+	+			+	+
AN7912	***orsC***, Tyrosinase, put.	+				+	+
AN7914	***orsE***, Alcohol dehydrogenase, put.	+		+		+	+
Penicillin (Pc) biosynthesis							
AN2621	***acvA***, Delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase*		+		+	+	+
AN2622	***ipnA***, Isopenicillin-N synthase, put.*	+	+		+	+	+
AN2623	***aatA***, Isopenicillin-N N-acyltransferase, put.*		+		+	+	+
AN7734	(bHLH) Transcription factor; repressor of the Pc biosynthesis gene *aatA* *		+				
AN0881	Isopenicillin synthase, CUE domain, put.		+	+			+
AN10026	Isopenicillin N synthase and related dioxygenases, put.	+	+		+	+	+
AN10028	Cytochrome P450	+	+			+	
AN2390	Isopenicillin N synthase, put., similar to PcbC	-					
AN9350	Isopenicillin N synthase, put., similar to PcbC	+	+			+	+

Transcriptional active and differentially expressed secondary metabolite gene clusters without hits in metabolome analysis. Among them are asperfuranone (Afo), orsellinic acid (Ors) and penicillin (Pc) gene cluster. Three genes AN1031, AN1033 and AN1035 from Afo gene cluster, which comprises nine genes are asexual (A) specific upregulated. In contrast, three genes AN7911, AN7912 and AN7914 from Ors gene cluster, comprising four genes are expressed at both development cycles. *A. nidulans* activates each ors gene at already at early asexual stage but not at early sexual (S) stage. Although four of nine genes of the Pc gene cluster are already expressed at early asexual stage. Pc genes are rather expressed at late stages of asexual and sexual development. Numbers behind A and S show hours after induction start for asexual and sexual development respectively. Data derived from transcriptome data of two independent biological replicates and two microarray platforms. put. = putative. Cut off was set to $\log2 \geq \pm1.5$. (*) validated gene.

In summary, nine genes involved in penicillin production are upregulated at both differentiation cycles. Expression of penicillin biosynthesis genes are restricted to late asexual and mid and late sexual development. At both early stages A24 and S24 expression is absent. Within this work genes were manually identified, which are homologues to genes encoding for enzymes involved in penicillin biosynthesis. Four homologues sequences of isopenicillin

synthase are encoded by AN0881, AN10026, AN2390 and AN9350. Each is still uncharacterized, functionally unknown and partially not described regarding their domains. Sequences were manually blasted and analyzed at NCBI/PFAM database. Interestingly, each isopenicillin synthase homologue reveals upregulation at late asexual and sexual development as well. In combination of their predicted function based on conserved domains and expressional pattern they might be involved in penicillin synthesis too. An additional cytochrome P450 monooxygenase is encoded by AN10028 and located in the chromosomal neighborhood of the putative isopenicillin synthase (AN10026) exhibiting similar expression pattern. It is upregulated at both asexual A24/A48 and late sexual stage S72. It forms an expressional unit and thus a tiny gene cluster comprises AN10026 and AN10028.

3.3.1.6 Identification of a novel secondary metabolite cluster in *A. nidulans*

Filamentous fungi produce numerous secondary metabolites without known function. Some of these compounds have antimicrobial and antibiotic properties and are important factors to provide a defense system against other competitive pro- or eukaryotic organisms in soil. The performed transcriptome analyses gave us opportunity to identify expressional hot spots on the eight fungal chromosomes. After manual identification and research for the putative function of the different parts of the cluster, prediction about their serious involvement in production of secondary metabolites in *A. nidulans* was finished. Here one gene cluster with potential connection to asexual development in *A. nidulans* is described (Fig 9).

A putative pigment generating cluster is formed by AN8433 – AN8438. The AN8433 gene product is a luciferase-like enzyme with amino acid adenylation domain. Luciferases are enzymes catalyzing the synthesis of high energy compounds with a high tendency for decay. With the decay of these compounds bioluminescence emission is detectable. Within the identified pigment cluster a tyrosinase (AN8435) is contained. Tyrosinases are known to catalyze pigment synthesis crucial among many eukaryotic organisms for UV-protection. Pigment product is most likely promoted by the AN8438 encoded cytochrome P450 monooxygenase. Each gene is upregulated at late sexual development S72/S96. Expression at asexual development is splitted. Whereas the luciferase gene is upregulated at both asexual stages, the AN8434 encoded protein with Willebrand factor type A (vWA) domain and ankyrin repeat and the tyrosinase are only asexually expressed at late asexual development A48. In contrast, the cytochrome P450 is already asexually upregulated at A24 but is absent at A48. Thus, pigment production could be different at both differentiation cycles.

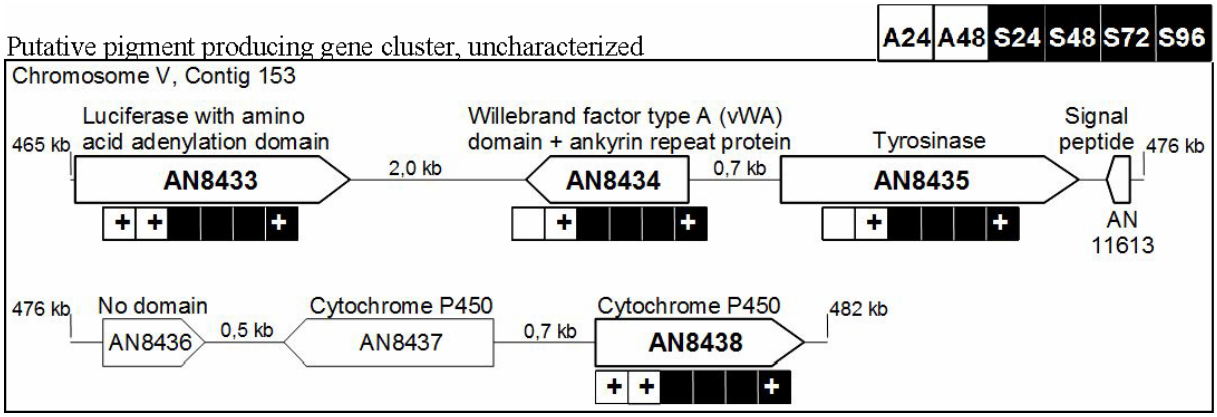


Figure 9: Transcriptionally identified gene cluster with connection to light-driven asexual development in *A. nidulans*. A novel gene cluster with a connection to light-dependent development was identified through the analysis of characteristic expression pattern during the examined stages of *A. nidulans* development. A putative pigment generating cluster is comprised by AN8433 – AN8438. Pigments assure the survival of numerous organisms suffering from e.g. UV radiation. For instance, tyrosinases are involved in melanin production that protects cells of higher eukaryotes from UV damage. Data derived from transcriptomes of two independent biological replicates and two microarray platforms (TIGR and AGILENT). Cut off was set to log2 ≥ ±1.5.

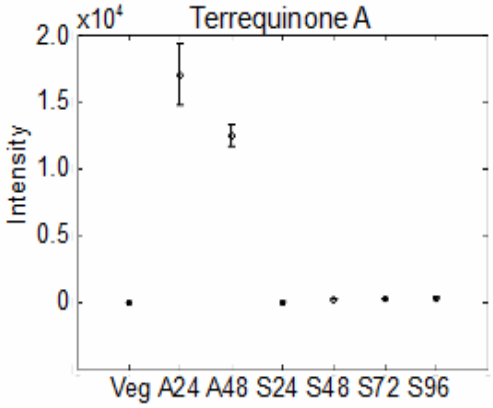
Interestingly, vegetative specifically expressed gene clusters could not be detected, which demonstrates expression of secondary metabolism gene cluster and consequently synthesis mainly takes place during both development cycles. The close connection between secondary metabolite production and development in *A. nidulans* is also reflected within the identification of this novel gene cluster.

3.3.2 Metabolome reveals light-dependent secondary metabolites in *A. nidulans*

3.3.2.1 Light-specific secondary metabolites in asexual development of *A. nidulans*

Since 1D-SOM clustering gives a summary of all present transcripts and metabolite marker candidates at the examined stages and creates a huge subset of data, we concentrated on intra-/extracellular kinetics of secondary metabolites and the genetic regulation of the corresponding gene clusters within this study.

Four out of five genes within the terrequinone (*tdi*) gene cluster (*tdiA* – *tdiE*; AN8513 - AN8517) are differentially expressed (Fig. 10). Whereas *tdiA* and *tdiB* are specifically expressed at both asexual stages, *tdiC* and *tdiE* are expressed at sexual development additionally. Since terrequinone A accumulates at asexual and is absent at sexual development, specific expression of each *tdi* gene shown here is essential for terrequinone synthesis. Thus, upregulation of the *tdiD* encoded aminotransferase is most likely not necessary for terrequinone biosynthesis.



tdi *gene cluster regulation*

Gene ID	Function	A24	A48	S24	S48	S72	S96
AN8513	***tdiA***, nonribosomal peptide synthetase, put.*	+	+				
AN8514	***tdiB***, Asterriquinone prenyltransferase*	+	+				
AN8515	***tdiC***, NADPH-dependent quinone reductase*	+	+			+	+
AN8517	***tdiE***, protein required for Tq synthesis*	+	+		+	+	+

Figure 10: Terrequinone A, a secondary metabolite specific for *A. nidulans* development in light. Terrequinone (Tq) A is synthesized at both asexual (A) stages of *A. nidulans* specifically. Although *tdi* gene expression required for Tq synthesis is partially present at sexual (S) development, its accumulation is strictly restricted to asexual development. Tq and its gene products are absent from vegetative (Veg) stage. Data from intracellular metabolome derived from two biological and three technical replicates. Relative intensities of metabolite markers are shown as error bars containing mean values (points) and standard deviations (bars). The metabolite fingerprinting analyses was performed by Dr. Kirstin Feussner, Department of Plant Biochemistry, Göttingen. Transcriptomic data derived from two biological and two technical replicates combined with a complete change of the used transcriptome platforms (TIGR and AGILENT). put. = putative. Cut off was set to log2 ≥ ±1.5. (*) validated genes.

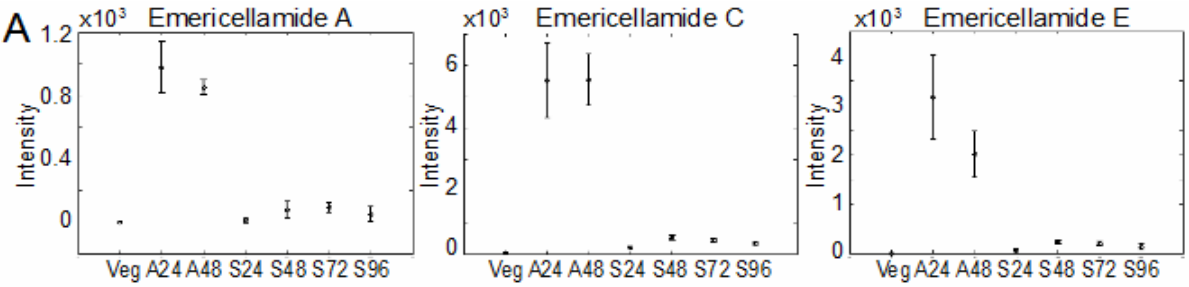
Further secondary metabolites partially described in *A. nidulans* are emericellamides (A-F) initially discovered in marine *Emericella sp.* (Oh, *et al.*, 2007). These compounds are also present in *Aspergillus nidulans* (Szewczyk, *et al.*, 2008). Here, we show intracellular and extracellular profiles of emericellamides during asexual, sexual and vegetative growth (Fig. 11 A). Emericellamide A, C and E are already present after 24 h of growth in light, whereas the content is extremely diminished after the same period in darkness at sexual development. Light seems to induce or derepress the production of these antibiotics. Emericellamide content increases slightly after 48 h sexual development but never achieves asexual amount. This is the first time emericellamides are described to be present at sexual development of *A. nidulans*. The emericellamide gene cluster comprises four genes *easA* (AN2545), *easB* (AN2547), *easC* (AN2548) and *easD* (AN2549) important for its synthesis (Chiang, *et al.*, 2008). Two *eas* genes encode a nonribosomal peptide synthetase (*easA*) and a polyketide synthase (*easB*) are constitutively expressed. In contrast, two genes code for a putative

acyltransferase (*easC*) and for an acyl-CoA ligase (*easD*) are expressed at both stages of asexual (A24; A48) and sexual (S24; A48) development, but emericellamides accumulate only during the asexual stage. All *eas* genes are involved in emericellamide synthesis and were shown to be indispensable for the production of all emericellamide species. In consequence, light controls the production of emericellamides on another level apart from genetic expression.

stc genes required for sterigmatocystin (ST) production in *A. nidulans* are mainly expressed at both asexual stages and late sexual development (Fig.11). 15 of 25 known *stc* genes are differentially expressed. Two exceptions were observed regarding *stc* gene expression. *stcF* (AN7818), a P450 monooxygenase is specifically expressed at both asexual stages and is absent at sexual cycle. A putative peroxidase encoded by *stcC* (AN7823) is expressed at S48 additionally. StcF converts averantin to hydroxyversicolone (Brown et al. 1996; Kelly et al. 2009). Function of StcC is not described and characterized yet. ST is highly accumulated at sexual development (S48 and S72, Fig. 11 B). At late sexual phase S96 ST content decreases.

While anthranilate is a primary metabolite in bacteria and single cell eukaryotes, e.g. in tryptophan biosynthesis, it is a non-proteinogenic aryl β-amino acid rather than a standard α-amino acid. However, in combination with known functions in other higher eukaryotes anthranilate has high secondary metabolite character and is therefore described in this secondary metabolite chapter. Anthranilate is present at both asexual stages A24 and A48 intracellularly. Beside additional functions anthranilate is an important precursor of melanin and thus is also involved in the protection of fungal mycelia and genetic material against damage from UV radiation at asexual development in light. In contrast to the broad knowledge about genetic regulation of secondary metabolite clusters less is known about it in anthranilate production.

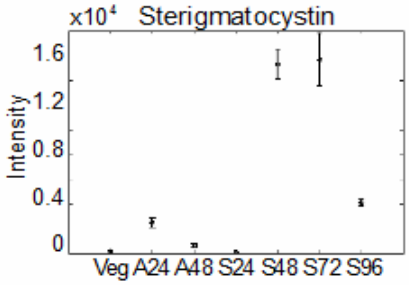
A



eas gene cluster regulation

Gene ID	Function	A24	A48	S24	S48	S72	S96
AN2548	***easC***, acyltransferase, put.*					-	-
AN2549	***easD***, acyl-CoA ligase, put.*					-	-

B



mdp gene cluster regulation

Gene ID	Function	A24	A48	S24	S48	S72	S96
AN7804	***stcW***, FAD-containing monooxygenase*	+	+			+	+
AN7806	***stcU***, versicolorin reductase, put.*	+	+			+	+
AN7807	***stcT***, translation elongation factor 1γ*	+	+			+	+
AN7810	***stcQ***,NADH-flavin reductase, put.	+	+			+	+
AN7812	***stcN***, versicolorin B synthase, put.	+	+			+	+
AN7816	***stcI***, lipase/esterase, put.*	+	+			+	+
AN7817	Uncharacterized dehydrogenase	+	+			+	+
AN7818	***stcF***, P450 monooxygenase, put.*	+	+				
AN7819	Uncharacterized w/o domain	+	+			+	+
AN7820	***aflR***, transcriptional activator*	+	+			+	+
AN7821	***stcE***, norsolorinic acid reductase, put.*	+	+			+	+
AN7822	Integral membrane protein	+	+			+	+
AN7823	***stcC***, peroxidase, put.	+	+		+	+	+

Figure 11: Secondary metabolites with preference for light or darkness regulated development of *A. nidulans*. (**A**) Emericellamide A/C/E reveal high intracellular accumulation at both asexual (A) stages. Each detected emericellamide is present at sexual (S) stages S48/S72 in lower concentrations. This is the first observation of emericellamide species present at sexual stage in *A. nidulans*. Though two *eas* genes are expressed even at vegetative (Veg) stage emericellamides are absent from vegetative growth. (**B**) Sterigmatocystin (ST) accumulates at sexual stages S48/S72/S96. ST gene expression reveals activation of the same *stc* genes at both asexual and sexual development, except for the expression of AN7818 and AN7823 encoding a cytochrome P450 monooxygenase and a peroxidase respectively. Relative intensities of metabolite markers are shown as error bars containing mean values (points) and standard deviations (bars). The metabolite fingerprinting analyses was performed by Dr. Kirstin Feussner, Department of Plant Biochemistry, Göttingen. Data from intracellular metabolome derived from two biological and three technical replicates. Transcriptomic data derived from two biological and two technical replicates combined with a complete change of the used transcriptome platforms (TIGR and AGILENT). Cut off was set to log2 ≥ ±1.5. (*) validated gene.

3.3.2.2 Secreted secondary metabolites during *A. nidulans* development in light

Protective secondary metabolites synthesized intracellular by *A. nidulans* has to be secreted to get to its target e.g. bacterial, fungal and other competitors Therefore, we performed extracellular metabolomics to get an global view on the kinetics of secondary metabolites already detected in intracellular metabolome. Analyses of extracellular metabolomes were restricted to end points of development. Thus, three stages were examined Veg, A48 and S96. This setup gives us a general overview concerning the secondary metabolites, which are either secreted or accumulated until the end of each development cycle of *A. nidulans*.

An asexual specific translocated compound is anthranilate (Fig. 12). As observed for emericellamide C, anthranilate also appears intracellular at both asexual stages and is present in the extracellular environment at the end of asexual differentiation A48.

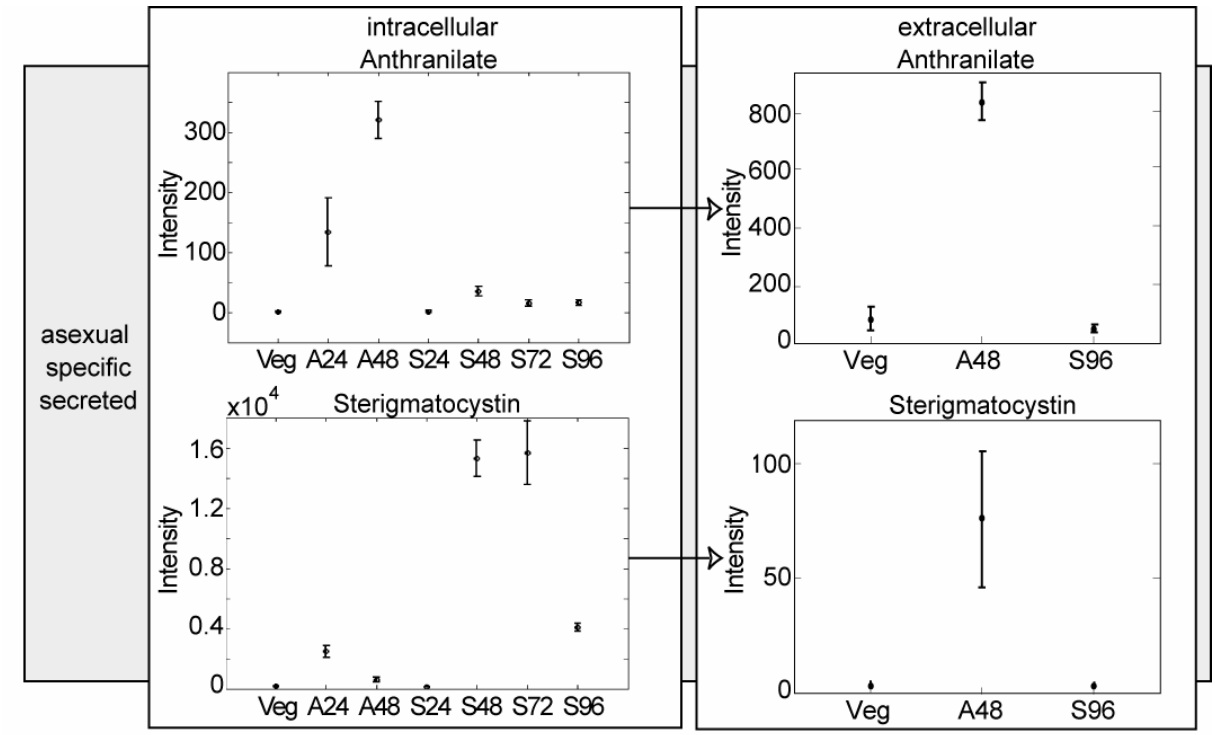


Figure 12: Light-dependent secretion of secondary metabolites at asexual development in *A. nidulans*
Intra- and extracellular accumulation of Anthranilate and Sterigmatocystin during fungal development. Intracellular accumulation of anthranilate is highest at asexual stages and is also produced at sexual cycle in minor amounts. It is translocated into the fungal environment at A48. In contrast, sterigmatocystin exhibits high intracellular abundance at sexual development. Sterigmatocystin could be detected only in trace amount in the supernatant of A48, which seems to be a problem of sterigmatocystin extraction out of aqueous media. The metabolite fingerprinting analyses was performed by Dr. Kirstin Feussner, Department of Plant Biochemistry, Göttingen. Results for the illustrated metabolites are based on two independent biological and six technical replicates. Relative intensities of metabolite markers are shown as error bars containing mean values (points) and standard deviations (bars).

These data conclude that anthranilate transport does not achieve a dynamic equilibrium. Since it is not secreted completely anthranilate might serve as UV-absorber and repellent beside its presumed function as a kind of toxin additionally.

Despite the high sexual sterigmatocystin (ST) accumulation, only very low amounts of ST could be detected in the culture supernatant. It seems that ST, a metabolite with lipophilic features, got lost during the extraction from supernatant (Fig. 12). Generally, ST content is highest in intracellular metabolome at S48 and. Its content at both stages of asexual development is significantly lower.

In summary, *A. nidulans* synthesis of secondary metabolites is less at vegetative stage in comparison to its asexual differentiation, so that secretion hardly takes place and secondary metabolites are not present at this stage. Although numerous secondary metabolites are produced at sexual development, only emericellamide C could be detected in the extracellular environment at S96 that will be discussed in chapter 3.4.2.2.3 (page 97).

3.4 Darkness-dependent sexual development in *A. nidulans*

3.4.1 Transcriptomics

3.4.1.1 Histone acetylation and light detection systems in early sexual development of *A. nidulans*

Fourty genes encode proteins with GCN5-related N-acetyltransferase domain are present in *A. nidulans.* Eight of them are differentially expressed at the analyzed stages (Tab. 10). The acetyltransferase domain was not shown for AN5053 and AN5330 at BROAD. Manual sequence analysis at NCBI exhibits the same domains and could be classified as proteins with GCN5-related N-acetyltransferase domain and thus integrated into table 10. Interestingly five of eight putative histone acetylases with GCN5-related N-acetyltransferase domain are expressed at early sexual development, which is most likely crucial for the fungus to control and initiate sexual development.

Table 10: Intensive histone acetylases expression at early sexual development of *A. nidulans.*

Gene ID	Function	A24	A48	S24	S48	S72	S96
Histone acetylases							
AN0969	***ngn17***, GCN5-related N-acetyltransferases*			+			
AN10234	***ngn10***, GCN5-related N-acetyltransferases*			+	+		
AN10238	***ngn21***, GCN5-related N-acetyltransferases*	+	+		+	+	+
AN5053	GCN5-related N-acetyltransferases, put.	-	-			-	-
AN5330	GCN5-related N-acetyltransferases, put.			+			
AN6411	***ngn28***, GCN5-related N-acetyltransferases*			+			
AN7944	***ngn3***, GCN5-related N-acetyltransferases*			+		+	+
AN9493	***ngn12***, GCN5-related N-acetyltransferases*					+	+

Expression of genes coding for putative GCN5-related N-acetyltransferases (GNAT) which have potential histone deacetylase activity at early sexual (S) development (S24) might be essential for the fungus to initiate expression of genes required for a proper sexual differentiation. AN10238 is also expressed from early asexual (A) development but is not specific for asexual differentiation. Expression of AN5053 is already initiated at vegetative growth and is still present in S24. Numbers behind A and S show hours after induction start for asexual and sexual development respectively. Data derived from transcriptome data of two independent biological replicates and two microarray platforms. put. = putative genes. Cut off was set to log2 > ±1.5, (*) validated genes.

Maybe *A. nidulans* waits for sensory kinases (Tab. 11) to be decomposited until sexual development can be induced properly. Nine histidine kinase families are described in *A. nidulans* whereas the fungus comprises 15 single histidine kinases generally. Among these, four histidine kinases of family 8 (AN2581, AN3214, AN4113, AN6820) are differentially expressed. Three are specifically downregulated at early sexual development S24. AN6820 is downregulated at S72 initially. Their molecular function is still unknown. Beside the histidine kinase proteins, three additional genes with connection to light transduction and sensing are expressed. *apyA* (AN3265) encodes an arrestin (or S-antigen) and PY-motif containing protein which was shown to interact with the carbon catabolite repressor CreD and with HulA (Boase & Kelly, 2004). On the other hand a close homologue to ApyA in mice (Sag an S-arrestin) is known to be essential for light transduction in eyes (Krishnan, *et al.*, 2008). *apyA* expression is decreased at A48, S48, S72 and S96. In case of this protein having a connection to light sensing and transduction in *A. nidulans*, it might be essential for the decision for asexual and sexual differentiation at vegetative growth and early stages of both development cycles. Opsin as a green light detecting protein is also present in *A. nidulans* and is encoded by *nopA*

(AN3361). Expression is present at vegetative growth as for all listed sensing complexes. *nopA* is specifically downregulated at S24 whereas it is still expressed at A24. Last protein in table 11 with light sensing connection is encoded by AN8959. Manual sequence analysis revealed a trp-rich sensory domain (TspO) that was numerously described for its light sensing properties.

Table 11: Light sensing complexes expression during *A. nidulans* development.

Gene ID	Function	A24	A48	S24	S48	S72	S96
Receptor systems, sensory kinases							
AN2581	Histidine kinase G7, GAF domain present in phytochromes (**)			-		-	
AN3214	TMAO reductase system sensor TorS (**)			-	-	-	
AN3265	***apyA***, Arrestin domains and PY motif-containing protein > essential for light transduction in eyes		-		-	-	-
AN3361	***nopA***, opsin			-		-	
AN4113	Hybrid sensory histidine kinase, put.			-		-	
AN6820	Sensor histidine kinase/response regulator, put. (*A. fumigatus*)					-	
AN8959	Trp-rich sensory protein (TspO) involved in light/oxygen sensoring			-			

Expression of the shown receptor systems already starts at vegetative growth and is already massively decreased for five of seven genes at early sexual (S) development. Light stimulates or darkness represses the expression of the corresponding genes at asexual (A) development. Numbers behind A and S show hours after induction start for asexual and sexual development respectively. Data derived from transcriptome data of two independent biological replicates and two microarray platforms. Put. = putative. Cut off was set to log2 > ±1.5. (**) identified but unknown function.

The functional meaning of light detecting systems in dark soil is at least the same than on the surface. In transition of mycelial growth to differentiation, illumination conditions result in an adaptation affecting fungal expression. In consequence, *A. nidulans* safes energy through decreased expression of e.g. light sensing systems it does not need in soil. Thereby the fungus avoids false induction through remaining light in upper layers of the soil.

3.4.1.2 Sexual regulators during *A. nidulans* growth in darkness

Beside asexual regulators shown in chapter 3.3.1.1, *A. nidulans* also possesses regulators specific for sexual development. Among them are differentially expressed sexual regulators like *choC* (AN1376) *lsdA* (AN2330), *matB* (AN2755), *gprD* (AN3387) and *cpcB* (AN4163) (Tab. 12). The phosphatidylethanolamine N-methyltransferase encoded by *choC* is linked to sexual development since its deletion results in an absent cleistothecia formation in *A. nidulans* (Tao, *et al.*, 2010). It is already expressed at vegetative growth and downregulated at S72 specifically. Although ChoC is involved in membrane synthesis that is important for maturation of cleistothecia as well, it is downregulated at S72. Beside this ChoC might have an additional function in sexual development. *lsdA* codes for the late sexual development protein that is a negative regulator of cleistothecia development (Lee, *et al.*, 2001). Deletion of *lsdA* increases the number of cleistothecia. It is expressed at late sexual development S72 and S96 and might be important to control and limit the number of cleistothecia at these stages. *A. nidulans* avoids nutrient starvation consequently since cleistothecia formation is a very energy consuming process.

The mating type protein encoded by *matB* is described to be expressed during sexual development and deletion results in sterile cleistothecia (Dyer, *et al.*, 2003, Paoletti, *et al.*, 2007). In contrast, overexpression of *matB* causes the complete absence of sexual cycle and cleistothecia formation. In this study *matB* is expressed at late sexual development S72. The fungus might elevate its expression to avoid another initiation of cleistothecia formation at this point of time.

Expression of *gprD* is decreased for all stages of sexual development. The G-protein coupled receptor *gprD* is one of two *gpr* genes out of nine present *gpr* genes in *A. nidulans* (Han, *et al.*, 2004) differentially expressed at the analyzed stages and occupies regulation on sexual development. It is a negative regulator of sexual development. *gprD* deletion results in an increased cleistothecia formation as it is described for the Δ*lsdA* strain. Furthermore an *nsdD* mRNA accumulation was observed. NsdD expression is constitutive at the analyzed stages. *A. nidulans* decomposites G-coupled receptor from early sexual development since its inhibitory effect on sexual development already starts at early sexual stage. A positive regulator of cleistothecia formation is *cpcB* encoding a guanine nucleotide-binding protein with seven WD repeats. Deletion has impact on cleistothecia formation that is blocked after microcleistothecia stage (Hoffmann, *et al.*, 2000). Interestingly, expression is always present except for late sexual stage S96.

Table 12: Sexual regulators & development specific genes expressed in *A. nidulans* as control for a proper sexual development.

Gene ID	Function	A24	A48	S24	S48	S72	S96
Sexual development regulators							
AN1376	***choC***, phosphatidylethanolamine N-methyltransferase *					-	
AN2330	***lsdA***, Protein involved in sexual development → VeA connection *					+	+
AN2755	***matB***, Mating-type, alpha-domain → mutants w/o cleisto/Hülle cells *					+	
AN3387	***gprD***, G-protein coupled receptor, put.* → negative regulator of sexual development			-	-	-	-
AN4163	***cpcB***, guanine nucleotide-binding* protein B, positive regulator of cleistothecia formation						-
Sexual specific genes							
AN4521	***fhpA***, Forkhead domain protein might be involved in sexual development					+	
AN7737	Serine/threonine protein kinase, put. MEK1 homologue → yeast meiosis					+	+
AN9121	***esdC***, VeA & FlbA regulated protein early sexual development				+	+	
Asexual & Sexual development regulators							
AN7553	***devR***, bHLH transcription factor → mutants with absent cleistothecia formation					+	
AN8333	***phiA,*** required for phialide development		-				

A. nidulans genome comprises numerous developmental regulators already described to be specific for asexual (A) and sexual (S) development and are serious controls for differentiation processes in *A nidulans*. For instance, *lsdA* (AN2330) a regulator in sexual differentiation known to be involved in sexual development and to possess VeA connection is expressed at late sexual stage S72 and S96. As negative regulator of sexual development *gprD* is downregulated at each sexual stage. Beside developmental regulators numerous genes specifically expressed at defined development stages were found. Among them are sexual specific genes like *fhpA* (AN4521, forkhead domain protein) or *esdC* (AN9121). *devR* (AN7553) codes for a bHLH transcription factor that was described to have impact on asexual and sexual development. It is upregulated at late sexual stage S72 merely. Numbers behind A and S show hours after induction start for asexual and sexual development, respectively. Data derived from transcriptome data of two independent biological replicates and two microarray platforms. put. = putative genes. Cut off was set to log2 > ±1.5

CpcB might be important for the formation of mature cleistothecia and not for initiation of sexual development and cleistothecia formation generally. Therefore, other regulators are required.

Sexual specific differentially expressed markers are forkhead domain protein FhpA (AN4521), the *veA* and *flbA* regulated GTP-binding protein EsdC (AN9121) and a homologue serine/threonine protein kinase (AN7737) to *MEK1* (YOR351C), which is involved in meiosis

in *S. cerevisiae* and not characterized in *A. nidulans* yet (Rockmill & Roeder, 1991). We could give another hint for *fphA* importance as positive regulator of sexual development through its specific expression at S72. FhpA is thought to have a possible role in sexual development (Lee, *et al.*, 2005, Bayram, *et al.*, 2010). It was shown that the *A. nidulans fphA* iRNA expressing strain is decreased in sporulation. EsdC is a positive regulator of sexual and a negative regulator of asexual development. The Δ*esdC* strain reveals an absent cleistothecia and increased conidiophore formation (Han, *et al.*, 2008). Although *esdC* is described to be involved in early sexual development we detected an initial elevated expression at S48 until S72 meaning that expression is already present at vegetative stage and increased at later sexual development again. This implies *esdC* to have either an additional role in sexual development or to be required for inhibition of asexual cycle during sexual development. AN7737 is specifically expressed at both late sexual stages S72 and S96. The still uncharacterized protein encoded by AN7737 shares high sequence similarity to Mek1p which is crucial for yeast meiosis. This might be essential for formation of ascospores that requires meiotic processes at S72 when cleistothecia maturation is not finished.

Regulators crucial for both a proper asexual and sexual development are *devR* (AN7553) and *phiA* (AN8333). Deletion of *devR* that encodes a basic helix-loop-helix transcription factor affects conidiophore and cleistothecia formation in *A. nidulans* mutants (Tuncher, *et al.*, 2004). Formation of both structures is absent completely. Interestingly, *devR* expression is increased at S72. Basic expressional strength of *devR* is constant at all stages of development, which might be sufficient for the fungus to develop asexual and sexual properly. Deletion of *phiA* results in a decreased conidiation and abnormal conidiophore morphology (Melin, *et al.*, 2003). Cleistothecia formation is absent in the Δ*phiA* strain. Expression of *phiA* is merely downregulated at A48, what might reveal the need for its expression at early asexual and complete sexual development. Phialidae and metulae formation is already initiated at earlier stages of asexual development e.g. A24 when *phiA* is still expressed. In contrast, cleistothecia formation and maturation is attended through *phiA* until late sexual stages of development S96.

In contrast, other known regulators of sexual development *veA*, *velB*, *imeB*, *nosA*, *nsdD* and for asexual development *abaA* are constitutively expressed at all analyzed stages, proposing that numerous crucial regulators possess and require only short expression periods to have impact on regulation of development in *A. nidulans*.

3.4.1.3 Heterokaryon incompatibility in *A. nidulans* is controlled by light

In filamentous fungi, heterokaryon incompatibility protein (HET) loci are believed to control and regulate self/nonself-recognition during vegetative growth. AN8923 encodes a response regulator of RpoS (PRK10693) with a HET domain in *A. nidulans* not characterized yet. Its sequence is shown without domain at BROAD but manual sequence analysis at NCBI/PFAM indicates the self/nonself-recognition function mentioned previously. Interestingly, AN8923 expression increases at both asexual and sexual development. Although *A. nidulans* differentiation is induced heterokaryon incompatibility control is present at both stages of asexual development A24 and A48. In contrast, AN8923 expression at sexual development is much later at S72 and S96. This implies that *A. nidulans* controls heterokaryon incompatibility much earlier at asexual stage than at sexual stage in a light dependent manner. It is most likely that *A. nidulans* is able to form heterokaryon at asexual stage much earlier or its formation is with less control at early sexual development stages S24 and S48 when AN8923 is not expressed yet. The heterokaryon incompatibility protein Het-C (AN2167) thought to be involved in heterokaryon incompatibility process is constitutively expressed at the analyzed stages (Davies, *et al.*, 2004). The found response regulator protein with comprised HET domain might be another still unknown crucial factor controlling heterokaryon formation in *A. nidulans.*

3.4.1.4 Amino acid metabolism gene regulation and translation reveal a broad downregulation at the end of *A. nidulans* sexual development in darkness

A. nidulans requires nutrient supply for proper development. Under laboratory conditions and also for the analyses in this work minimal medium was used. It contains components such as glucose, sulphate, nitrate, phosphate and trace elements essential for the successful performance of asexual and sexual development. Within few days of growth on minimal medium and in the course of development nutrient concentration decreases and the fungus suffers from nutrient limitation. On the other hand numerous known protective secondary metabolites e.g. antibiotics, mycotoxins and further compounds are synthesized and secreted by the fungus to decrease the number of pro- and eukaryotic competitors in its native habitat. Therefore, the fungus limits nutrient waste within the soil and avoids nutrient limitation consequently.

Transcriptome analysis reveals massive downregulation of numerous genes reaching its maximum at S96 (Tab. 13). Specifically numerous amino acid synthesis genes are downregulated at S72 and S96.

Table 13: Global downregulation in the expression of amino acid biosynthesis genes during late sexual development in *A. nidulans.*

Gene ID	Function	A24	A48	S24	S48	S72	S96
Cysteine & Methionine biosynthesis							
AN1222	***mecC***, AdoMet synthetase *		-				-
AN2882	Homoserine dehydrogenase *					-	-
AN4793	Aspartate-semialdehyde dehydrogenase, put.					-	-
AN8057	***cysB***, Cysteine synthase *					-	-
AN8277	***cysD***, Homocysteine synthase *					-	-
Alanine, Aspartate & Glutamate biosynthesis							
AN1883	***arg3***, Argininosuccinate synthase *					-	-
AN1891	L-asparaginase, put.		+				
AN1923	Alanine transaminase *		-			-	-
AN2243	***cpa***, Carbamoyl-phosphate synthase, put.						-
AN2914	***arg1***, Argininosuccinate lyase *					-	-
AN4159	***glnA***, Glutamine synthetase *				-		-
AN4376	***gdhA***, NADP-specific glutamate dehydrogenase *		-		-	-	-
AN4401	Asparagine synthase (glutamine-hydrolysing),					-	-
AN5134	***gltA***, Glutamate synthase *			-	-		-
AN5447	Glutamate decarboxylase, put.	-	-		-	-	-
AN7451	***gdhB***, Glutamate dehydrogenase *	+	+			+	+
Glycine, Threonine & Serine biosynthesis							
AN2284	***hemA***, 5-aminolevulinic acid synthase, mitochondrial precursor, put.		-		-	-	-
AN2532	Primary-copper amine oxidase 1 , put.			-			
AN2882	Homoserine dehydrogenase *					-	-
AN3031	Threonine synthase, put.					-	-
AN3291	Monoamine oxidase, put.					+	
AN4793	Aspartate-semialdehyde dehydrogenase, put.					-	-

Table 13 continued

Gene ID	Function	A24	A48	S24	S48	S72	S96
Glycine, Threonine & Serine biosynthesis continued							
AN5444	D-3-phosphoglycerate dehydrogenase, put.		-			-	-
AN5690	Primary-amine oxidase, put.				+		
AN6943	Glycerate kinase, put.		+				
AN7641	Primary-amine oxidase, put.	+	+		+	+	+
AN8843	Homoserine kinase, put.					-	-
AN8866	D-3-phosphoglycerate dehydrogenase, put.					-	-
Valine, Leucin & Isoleucin biosynthesis							
AN0705	Isoleucyl-tRNA synthetase, put.						-
AN0840	2-isopropylmalate synthase, put.		-			-	-
AN0912	3-isopropylmalate dehydrogenase A, put.					-	-
AN2526	ketol-acid reductoisomerase, put.					-	-
AN4323	Branched-chain amino acid aminotransferase, put.		-			-	-
AN5162	***pdhB***, pyruvate dehydrogenase E1 subunit alpha*						-
AN6364	***sudA***, Dihydroxy-acid dehydratase *					-	-
AN9403	***pdhC,*** pyruvate dehydrogenase E1 subunit beta*						-
Lysine biosynthesis							
AN2882	Homoserine dehydrogenase *					-	-
AN4793	Aspartate-semialdehyde dehydrogenase, put.					-	-
AN5206	***lysB***, Homoisocitrate dehydrogenase *					-	-
AN5601	Saccharopine dehydrogenase, put.					-	-
AN6521	***lysF***, Homoaconitase, mitochondrial precursor *						-
Phenylalanine, Tyrosine and Tryptophan biosynthesis							
AN1137	***qutB***, Quinate dehydrogenase *	-	-			-	-
AN5444	Tryptophan synthase beta chain, put.		-			-	-
AN5731	Chorismate synthase		-		-	-	-
AN6403	3-dehydroquinate synthase	+	+				

A. nidulans decreases expression of amino acid biosynthesis genes at the end of sexual (S) development S72 and S96 massively. This effect is not very obvious during asexual (A) development A48. Numbers behind A and S show hours after induction start for asexual and sexual development respectively. Data derived from transcriptome data of two independent biological replicates and two microarray platforms. put. = putative. Cut off was set to $\log2 \geq \pm1.5$. (*) validated gene.

Amino acids are sorted into specific groups regarding their common metabolism. One prominent group is the alanine, aspartate and glutamate group. 11 genes involved in metabolism of these amino acids are differentially expressed.

Table 14: Downregulation of translation genes as consequence of the amino acid metabolism shutdown at late sexual development of *A. nidulans.*

Gene ID	Function	A24	A48	S24	S48	S72	S96
Translation							
AN0470	30S ribosomal protein S7					-	-
AN0705	isoleucyl-tRNA synthetase						-
AN0776	60S ribosomal protein L17						-
AN10210	Asp-tRNAAsn/Glu-tRNAGln amidotransferase A					+	
AN10459	translation initiation factor eIF-2b epsilon					-	-
AN10475	tryptophanyl-tRNA synthetase						-
AN1270	translation initiation factor eIF-3h						-
AN2662	Asp-tRNAAsn/Glu-tRNAGln amidotransferase A	+			+	+	+
AN2879	Asp-tRNAAsn/Glu-tRNAGln amidotransferase A					+	
AN2907	translation initiation factor eIF-3e						-
AN3156	translation initiation factor eIF-2a						-
AN3635	Ribosomal protein L10 family					-	-
AN4202	***rpl16a***, 60S ribosomal protein L16*						-
AN4251	40S ribosomal protein S2, put.					-	
AN4434	Mitochondrial 37S ribosomal protein S25						-
AN4452	60S ribosomal protein L36	+	+			+	+
AN4893	eIF-2C4, put > Piwi-like: PIWI domain found in					+	+
AN4954	Mitochondrial 54S ribosomal protein L28						-
AN5014	60S ribosomal protein L22, put.						-
AN5164	30S ribosomal subunit S4, put.						-
AN5520	large ribosomal subunit protein L7A						-
AN5979	40S ribosomal protein S17, put. (*rpl3*)						-
AN6202	60S ribosomal protein L3						-
AN6500	60S ribosomal protein L28						-
AN7003	60S ribosomal protein L13						-
AN7540	translation initiation factor eIF-3d						-
AN8176	60S ribosomal protein L4						-
AN9108	Indoleamine 2,3-dioxygenase > local usage of Trp	+	+		+	+	+
AN9138	Asp-tRNAAsn/Glu-tRNAGln amidotransferase A	+	+		+	+	
AN9138	Asp-tRNAAsn/Glu-tRNAGln amidotransferase A	+	+		+	+	
AN9157	Glutaminyl-tRNA synthetase						-
AN9304	***elfA***, translation elongation factor eEF-1B gamma			-	-		
AN9465	60S ribosomal protein L9, put.						-

Numerous genes involved in translation are downregulated at late sexual stage. For example expression of 15 genes coding for ribosomal proteins of the small and large subunit are decreased at S72 and/or S96. An additional upregulation of genes involved in protein degradation takes place at late sexual development. Numbers behind A and S show hours after induction start for asexual and sexual development respectively. Data derived from transcriptome data of two independent biological replicates and two microarray platforms. put. = putative. Cut off was set to log2 ratio > ±1.5 (*) validated genes.

A broad downregulation of amino acid genes required for the proper performance of amino acid synthesis has direct impact on amino acid homeostasis in *A. nidulans* and results in decreased amino acid concentrations at late sexual stage as it is going to be described in more detail in chapter 3.4.2.1.1 (page 88).

In agreement with the observed downregulation of numerous genes involved in amino acid metabolism also many genes required for translation in *A. nidulans* reveal decreased expression in the end of sexual development (Tab. 14). 20 of 33 differentially expressed translation genes show specific downregulation at S96 when *A. nidulans* cleistothecia are already mature and sexual development is completed. Among them are several genes encode for ribosomal subunits indispensable for the composition of proteins.

3.4.1.5 *A. nidulans* aging through controlled cell death mediated by regulators of apoptosis and reactive oxygen generating factors in darkness

In contrast to upregulation of apoptosis initiation genes in light, their expression could not be detected at early sexual development S24 and S48 of *A. nidulans*. After three days of sexual induction in darkness (S72), AN0394, most likely a positive regulator of apoptosis, is upregulated (Tab. 15). AN0394 encodes a still uncharacterized flavoprotein oxidoreductase and is a close homologue of the apoptosis-inducing factor 2 in humans (Horikoshi, *et al.*, 1999, Wu, *et al.*, 2002, Wu, *et al.*, 2004). The expression of this gene was also found to be induced by tumour suppressor protein *p53* in colon cancer cells in humans.

After four days of sexual growth (S96), *aifA* (AN9103), a putative apoptosis-inducing factor (*AIF*)-like mitochondrial oxidoreductase, is upregulated. *aifA* was shown to accumulate transcriptionally in response to farnesol that inhibits proliferation and induce apoptosis (Savoldi, *et al.*, 2008, Dinamarco, *et al.*, 2010). Exposed to farnesol, *aifA* deletion strains exhibit increased reactive oxygen species (ROS) production. Therefore, *aifA* seems to trigger ROS concentration inside the fungus. We could show that the *aifA* expression is regulated in a development-dependent manner and takes place at a very late stage of sexual development in *A. nidulans*. Farnesol is not detectable at the analyzed stages in the metabolome. This result complies with already described lack of farnesol in *A. nidulans* though it is present in *C. albicans* (Semighini, *et al.*, 2006). ROS triggered programmed cell death is influenced through numerous different enzymes like NADPH oxidases (Nox), catalases, peroxidases, superoxide-dismutases.

Table 15: *A. nidulans* genes important for development, programmed cell death and oxidative stress response through triggering reactive oxygen levels in darkness.

Gene ID	Function	A24	A48	S24	S48	S72	S96
Apoptosis genes							
AN0394	Putative positive regulator of apoptosis					+	
AN9103	***aifA***, Apoptosis-Inducing Factor (AIF)-like mitochondrial oxidoreductase*						+
NADPH oxidases (Nox)							
AN4906	Nox, uncharacterized					+	
AN8683	Nox, uncharacterized					+	
Superoxide dismutases (Sod)							
AN0785	***sodM***, Sod, Mn, uncharacterized (*)				+		

Programmed cell death is induced through apoptosis genes generally. *A. nidulans* activates several development specific apoptosis genes. In contrast to both asexual (A) specific apoptosis genes shown in table 10, both sexual (S) specific apoptosis genes AN0394 and AN9103 are activated at late sexual stages S72 and S96 in *A. nidulans* merely. AN0394 encodes a putative positive regulator of apoptosis and AN9130 (*aifA*) an apoptosis-inducing factor (AIF)-like mitochondrial oxidoreductase. Before ascospore formation is finished completely *A. nidulans* induces programmed cell death at S72 and S96. *A. nidulans* expresses additional genes essential for development and oxidative stress response like NADPH oxidases (Nox) and superoxide dismutases (Sod). AN4906 and AN8683 code for sexual (S) specifically expressed and still uncharacterized Nox. Asexual (A) specifically expressed Nox are not present at the analyzed stages. NoxA involved in sexual development and cleistothecia formation was constitutively expressed (Lara-Ortiz et al. 2003). *sodM* is also still uncharacterized. Numbers behind A and S show hours after induction start for asexual and sexual development, respectively. Data derived from transcriptome data of two independent biological replicates and two microarray platforms. Cut off was set to $\log2 \geq \pm1.5$. (*) validated gene.

Hence, the importance of ROS as signals is not restricted to induction of programmed cell death but spans regulation of diverse cellular processes such as defense, growth and development. For instance, NoxA was described to have impact on sexual development (Lara-Ortiz, *et al.*, 2003). Nox catalyzes the generation of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide that was also shown by Lara-Ortiz and co-workers being crucial for proper sexual development. The Δ*noxA* strain reveals decreased cleistothecia formation. Since ROS trigger development or apoptosis a sensitive balance is basis for proper signalling mediated by the mentioned enzymes with impact in ROS homeostasis. In contrast to asexual development, *A. nidulans* expresses two sexual specific Nox encoded by AN4906 and AN8683 that are still uncharacterized. Interestingly, both are expressed at S72, which might be a hint that these Nox are also involved in cleistothecia formation or they are expressed for promotion of programmed cell death at the end of sexual development.

Additionally, superoxide-dismutases (Sod), catalyzing conversion of superoxide to hydrogen peroxide, reveal development specific expression as well. Already identified but

uncharacterized SodM (AN0785) involved in the glutathione system (Sato, *et al.*, 2009) is a close homologue to the manganese superoxide dismutase Sod2p in *S. cerevisiae*, which is known to have impact in chronic cell aging in yeast (Balzan, *et al.*, 2004). *sodM* is the only Sod specifically expressed at S48.

Since Nox generates superoxides from hydrogen peroxides and Sod catalyzes the reverse reaction we can assign development specific, antagonistic Nox and Sod based on our transcriptome data. Thus, vegetative Nox (AN0773; AN10893) are counterparts of the Fe Sod (AN5148), the sexual Nox (AN4906; AN8683) of the Mn Sod *sodM* (AN0785). Contrary, in asexual development the asexual specific Cu/Zn Sod (AN1131) has no opposite Nox. This results most likely in different specific H_2O_2 / O_2^- ratios characteristic for asexual and sexual development. Since sexual cycle initiates specific Nox, O_2^- formation is presumably much higher than at asexual stage. A positive side effect might be a much more efficient protection against competitors in soil with superoxide, which is more reactive and toxic compared to hydrogen peroxide.

In contrast to asexual development we could not find sexual specific catalases in *A. nidulans*. Since catalases convert hydrogen peroxide into water and oxygen (Chelikani et al. 2004), hydrogen peroxide level remains high in sexual structures. Again, this might be an important signal for the formation of fruit bodies and a sexual cycle performed properly. Beside superoxide, even hydrogen peroxide level is most likely higher than in asexual structures, which marks another ROS for protection to competitors.

In contrast to development in light when the fungal structures suffer in a high oxidative burst due to the high oxygen partial pressure and UV-mediated ROS formation, *A. nidulans* faces a low oxidative burst in soil. Under these conditions the fungus might induce controlled accumulation of different ROS as development signal on the one hand and as protection against competitors on the other hand.

3.4.1.6 Constitutive resistance towards xenobiotics through development by specific glutathione-S-transferases (GST) in *A. nidulans*

GSTs are cytosolic proteins involved in cellular detoxification by catalyzing the conjugation of glutathione (GSH) with a wide range of endogenous and xenobiotics alkylating agents, including oxidative stress products and environmental toxins (Fraser, *et al.*, 2002, Nebert & Vasiliou, 2004, Hayes, *et al.*, 2005).

GstA (AN4905) was shown to be essential for resistance to several xenobiotics e.g. diamide in *A. nidulans* (Fraser, *et al.*, 2002). Interestingly, *gstA* is a neighbor gene of AN4906, which

codes for Nox expressed specifically during sexual development, which was described in chapter 3.3.1.4 (page 60). Both genes form a gene cluster required for oxidative stress response. Expression at S72 is common to both genes. Additionally, *gstA* is also expressed at A24 (Tab. 16). *gstB* (AN6024) is involved in oxidative stress response and is induced at menadione treatment. (Sato, *et al.*, 2009, Pusztahelyi, *et al.*, 2011). Menadione is a superoxide generating compound.

Table 16: Glutathione-S-transferase genes involved in detoxification and stress response during development of *A. nidulans*.

Gene ID	Function	A24	A48	S24	S48	S72	S96
Glutathione-S-transferases							
AN4905	***gstA***, gluthatione-S-transferase *	+				+	
AN6024	***gstB***, gluthatione-S-transferase *	+			+	+	+
AN10695	Glutathione-S-transferase, put.						+
AN3299	Glutathione-S-transferase, put.				+		

Expressed glutathione-S-transferases at the analyzed asexual (A) and sexual (S) development stages in *A. nidulans. gstA* (AN4905) and *gstB* (AN6024) are expressed at early asexual stage A24. Except for A48 and S24 stages two GST are always upregulated, suggesting its important presence for asexual and sexual development. Data derived from transcriptome data of two independent biological replicates and two microarray platforms. put. = putative. Cut off was set to log2 ratio ≥ ±1.5

In contrast to *gstA* and *gstB*, which are expressed at both differentiation pathways, two novel uncharacterized GSTs specifically expressed at sexual development have been found in this work. AN3299 is already expressed at S48, whereas AN10695 is expressed at late sexual development specifically. The fungus mobilizes more GST at sexual compared to asexual development. Starting from S48 at least two of the four identified GSTs are expressed until the end of sexual cycle. GSTs are not expressed at early sexual point of time S24. In contrast, *gstA* and *gstB* are already expressed at early asexual point of time A24. GST expression is absent at late asexual development A48. Detoxification is not necessary at late asexual development since proliferation units are not in direct contact with competitors or harming conditions. At long-lasting sexual development, *A. nidulans* is always in direct contact with competitors, toxins and other damaging xenobiotics. Therefore, *A. nidulans* requires factors diminishing harming conditions like GSTs.

3.4.1.7 Degradation and remodelling of fungal cell wall in darkness is much more intensive revealing a higher need for energy at sexual development of *A. nidulans*

As a probable reflection of a response to overcome nutrient limitation in the soil *A. nidulans* expresses numerous development-specific genes encoding for enzymes required for degradation of its own, plant and bacterial cell wall as well as other exogenous polysaccharides stored by plants, moos and lichen. Hydrolyzed sugars serve as energy and additional units for construction and remodelling of the fungal cell wall. An essential enzyme necessary for the mobilization of energy from glucans during sexual development of *A. nidulans* is the alpha-1,3-glucanase encoded by *mutA* (AN7359) that is expressed at sexual development S48, S72 and S96 as it was described by Wei et al. 2001. Transcriptome analyses on different stages of the two developmental cycles of *A. nidulans* gives us opportunity to identify further glucanases and to sort them for their asexual and sexual specificity. In total 47 genes encoding for fungal cell wall degradation enzymes are differentially expressed at the seven analyzed stages (www.dropbox.com/sh/yj8r6dx5z2n5zqp/PVpPVIV0mN). 14 genes are present in both asexual and sexual development. Six genes for cell wall hydrolysis are expressed at asexual whereas 21 genes are specifically upregulated at sexual differentiation, suggesting that sexual development requires much more cell wall degrading enzymes than the asexual pathway. A mannosidase (AN3336) is the only glucan hydrolase, which is specifically upregulated at vegetative growth Veg. The expression of five more glucan hydrolases starts already at vegetative growth and remains unchanged at different stages of development.

3.4.1.8 *A. nidulans* activates numerous plant and bacterial cell wall hydrolyzing as well as polysaccharide hydrolyzing enzymes during sexual development in soil.

Interestingly, 5 out of 11 of these saprophytic genes already expressed at vegetative stage are downregulated at asexual development A24, whereas each gene is still expressed at the corresponding point of time in darkness S24 (chapter 3.3.1.2). *A. nidulans* seems to maintain the expression of these genes for a longer and more energy consuming sexual cycle. The delay on genetic stage in *A. nidulans* differentiation at darkness also affects this category, demonstrating an exact light-dependent coordination of genes from different categories assuring successful sexual propagation.

In contrast, the fungus expresses two additional bacterial cell wall hydrolases (AN0543; AN8466) at late sexual development S72 and S96 (Tab. 17). Both uncharacterized enzymes contain a lysine domain (LysM motif) known from peptidoglycan hydrolases with peptidoglycan binding site (PFAM) (Joris, *et al.*, 1992, Bateman & Bycroft, 2000).

Table 17: Selected genes mediating plant and bacterial cell wall & plant polysaccharide hydrolysis to assure energy supply during *A. nidulans* development.

Gene ID	Function	A24	A48	S24	S48	S72	S96
Sexual specific							
AN0543	Lysine domain protein, bacterial cell wall degradation						+
AN0711	alpha-L-rhamnosidase, put.			+			
AN1477	beta-1,4-xylosidase, GH43						+
AN2569	Pectin lyase, put.			+			
AN5320	Endoglucanase E-like members of the SGNH hydrolysis → cellulose or lechnin hydrolysis						+
AN8453	Pectin lyase, put.			+			
AN8466	Lysine domain protein, bacterial cell wall degradation					+	+
AN8891	Exopolygalacturonase				+		+
Asexually + Sexually expressed							
AN0245	Extracellular endo-1,3(4)-beta-Glucanase, put. GH 16	+	+			+	
AN5282	***cbhC***, 1,4-beta-Cellobiosidase *		+			+	+
AN5309	Cutinase	+	+				+
AN6518	Endoxylanase, put.	+	+		+	+	+
AN7828	Unsaturated Rhamnogalacturonane hydrolase, GH 88	+	+			+	+
AN8007	***abnC***, endo-1,5-alpha-L-Arabinosidase *	+	+		+	+	+
AN8890	Cellobiose dehydrogenase	+	+			+	+
AN9383	Unsaturated Rhamnogalacturonan hydrolase, GH 88		+	+	+	+	
Other energy sources (e.g. plant polysaccharides)							
Already at vegetative growth							
AN2385	***xgeA***, Licheninase, put. GPI-anchored *		-				
AN3308	***amyD***, Amylase		-				
Asexually + Sexually expressed							
AN11143	***glaA***, Glucoamylase		+			+	+
AN3402	***amyB***, Amylase *	+	+		+	+	+
AN5463	Glucoamylase (1,4-alpha-Glucosidase)		+			+	+

A. nidulans expresses sexual specific plant xylan hydrolysing enzymes like the AN1477 encoded beta-1,4-xylosidase classified as glycosyl hydrolase (GH) 43. AN1477 is expressed in the end of sexual differentiation S96 merely. Among the genes coding for plant and bacterial cell wall hydrolyzing enzymes expressed at both asexual and sexual stage only one out of eight is expressed at S24 whereas already six genes are expressed at A24. Expression of these non-development-specifically expressed genes is strongly increased in the end of sexual development. Transcriptome analyses exhibit expression of five plant polysaccharide hydrolyzing enzymes. Interestingly, *A. nidulans* does not activate an asexual or sexual specific gene for this category. *xgeA* and *amyD* are already expressed at vegetative stage and *glaA*, *amyB* and another still unknown amylase (AN5463) are expressed at both differentiation cycles. Numbers behind A and S show hours after induction start for asexual and sexual development respectively. Data derived from transcriptome data of two independent biological replicates and two microarray platforms. put. = putative. Cut off was set to log2 > ±1.5. (*) validated gene.

Among the eight sexual specific saprophytic genes are four pectin hydrolyzing enzymes (AN0711; AN2509; AN8453; AN8891). Three of them are already expressed at early sexual development that exhibits the importance for a quick usage of pectin as energy source for *A. nidulans*. Xylan and cellulose hydrolysis through sexual specific enzymes encoded by AN1477 and AN5320, respectively, is merely induced at late sexual development S96.

Beside hydrolysis of external cell wall material *A. nidulans* exploits polysaccharides stored by plants and bacteria e.g. plant lichenins and starch. Hydrolases of starch requires amylases. Three of seven (*amyA – amyF, glaA, glaB*) known amylases comprised by *A. nidulans* are upregulated at the analyzed stages. An amylase already expressed at vegetative stage is encoded by *amyD* (AN3308). It is downregulated at A48 but remains activated the whole sexual cycle. Since *A. nidulans* requires further starch mobilization the fungus expresses three additional amylases (*glaA*, *amyB*, AN5463) at both developmental cycles. The glucoamylase encoded by AN5463 is upregulated at late stage of both development cycles (A48 and S72, S96). *glaA and amyB* reveal similar activation pattern, demonstrating requirement for the usage of starch at the end of both development stages when the fungus might be confronted with starvation. Interestingly, *A. nidulans* neither activates an asexual or sexual specific for starch hydrolysis, revealing generally a broad and increasing energy consumption at middle and late sexual development. Additionally, *xgeA* (AN2385) encodes a GPI-anchored licheninase already expressed at vegetative stage of *A. nidulans*. Lichenins are polysaccharides found in lichen, moos and few plants. Lichenins serve as energy storage. A gene required for starch hydrolysis (AN5463) is expressed at both late asexual and sexual development.

Even for saprophytic genes expressed at both asexual and sexual development only plant cell wall hydrolyzing are found in this category (AN0245; AN5282; AN5309; AN6518; AN8890), proposing that expression of bacterial cell wall hydrolase genes are inhibited through light and stimulated at darkness specifically.

3.4.1.9 Identification of novel secondary metabolite clusters of *A. nidulans* activated in darkness

Filamentous fungi produce numerous secondary metabolites without known function. Some of these compounds have antimicrobial and antibiotic properties and are important factors to provide a defense system against other competitive pro- or eukaryotic organisms in soil.

All yet identified secondary metabolite cluster in *A. nidulans* are based on a genome

wide search and identification of polyketide synthases or non-ribosomale peptide synthetases necessary for the formation of secondary metabolites. Based on similar expression pattern at the analyzed stages of development and a similar composition of gene clusters, except for the lacking polyketide synthase or the non-ribosomale peptide synthetases, required for secondary metabolite production we were able to identify two novel potential secondary metabolite clusters activated during development in darkness (Fig. 13).

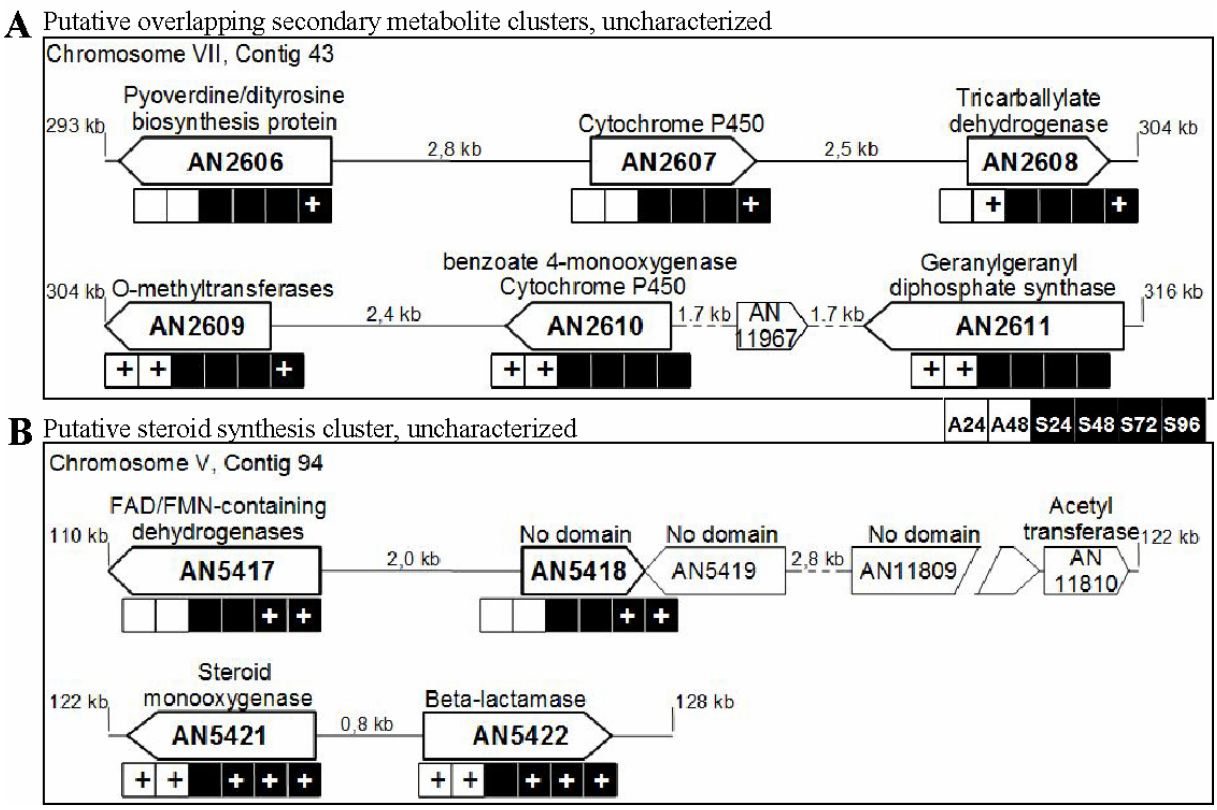


Figure 13: Transcriptionally identified, gene clusters with connection to sexual development of *A. nidulans*
Novel gene clusters in *A. nidulans* were identified through intensive transcriptome analyses derived from two independent biological and two biological replicates on two different platforms (TIGR and AGILENT) demonstrating a potent tool for the identification of gene clusters. Gene clusters with identical expression are present at each analyzed stage of growth and development. **(A)** AN2606 - AN2611 consists of two overlapping gene clusters sharing a tricarballylate dehydrogenase (AN2608) and an o-methyltransferase (AN2609) that reveal an upregulation at asexual and sexual differentiation. AN2606 encodes a homologue to a dityrosine synthesis protein in *S. cerevisiae* expressed at late sexual development. Dityrosin protects yeast ascospores. Additionally the cluster involves a transcription factor and two cytochrome P450 monooxygenases present in numerous secondary metabolite clusters. **(B)** The AN5417 – AN5422 cluster contains four genes that are preferentially expressed at middle and late sexual stages S48/72/96 and are most likely required for steroid biosynthesis. Cut off was set to $\log2 \geq \pm1.5$.

The first cluster might be an overlapping cluster sharing a tricarballylate dehydrogenase (AN2608) and an O-methyltransferase (AN2609). Both are expressed at A48 and S72. AN2608 is expressed at S96 and AN2609 at A24 additionally. AN2608 belongs to cluster comprising three genes AN2606 – AN2608. AN2606 codes for a pyoverdine/dityrosine

biosynthesis protein that is expressed at late sexual development. *S. cerevisiae* contains a homologous protein which is described to produce pyoverdine that protects ascospores. Pyoverdine might also be produced inside ascospores of *A. nidulans* and protect them from environmental damage. The S72 expressed cytochrome P450 is encoded by AN2607. Another cytochrome P450 is part of the second overlapping cluster and is expressed at both asexual stages. The putative geranylgeranyl diphosphate synthase (AN2611) is expressed at A24 and A48 as well and is important for the isoprenoid biosynthesis. Isoprenoids serve as basis for the formation of several terpene-derived secondary metabolites. For instance, paxilline is a prominent toxic indol diterpene alkaloid produced by another fungus *Penicillium paxilli*. Many formed pheromones are also isoprenoid-based compounds and involved in the regulation of development of *Aspergilli* and other ascomycetes (Gooday, 1974, Kappas, 1983, Cerda-Olmedo*, et al.*, 1994, Semighini*, et al.*, 2008)

Furthermore AN5417 – AN5422 might also encode a steroid synthesis gene cluster. Four genes are upregulated at late sexual stages S72/S96. A steroid monooxygenase (AN5421) and a beta-lactamase (AN5422) exhibit additional upregulation at both asexual stages A24/48 and S48. Within this cluster a constitutively expressed acetyltransferase (AN11810) and two genes without domains (AN5429 and AN11809) are located. This cluster might be bigger than shown in figure 13. AN5430 – AN5433 are also upregulated at late sexual development and might belong to this cluster. AN5432 codes for a lipase/esterase and could be necessary for steroid modification/synthesis as well. Cytochrome P450 monooxygenases, as the AN5433 encoded are often part of secondary metabolite clusters and could also participate in steroid synthesis. The AN5417 – AN5422 might be crucial for the synthesis of another secondary metabolite, for example a hormone, regulating sexual differentiation processes like *psi* factors in *A. nidulans*.

3.4.2 Metabolome reveals sexual-specific metabolites in *A. nidulans*

3.4.2.1 Primary metabolites

3.4.2.1.1 Intracellular amino acid concentrations decrease in the end of sexual development of *A. nidulans*

In developmental metabolome, the intracellular amino acid content of *A. nidulans* show specific accumulation to defined stages of development and depletion at late sexual development starting from S72 (Tab. 18), confirming that the fading transcription of amino acid metabolism genes has direct impact on amino acid homeostasis.

Thereby, only defined members of the different amino acid groups are affected and

show specific accumulation pattern at different stages of development. As intermediate in the synthesis of the plant hormone ethylene, of cysteine, carnitine, taurine, lecithine, phosphatidylcholine and other phospholipids, methionine serves as methyl donor in its derivative S-adenosyl methionine (SAM) as well. It participates also in histone methylation and transcriptional regulation indirectly (Bartova, *et al.*, 2008). Methionine accumulates at S24 specifically. At this stage *A. nidulans* contains most free methionine, which could be explained by a delay in the formation of the methionine-based compounds inhibited in darkness.

Table 18: Abundance of amino acids during development of *A. nidulans*

Amino acids	Veg	A24	A48	S24	S48	S72	S96
D-Met				+			
L-Ala	+			+			
L-Glu	+	+					
L-Asp		+			+	+	
L-Gln		+			+	+	
L-Thr	+	+		+			
L-Val			+				
L-Lys		+		+	+		
L-Phe	+	+	+	+	+	+	+
L-Asn		+	+	+	+		
L-Orn	+			+			
L-Pro		+		+	+		
L-His		+			+	+	

Accumulation of various amino acids in the intracellular metabolome at defined stages of *A. nidulans* development. Generally, amino acids increase at early stages of asexual (A) and sexual development (S). Amino acids are separated regarding their synthesis (black lines). Amino acids like alanine, glutamate, threonine, phenylalanine and ornithine already accumulate at vegetative (Veg) stage. Only few amino acids like asparagine or phenylalanine are still accumulated at late development. Valine content increases at late asexual development A48 specifically, whereas methionine content is only increased at early sexual stage S24. Ornithine, the starting compound in polyamine synthesis, is increased at vegetative and early sexual stage. Spermine, spermidine and putrescine are polyamines, which have signalling properties and are partially toxic and apoptosis-inducing agents in the highly competitive habitat. Expression of various potential polyamine transporters is activated during the course of development and mediates an active polyamine secretion in *A. nidulans* probably. *A. nidulans* FGSC A4 wild type was grown in minimal medium without amino acid supplements generally. The metabolite fingerprinting analyses was performed by Dr. Kirstin Feussner, Department of Plant Biochemistry, Göttingen.

The content of the alanine, glutamate and aspartate group are dispensable at both late stages. Both alanine and glutamate are present at vegetative stage whereas alanine is also detectable at early sexual stage S24 and glutamate at early asexual stage A24 specifically. Apart from the major destination for the protein biosynthesis (Bouhired, *et al.*, 2007), some amino acids have additional function in secondary metabolite formation. In higher eukaryotes aspartate and glutamate serve as neurotransmitter in vertebral neural systems (Chen, 2005). A signalling function was not observed for both amino acids in filamentous fungi yet. Alanine is part of some peptide antibiotics, which might be specific for vegetative growth and early sexual stage S24 since alanine accumulates at these stages.

Phosphorylation is one of numerous possible posttranslational protein modifications and requires threonine when it is mediated through threonine kinases. Thereby, its side chain can undergo glycosylation. Threonine accumulation is highest at vegetative growth and both early development stages A24 and S24.

Lysine is involved in numerous protein modifications and can be acetylated, methylated, ubiquitinated, sumoylated, neddylated, biotinylated, pupylated and carboxylated, which can affect protein function. Furthermore, lysine is needed for the synthesis of several hormones. Lysine accumulation is restricted to both early development stages A24 and S24 as well as to S48. Lysine derived hormones might also control development at early stages.

Phenylalanine is the starting compound used in flavanoid biosynthesis and is the precursor of several mammalian hormones and of the pigment melanin as well. Melanin also protects fungal tissue from UV radiation damage. Phenylalanine is present at all analyzed stages and might be indispensable for *A. nidulans.* Although melanin is of major importance for asexual cultures suffering from UV radiation, the fungus might also protect its genetic material from a possible UV mediation constitutively.

Asparagine provides the key site for N-linked glycosylation. It accumulates at both 24 h and 48 h stages of sexual and asexual differentiation. As for lysine protein modification sites are of high importance for the organism without initiation of another translational round or proteasomal impact to control protein function.

As a non-proteinogenic amino acid, ornithine is the starting compound of polyamine synthesis. Its increase in concentration is restricted to vegetative and early sexual stage. As already described previously polyamines are involved in signalling, important for growth and differentiation in numerous organisms. Thereby, *A. nidulans* might control growth of its prokaryotic competitors in soil since the polyamine putrescine is toxin in large doses and is an apoptosis-inducing agent. Despite the absence of these polyamines at the analyzed

developmental stages, expression of various potential polyamine transporters might hint for their presence. They mediate an active polyamine exchange in *A. nidulans* and emphasize a possible meaning of polyamines in the interaction with its environment and survival finally.

The imidazole group containing histidine is involved in oxidative stress response. On the one hand histidine is a precursor in carnosine biosynthesis. Carnosine has been proven to scavenge reactive oxygen species (ROS) as well as alpha-beta unsaturated aldehydes formed from peroxidation of cell membrane fatty acids during oxidative stress. On the other hand in Actinomycetes or filamentous fungi like *Neurospora crassa* histidine is converted into the antioxidant ergothioneine (Fahey, 2001). Histidine accumulates at early asexual A24 and mid-sexual development S48, S72. *A. nidulans* is known to trigger ROS levels crucial for development that might also harm the fungus in case of an imbalanced state. Histidine might also participate in these processes.

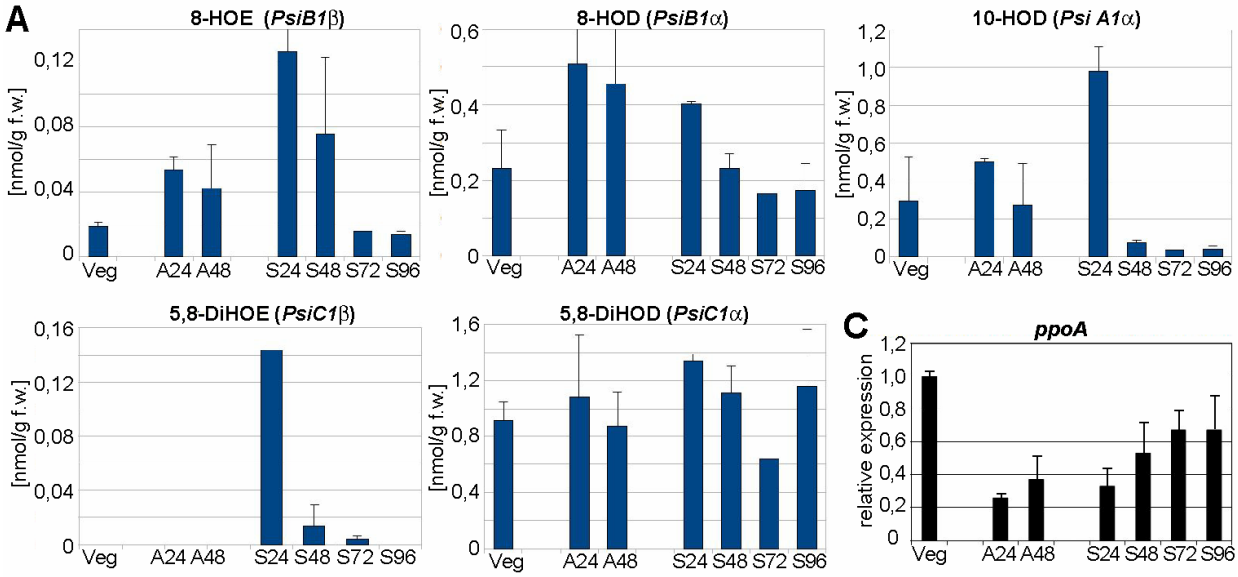
Fading amino acid content reveal that *A. nidulans* strictly controls and regulates energy requirement and consumption during a long lasting sexual development. Thereby, the fungus achieves a very economical usage of nutrients and assures its survival even at extreme nutrient limitation conditions.

3.4.2.2 Secondary metabolites

3.4.2.2.1 Development in *A. nidulans* reveals characteristic linoleic and oleic acid derived *psi*-factor ratios and dioxygenase expression

A. nidulans development is accompanied by the synthesis of characteristic linoleic and oleic acid derived *psi*-factors and development specific expression of the dioxygenases *ppoA* (AN1967) and *ppoC* (AN5028) catalyzing conversion of these *psi*-factors (Champe, *et al.*, 1987, Champe & el-Zayat, 1989, Mazur, *et al.*, 1990, Mazur, *et al.*, 1991, Calvo, *et al.*, 2001, Tsitsigiannis, *et al.*, 2004, Garscha, *et al.*, 2007, Andreou, *et al.*, 2009, Brodhun, *et al.*, 2009, Brodhun, *et al.*, 2010). *psiB1α* (8-HOD) and *psiC1α* (5,8-DiHOD) are hydroxylated linoleic acid derivatives reported to stimulate sexual spore development whereas *psiA1α* (lactone ring of *psiC* at position 5) inhibits sexual spore development (Champe & el-Zayat, 1989). Impact of *ppoA* ((8R)-dioxygenase with hydroperoxide isomerase activity) and *ppoC* ((10R)-dioxygenase) on *A. nidulans* development, on spore formation and *psi*-factor ratio was already examined. Deletion of *ppoA* led to prominent reduction of 8-HOD and complete loss of 5,8-DiHOD biosynthesis. Decreased 8-HOD concentration results in a changed ratio of 8-HOE:8-HOD in which leads to cultures that exhibit more asexual structures in Δ*ppoA*. Meaning that *ppoA* promotes sexual development. Expression of *ppoA* is highest at vegetative growth,

indicating that the fungus already prepares for induction of sexual development at vegetative growth before it reaches developmental competence (Fig. 14). The following measurements for *psi*-factors and their analysis was performed by Dr. Cornelia Herrfurth, Department of Plant Biochemistry, Göttingen.



B

	8-HOE:8-HOD	8-HOD:10-HOD	5,8-DiHOE:5,8-DiHOD
A24	9,4:1	1:1	only 5,8-DiHOD
A48	10,8:1	1,7:1	only 5,8-DiHOD
S24	**3,2:1**	**1:2,5**	**1:9,3**
S48	**3,1:1**	3:1	1:79,6
S72	10,3:1	4,6:1	1:159,8
S96	13,4:1	4,4:1	only 5,8-DiHOD
Veg	12,9:1	1:1,3	only 5,8-DiHOD

Figure 14: Targeted *psi*-factor analysis during development of *A. nidulans* offers novel data about *psi*-factors characteristic for early sexual development (A) Concentration of *psi*-factors during the analyzed stages of development. Vegetative growth (Veg); asexual (A); sexual (S) (**B**) Ratio between 8-HOE:8-HOD; 8-HOD:10-HOD; 5,8-DiHOE:5,8-DiHOD. Higher 8-HOD and 10-HOD concentrations at 24 h and 48 h are characteristic for sexual development. 5,8-DiHOE is present only in sexual differentiated *A. nidulans*. Abbreviations: 8-HOE, 8-hydroxy oleic acid, *psiB1β*; 8-HOD, 8-hydroxy linoleic acid, *psiB1α*; 5,8-DiHOE, 5,8-dihydroxy oleic acid, *psiC1β*; 5,8-diHOD, 5,8-dihydroxy linoleic acid, *psiC1α*. (**C**) Relative *ppoA* (AN1967) and *ppoC* (AN5028) expression for the analyzed stages of *A. nidulans* development derived from transcriptomes. *psi*-factors measurements and their analysis was performed by Dr. Cornelia Herrfurth, Department of Plant Biochemistry, Göttingen. Data derived from transcriptomes of two independent biological replicates and two microarray platforms. Cut off was set to log2 ≥ ±1.5.

ppoA expression is similar at both early stages of development A24 and S24, whereas it is higher in S48 compared to A48. In the course of sexual development *ppoA* expression increases continuously and reaches its highest expression through S72 and S96. 8-HOE:8-HOD ratio is around 3-fold higher at A24 compared to S24 (9.4:1 vs. 3.2:1). At A48 the ratio is 10.8:1 compared to 3.1:1 at S48. These results are consistent with the observation described

by Tsitsigiannis *et al*. (2004). At sexual development S72 and S96 8-HOE:8-HOD ratio reaches to asexual level (10.3.1 and 13.4:1), which might be consistent with the observations that *A. nidulans* secondarily forms asexual structures when sexual development is already completed. *A. nidulans* prepares most likely for another round of asexual differentiation. Deletion of *ppoC* results in an increased number of ascospores and causes biosynthesis of traces of racemic 10-HOD but did not affect the biosynthesis of other oxylipins (Garscha, *et al.*, 2007). In contrast, 10-HOD concentration is increased in the Δ*ppoA* strain. Expressional and metabolomic analyses within this work are in agreement with the published data in former publications and reveal a much more detailed insight into the *ppoA*/*ppoC* expression combined with the kinetic of *psi*-factors during *A. nidulans* development. In the light when *ppoC* expression is increased and the fungus is induced for asexual development 10-HOD concentration is less than at early sexual development S24. 10-HOD concentration decreases much faster at sexual than the asexual development. When 10-HOD is double concentrated at S24, it is only half concentrated at S48 compared to the corresponding asexual stages and is rarely enriched at S48, S72 and S96 when the expression of *ppoA* increases. Thus, 10-HOD presents a marker specific for early sexual development and might be required for a proper ratio of asexual to sexual spores in *A. nidulans*.

Hydroxylated derivatives of oleic acid (*psiA1β* (*psiC1β* with lactone ring at C-5), *psiB1β* (8-HOE), and *psiC1β* (5,8-DiHOE)) have also been isolated from *A. nidulans* (Mazur, *et al.*, 1990, Mazur, *et al.*, 1991). Impact of oleic acid derived *psi*-factors on *A. nidulans* development is mostly unknown. Calvo and co-workers (2001) analyzed a desaturase *odeA* deletion strain and could show that this strain is depleted of polyunsaturated fatty acids (18:2 and 18:3) but increased in oleic acid (18:1). The total percent fatty acid content of the linoleic acid derived *psi*-factors exhibit massive decrease whereas oleic acid derived *psi*-factors concentration is increased. Development of the deletion strain shifted to sexual direction since ascospore formation was elevated so that they concluded that oleic acid derivatives have an effect on the ratio of asexual to sexual spores in *A. nidulans*.

Beside the already characterized desaturases *odeA* (AN1037) another monofunctional oleoyl-Delta12 desaturase encoded by *an2* (AN7204) reveals the same expression pattern as *odeA*. An2 was biochemically and structurally characterized but the effect of an *an2* deletion on *A. nidulans* development was not analyzed yet (Hoffmann, *et al.*, 2007). Interestingly expression is already present at vegetative growth when the fungus requires developmental competence to be able to differentiate asexual or sexually. Their expression is still increased at early asexual and sexual development A24, S24, S48, suggesting that their presence might

accompany or control asexual and sexual structure formation and is not merely important for the decision of the development induced by light. Additionally *an2* is again expressed at S96 when the fungus prepares for another development cycle. Deletion of *odeA* is most likely similar to the state when *odeA* is less or even not expressed.

Even more interesting is the finding that the oleic acid derived 5,8-DiHOE is only present in early sexual development S24 specifically and its concentration decreases massively and is not detectable at late sexual development. Calvo and co-workers (2001) could detect 5,8-DiHOE in the sexually induced Δ*odeA* strains merely. Combined with the specific appearance of 5,8-DiHOE at S24 it indicates that 5,8-DiHOE is crucial for the induction of sexual development in *A. nidulans*.

Furthermore even 8-HOD:10-HOD ratio is development specific in *A. nidulans*. In this study ratio of 8-HOD to 10-HOD is almost similar at A24, A48, S24 and Veg (1.7:1 – 1:2.5), whereas it is transposed from 1:2.5 at S24 to 4.6:1 at S96 in 8-HOD direction. This is consistent with the findings of Garscha and co-workers (2007), who revealed that the absence of *ppoA* results in a massive decrease of 8-HOD whereas the amount of 10-HOD is not effected.

Preparation for *psi*-factor synthesis requires different initial steps starting from fatty acid beta-oxidation located in peroxisomes, long chain fatty acid synthesis and oleic – and linoleic acid synthesis specifically (Dimitrios 2004, PhD thesis). Initial fatty acid oxidation in *A. nidulans* is mediated through the peroxisomal multifunctional enzyme encoded by *foxA* (AN7111) (Maggio-Hall & Keller, 2004). It was shown to be essential for growth on very long-chain fatty acid medium. Its expression is induced by fatty acids. Here *foxA* transcription is increased at S72 specifically. In contrast, *fasA* (AN9407) encodes a fatty acid synthase that is already expressed at vegetative growth and early sexual development S24 and S48 (Tab. 19). Deletion of *fasA* was described to be lethal for *A. nidulans*. (Brown, *et al.*, 1996, David, *et al.*, 2008), concluding that *fasA* is crucial for the fungal long chain fatty acids supply required for membrane formation during growth generally and for *psi*-factor formation specifically. Palmitic- and stearic acids are among long fatty acids. The putative delta-9-stearic acid desaturase SdeB (AN4135) converts both fatty acids to palmitoleic acid and oleic acid, the precursor of oleic acid derived *psi*-factors. As observed for *fasA* also *sdeB* is specifically expressed at vegetative growth and early sexual stage S24.

Table 19: Genes involved in linoleic- and oleic acid conversion and formation of polyunsaturated fatty acids expressed during *A. nidulans* development.

Gene ID	Function	A24	A48	S24	S48	S72	S96
Desaturases & other enzymes involved in fatty acid synthesis							
AN7111	***foxA***, peroxisomal multifunctional enzyme involved in fatty acid oxidation*					+	
AN9407	***fasA***, fatty acid synthase, alpha subunit*	-	-			-	-
AN4135	***sdeB***, delta-9-stearic acid desaturase *	-	-		-	-	-
AN1037	***odeA***, oleate delta-12 desaturase *		-			-	-
AN7204	***an2***, oleoyl-delta12 desaturase, put *		-			-	

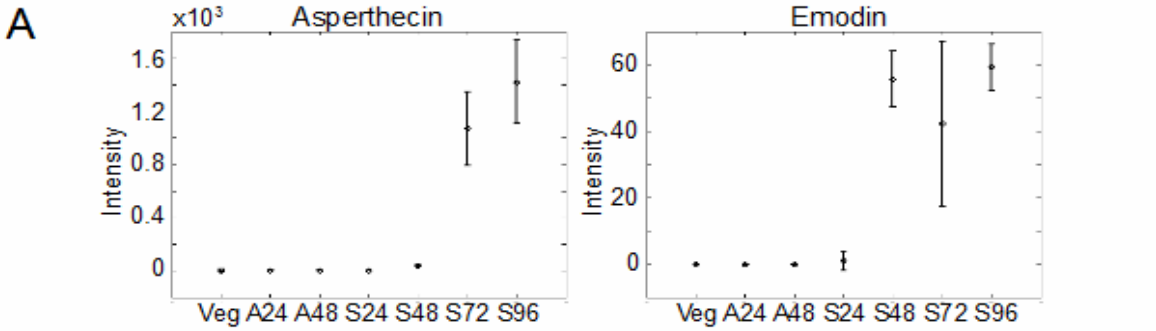
Each desaturase *sdeB*, *odeA* and *an2* is already expressed at vegetative (Veg) growth and still present at early asexual (A) and sexual (S) development. OdeA was shown to be important for the proportion between linoleic- and oleic acid-derived *psi*-factors. Linoleic acid-derived *psi*-factors were absent in Δ*odeA* strain but oleic acid-derived *psi*-factors were increased relative to wild type (Calvo et al. 2001). Data derived from transcriptome data of two independent biological replicates and two microarray platforms. put. = putative. Cut off was set to log2 ≥ ±1.5. (*) validated gene.

A double deletion with another stearic acid desaturase *sdeA* is lethal for *A. nidulans* emphasizing the importance of desaturases (Maggio-Hall & Keller, 2004). The function of both genes is not suppressed by other gene products. In summary, *A. nidulans* requires polyunsaturated fatty acids for membrane formation as well as for *psi*-factor formation and already expresses crucial genes for this synthesis at vegetative stage. The only exception is *foxA* that is upregulated at S72 when each other gene involved in polyunsaturated fatty acid synthesis is not expressed long ago (Tab. 19). This remains to be discussed.

3.4.2.2.2 Secondary metabolites specific for sexual development of *A. nidulans*

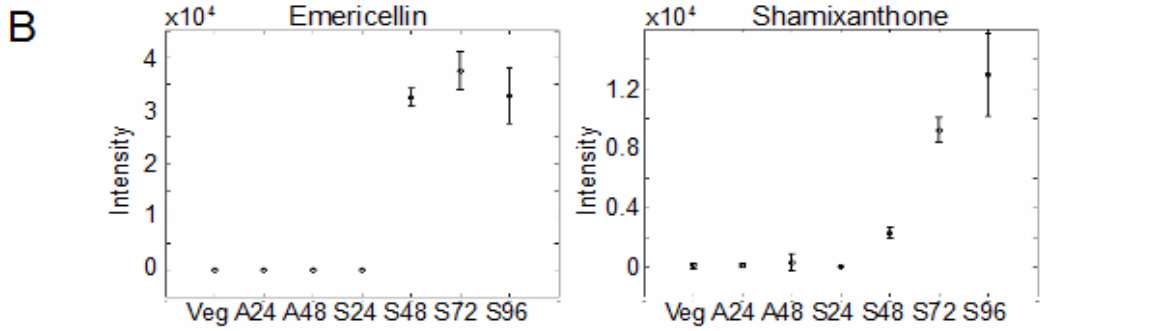
As for asexual specific secondary metabolites, already described in chapter 3.3.2.1 (page 65) intracellular secondary metabolites characteristic for *A. nidulans* sexual development in darkness were also detected. In combination with our transcriptomic data we try to understand how stage specific expression pattern influence synthesis of the corresponding secondary metabolites.

Numerous genes required for asperthecin (*apt*) and monodictyphenone (*mdp*) synthesis are rather sexual specific (Fig. 15). The *apt* genes within the asperthecin cluster are reported not being expressed at laboratory conditions (Szewczyk, *et al.*, 2008). *apt* gene expression is increased and asperthecin accumulates in the *A. nidulans* Δ*sumo* strain.



apt *gene cluster regulation*

Gene ID	Function	A24	A48	S24	S48	S72	S96
AN6000	***aptA***, polyketide synthase*					+	
AN6001	***aptB***, hydrolase, put.*					+	+
AN6002	***aptC***, monooxygenase, put.*					+	+



mdp *gene cluster regulation*

Gene ID	Function	A24	A48	S24	S48	S72	S96
AN0146	***mdpC***, versicolorin ketoreductase*					+	
AN0147	***mdpD***, flavin-containing monooxygenase*					+	+
AN0148	***mdpE***, protein homologue to AflR*					+	+
AN0149	***mdpF***, zinc-dependent hydrolase, put.					+	+
AN10022	***mdpH***, DUF 1772 superfamily					+	+
AN10023	***mdpL***, no domain detectable*					+	
AN10035	***mdpI***, AMP-binding CoA ligase, put.					+	
AN10038	***mdpJ***, glutathione-S-transferase*					+	+
AN10044	***mdpK***, oxidoreductase*					+	
AN6784	***xptA***, prenyltransferase*					+	

Figure 15: Secondary metabolites specific for *A. nidulans* development in darkness.
(A) Asperthecin, emodin, (**B**) emericellin and shamixanthone are secondary metabolites enriched at sexual (S) development specifically. Emericellin and shamixanthone content increases at S48 merely whereas it is hardly detectable at early sexual stage S24. Asperthecin accumulation is highest at both late sexual stages S72/S96. Generally expression of each gene cluster coincides with the intracellular metabolomic findings (corresponding tables beneath the intracellular profiles). Emericellin and shamixanthone biosynthesis share *mdp* gene products required for monodictyphenone synthesis, which was not detected in the metabolome. All compounds are absent from vegetative (Veg) and asexual (A) stages. Numbers behind A and S show hours after induction start for asexual and sexual development respectively. The metabolite fingerprinting analyses was performed by Dr. Kirstin Feussner, Department of Plant Biochemistry, Göttingen. Data from intracellular metabolome derived from two biological and three technical replicates. Relative intensities of metabolite markers are shown as error bars containing mean values (points) and standard deviations (bars).. Transcriptomic data derived from two biological and two technical replicates combined with a complete change of the used transcriptome platforms (TIGR and AGILENT). Cut off was set to log2 ≥ ±1.5. (*) validated gene.

Our data show *apt* gene upregulation and an intracellular asperthecin accumulation at late sexual development. Asperthecin is already detectable at S48 in low contents when the cleistothecia start to form. It accumulates at S72 more than 10-fold and reaches its maximum at S96 when sexual development is complete. Emodin as precursor of asperthecin is also present at sexual stages S48 - S96. Because AptC, which catalyzes the conversion of emodin to asperthecin is merely expressed at S72, emodin accumulates already at S48 whereas asperthecin content is still low at this point of time. This example clearly demonstrates connection between gene expression and its influence on metabolome level.

mdpC, *mdpL*, *mdpI* and *mdpK* are specifically expressed at S72. MdpC is a protein with high homology to versicolorin ketoreductases. The specific function of MpdL is unknown yet. MdpI is a putative AMP-binding CoA ligase and MpdK a putative oxidoreductase. It was reported that MdpC, MdpL and MdpK are required for monodictyphenone but not for emodin and other intermediates production (Chiang, *et al.*, 2010). MdpI is not necessary for the synthesis of emodin, derivatives or monodictyphenone. Though three of five *mdp* genes needed for monodictyphenone synthesis are upregulated at S72, we could not detect monodictyphenone. In contrast, compounds that share the same biosynthesis pathway with monodictyphenone like emericellin and shamixanthone could be detected at mid and late sexual development S48, S72 and S96.

Emericellin and shamixanthone belong to the xanthone family like monodictyphenone and asperthecin. Their biological function is still not known, but they are thought to be antimicrobial compounds (Sanchez, *et al.*, 2011). Two genes required for emericellin and shamixanthone synthesis like *mdpD*, *xptA* are upregulated at late sexual development. Thereby, MdpD converts paeciloxanthone to variecoxanthone and XptA catalyzes the conversion from variecoxanthone to emericellin. Paeciloxanthone and variecoxanthone are converted to emericellin, which accumulates at mid and late sexual development in *A. nidulans*. This finding explains accumulation of emericellin in sexual development but not the shamixanthone accumulation, since the *xptC* gene product, responsible for the conversion of emericellin to shamixanthone, is constitutively expressed.

3.4.2.2.3 Secreted secondary metabolites during *A. nidulans* development in darkness

Protective secondary metabolites synthesized intracellular by *A. nidulans* have to be secreted to get to its target e.g. bacterial, fungal and other competitors. Therefore, we analyzed the extracellular metabolome to get a global view on the content of secondary metabolites already detected in intracellular metabolome. Analyses of extracellular

metabolomes were restricted to end points of development. Thus, three stages were examined Veg, A48 and S96. This setup gives us a general overview concerning the secondary metabolites, which are either secreted or accumulated until the end of each development cycle of *A. nidulans*.

Although several secondary metabolites like asperthecin, emodin, emericellin and shamixanthone could be detected intracellularly, none of them could be detected in the extracellular environment at the end of sexual development S96. Surprisingly, emericellamide C, a secondary metabolite strongly intracellularly accumulated at both asexual stages, is present extracellularly at vegetative growth, A48 and S96. Emericellamide C exhibits its strongest extracellular accumulation at asexual development A48 what coincides with its increased synthesis at asexual development. Additional secretion during vegetative growth makes emericellamide C a constitutively secreted secondary metabolite (Fig. 16). It was reported to be secreted into liquid medium after 4 d of vegetative growth and in solid complex medium after 5 d of growth in light, indicating that emericellamides are most likely not secreted during complete asexual differentiation. Emericellamide C secretion is obviously driven by light since its extracellular concentration is strongest at A48. This is the first time emericellamide species were shown also to be intracellularly present at sexual stage and an emericellamide species secreted at sexual stage. Why emericellamide A and E could not be detected extracellularly, remains to be discussed.

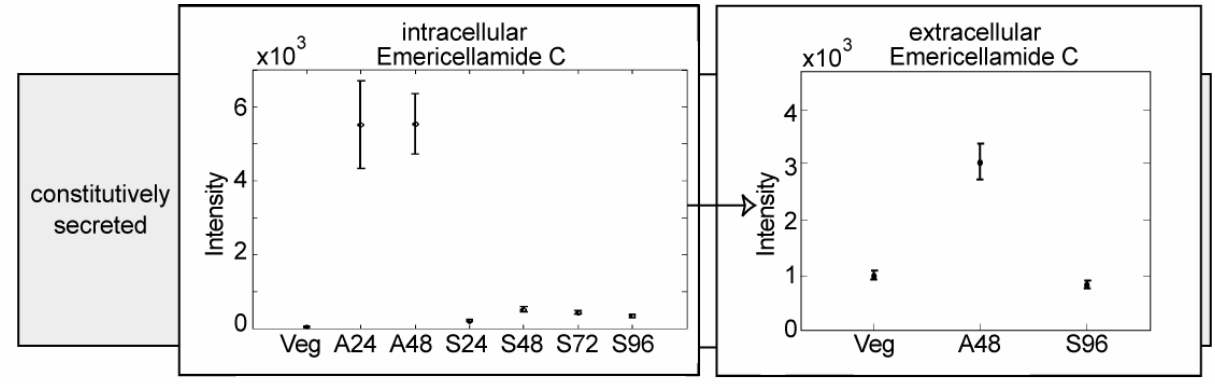


Figure 16: Emericellamide C is secreted at sexual development in *A. nidulans*
Emericellamide C, a secondary metabolite, was detected in the intra- and extracellular environment. Extracellular end point analyses reveal its accumulation at Veg, A48 and S96. Although several secondary metabolites exhibit intracellular accumulations even at late sexual stage, like asperthecin, emericellin and some more, no additional candidate could be detected at extracellularly. This serves as control for the detectable extracellular substances not being the consequence of a lytic fungus concurrently. Emericellamide A/C/E accumulate at both asexual development stages A24/48 and at S48/S72 in lower amount. While emericellamide A and E are not present in extracellular milieu emericellamide C is translocated at each analyzed extracellular phase Veg / A48 / S96. Thereby, its amount is highest at A48, what coincides with its high asexual intracellular enrichment. The metabolite fingerprinting analyses was performed by Dr. Kirstin Feussner, Department of Plant Biochemistry, Göttingen. Results for the illustrated metabolites based on two independent biological and six technical replicates. Relative intensities of metabolite markers are shown as error bars containing mean values (points) and standard deviations (bars).

Secondary metabolites with extracellular destinations at sexual stage might be already secreted at an early stage of sexual development. Sexually secreted secondary metabolites might also have a shorter life span and face a more quick decay generally.

4. Discussion

4.1. Reflection of delayed *A. nidulans* asexual spore formation in darkness on genetic level

A. nidulans differentially expresses one fifth of its comprised genes in transition of vegetative growth to asexual and sexual development. This does not necessarily mean that the remaining and constitutively expressed genes are not connected to regulation of development in *A. nidulans*. The chosen setup gave us inside into expression of light-dependent asexual and sexual specific genes after achieving developmental competence.

A. nidulans also forms asexual conidiospores during sexual development secondarily. The number of secondarily formed is lower than of primary conidiospores formed under asexual condition in light. An explanation for a lower number of secondarily formed conidiospores at the end of sexual cycle might be the decreased nutrient supply. Under steady-state conditions, when the fungus does not have to face nutrient limitation, conidiospore number could be much higher or even equal to primarily formed conidiospores at asexual differentiation in light. An important question is whether *A. nidulans* forms conidiospores secondarily due to nutrient starvation as a kind of emergency program for a quick proliferation of its genetic material or whether it is constitutively induced during sexual differentiation. This can be easily proofed by using thicker agar plates. In case of lesser conidiospores under this condition the fungus indeed induces an emergency program by forming conidiospores on standard plates.

Since *A. nidulans* even induces asexual differentiation during sexual development, complete differentially expressed gene sets of both asexual stages were compared to gene sets of at least 24 h delayed sexual stages. Especially, the comparison of the early asexual set with the corresponding delayed sexual gene sets reflect an increasing similarity through elevating numbers of differentially expressed genes on genetic level.

The decision for the development, *A. nidulans* is going to perform starts with light. Thereby, histidine sensor kinases might also play an important part in the delayed asexual induction at sexual stage. Four of seven differentially expressed histidine sensor kinases are downregulated at early sexual stage S24, upregulated at S48 and again downregulated at S72. The small expressional activation window at S48 might be crucial for the induction of asexual differentiation during *A. nidulans* sexual development.

As reflection for a proper asexual induction by light, asexual specific genes like the conidiation protein 6 were among the delayed genes. Another already verified asexual specific

gene is *tmpA* encoding a transmembrane flavoprotein involved in conidium formation (Soid-Raggi, *et al.*, 2006). Its expression exhibits a 48 h delay in darkness.

Among the delayed are secondary metabolite genes encoding enzymes required for pigment or phytoalexine-like compounds for a direct answer to bacterial or fungal infection and attacks. Thereby, the pigment formation genes are merely expressed during asexual and sexual development accounting for its specific localization in developmental structures like conidiophores or –spores. In contrast, the transferase most likely involved in the synthesis of phytoalexine-like compounds is already present at vegetative stage to react on infections of mycelia, the fungal backbone.

Several genes encode factors with a possible connection to the delay of *A. nidulans* asexual development during sexual differentiation. These are involved in transcriptional control, signal transduction and development generally. The catalase-peroxidase CpeA is an important enzyme that is involved in development through triggering ROS levels (Scherer & Fischer, 1998, Scherer, *et al.*, 2002). It was shown to be important for sexual reproduction. In this thesis, *cpeA* is also expressed at both asexual stages and thus might also participate in asexual reproduction.

OdeA encodes a Delta-12 desaturase that converts oleic acid to linoleic acid which controls differentiation in *A. nidulans* (Calvo, *et al.*, 2001, Hoffmann, *et al.*, 2007). The delta *odeA* strain is reduced in conidial production and mycelial growth. *odeA* reveals an 24 h expressional delay at sexual cycle. Since the deletion strain is reduced in conidiation it is most likely that *odeA* is crucial for the performance of asexual reproduction even at sexual stage.

Even the process of fusing filaments of the same wild type, restricted to ascomycetes, might be an asexual specific process since a heterokaryon incompatibility protein (AN8923) is delayed for 48 h. Maybe the fusion is an accompaniment of asexual differentiation. It would be interesting to analyze the formation of heterokaryon mycelia in strains deficient for or inhibited in proper asexual development. Concurrently expression of this gene should be tested.

Protein degradation also has high impact on development in *A. nidulans* (Busch, *et al.*, 2003, Busch, *et al.*, 2007, Nahlik, *et al.*, 2010, Bayram & Braus, 2012, von Zeska Kress, *et al.*, 2012). Before proteins are degraded through the 26S proteasome, proteins have to be marked for degradation. Proteins are marked for degradation through Cullin RING finger ubiquitin ligases (CRL). Thereby, the composition of CRL is controlled by the COP9 signalosome which consists of eight Csn subunits in *A. nidulans*. Among the CRLs is the well-investigated Skp1-Cullin/Cdc53-F-box protein (SCF). The substrate recognition complex

consists of the Skp1 protein, mediating binding to the N-terminal stalk of Cul1 and one of several F-box proteins that bind substrate directly. One F-box gene (AN6625) features a 24 h delay. Since it is expressed at early asexual stage and at S48 it might participate in the initiation of conidiation and probably degrades repressors of asexual development.

ChiB a classV chitinase was shown to be responsible for age-dependent autolysis in *A. nidulans* (Erdei, *et al.*, 2008, Karlsson & Stenlid, 2008). Finally ChiB assures fungal nutrient supply and survival even in a nutrient exhausted environment. As many other observations autolysis and aging mediated by ChiB might also be an asexual specific accompaniment at sexual development misleadingly described as sexual specific behaviour (Yamazaki, *et al.*, 2007). The performance of two different, temporal-spatial not separated developmental processes sometimes might result in wrong conclusions. Only targeted examination of defined and separated asexual and sexual structures will assure differentiated and reliable results.

4.2 Saprophytic activity during development of *A. nidulans*

A. nidulans reveals active protection against nutritional competitors and comprises numerous genes for the usage of external energy sources like plant and bacterial polysaccharides. Under laboratory conditions the fungus has neither to face competitors nor plant material. Nevertheless, *A. nidulans* comprises and constitutively activates numerous polysaccharide hydrolyzing enzymes to each stage of development. The fungus possesses a high energy requirement at vegetative growth reflected in the number of cell wall hydrolyzing genes at this stage. *A. nidulans* seems to have preference for plant cell wall material since only two muramidase genes (AN6470; AN8969) for hydrolyzing bacterial peptidoglycan are activated. Most of them still remain activated at early developmental stages, proposing the importance for steady energy supply at the transition of vegetative, undifferentiated and differentiated growth. Especially, this behaviour can be observed at early sexual stage.

A clear preference for bacterial cell wall hydrolysis is detectable at sexual stage since two additional genes (AN0543; AN8466) with this function are expressed at both late sexual stages (S72 and S96). Bacterial cell wall hydrolysis in *A. nidulans* is inhibited through light and stimulated at darkness specifically. The fungus faces a much higher bacterial concentration within soil compared to the surface. For this purpose the fungus avoids expression of bacterial cell wall hydrolyzing enzymes on the surface controlled by light.

Beside plant and bacterial material, *A. nidulans* also hydrolyzes lichen polysaccharides, revealing a broad spectrum for external polysaccharides. *A. nidulans* is classified as saprophytic fungus that is able to use dead plant material as external energy

source. It might be much more than this. Its constitutive expressional activity regarding external polysaccharide hydrolyzing genes and the selective expression of bacterial cell wall degradation might also be an expression of aggression to defend its habitat. As secreted toxins, antibiotics and bacteriostatics, expression of such plant and bacterial cell wall and polysaccharide hydrolyzing genes could also be a kind of active defense against all competitors, to which plants belong as well.

4.3 Asexual vs. Sexual development (w/o secondary metabolism)

The performed transcriptome analyses give a deep insight into gene regulation during *A. nidulans* asexual and sexual development. Thereby, special interest and effort is spent on identification of novel not yet identified factors, which possess development-specific expression pattern and might have impact on the regulation of developmental processes. Our transcriptome analyses reveal that light stimulates gene expression generally. The number of upregulated genes at asexual stage is much higher compared to early sexual stage. Independently of whether genes required for the preparation of sexual development are already expressed at vegetative stage or not, phenotypical similarity of vegetative and early sexual cultures is reflected on genetic level. Even the number of downregulated genes is higher at light exposure, demonstrating that gene activation and derepression are more intensive and take place much earlier in light. Cultures growing in darkness are delayed in gene regulation. Thereby, *A. nidulans* generates a temporal separation of asexual and sexual propagation to avoid most likely running out of energy required for a very energy consuming spore formation process.

Especially genes for transcriptional- and translational machinery are activated at both asexual stages and both late sexual stages. This is surprising for sexual differentiation since the majority of transcriptional and translational genes are merely expressed when sexual development is initiated long ago and even cleistothecia are formed but not mature. Either initiation of sexual development is performed to a high extend already at vegetative stage or differentially expressed genes got lost at a 96 h consuming sexual differentiation with 24 h analyzed stages. Upregulation of the corresponding categories take place and might be essential for additional processes like formation of secondary metabolites and transporters.

The highest numbers of downregulated genes were present at the end of asexual and sexual development, respectively. Both developmental pathways reached to the final stage and gene expression needed in the asexual and sexual development were not needed at the end of these stages and were subsequently downregulated. There, especially genes for transcription

and translation are affected. Transcriptional– and translational machinery is shut down since asexual and sexual structure formation is finished and the fungus has to save energy finally.

For the direct comparison of both developmental stages at 24 h and 48 h only asexual and sexual specific genes were used. Thereby, numbers of asexual specific genes expressed at both asexual stages are higher than at the corresponding sexual stage. Ratio between up- and downregulated genes is different at asexual stages. Whereas the numbers of downregulated genes are higher at sexual stage much more genes are upregulated at asexual stages. Thus, regarding its proliferation and survival *A. nidulans* seems to have priority for asexual cycle driven by light. Even in soil, remaining light enters deep soil layers and has impact on gene expression. The fungus prepares for sexual sporulation within the first 48 h but always has asexual priority. First high sexual expression burst is detectable after 72 h when asexual cycle is completed long ago. This preference is the central reason for *A. nidulans* asexual cycle being performed much faster. It is important for the fungus to answer to changing environmental conditions quickly. Asexual spores are ready for proliferation in around 48 h. Maturation of sexual ascospores is finished after around 96 h. They remain within the soil as persistence units.

4.3.1 Identification of stage specific genes synchronizing *A. nidulans* development

Development in *A. nidulans* is a light-dependent process. Light required for initiation of asexual development is detected by light receptors like phytochrome (*fphA*), cryptochrome (*cryA*) and opsin (*nopA*) (Blumenstein, *et al.*, 2005, Lafon, *et al.*, 2006, Bayram, *et al.*, 2008, Purschwitz, *et al.*, 2008). Deletion in the corresponding genes results in developmentally defective phenotypes. Within this study seven differentially expressed proteins with connection to light sensing are present. Some of them have never been described before and have to be identified manually since sequence information and domain description are incomplete on some platforms. Opsin, encoded by *nopA*, is among these light sensing proteins. Like the other light sensing candidates, opsin is already expressed at vegetative stage, demonstrating that *A. nidulans* detects light already at vegetative stage and might be prepared for asexual differentiation to a much higher extend. Interestingly, expression of five of seven light sensing proteins is inhibited at early sexual stage (S24). *A. nidulans* reacts on the absence of light and represses expression of light sensory complexes in darkness. An interruption of the signal transduction cascade essential for the proper initiation of developmental cycles is the consequence. Whether *A. nidulans* would exhibit a similar expression pattern for the light sensory proteins in case of vegetative cultures, growth in

darkness remains an open and interesting question. Expression of only two genes is decreased at S48, suggesting the presence for a further signal within the signal cascade, which might have another origin apart from light. Six of seven genes are inhibited at late sexual stage (S72). At the end of sexual development (S96) expression of only one light sensory gene is inhibited. *A. nidulans* prepares for another developmental cycle and the light sensory apparatus for detection of a possible change in light conditions. Expression of these genes might also be restricted to ascospores so that they are prepared for quick light reception, initiation of vegetative growth and asexual proliferation finally.

Light reception has direct impact on transcription of genomic areas required for developmental initiation and processes in fungi. One prominent example of transcriptional control in *A. nidulans* is CryA. CryA senses UVA and blue light and represses sexual development by regulating factors such as VeA, NsdD, and RosA (Bayram, *et al.*, 2008, Bayram, *et al.*, 2008). Deletion of *cryA* results in abnormal Hülle cell formation in submerged vegetative cultures and decreased response to light. In consequence *A. nidulans* development is transposed to sexual development generally. Transcriptional control is also mediated by histone modification. Histone acetylation is performed through histone acetylases and results in a release of histones from DNA, which can be transcribed finally. Thereby, histone acetylation can be restricted to defined genomic areas, ensuring a directed transcriptional control. Fourty genes encoding for proteins with GCN5-related N-acetyltransferase domain are present in *A. nidulans.* For some *ngn* genes, encoding for putative GCN5-related N-acetyltransferases, an impact on toxin production in co-cultivation with a *Streptomycetes* ssp. could be shown (Nutzmann, *et al.*, 2011). Since secondary metabolite production is closely connected to developmental processes, some of these histone acetylases most likely possess developmentally regulatory properties as well. Eight GCN5-related N-acetyltransferases reveal differential expression at the examined stages. One shows already a high expression at vegetative growth (AN5053), revealing transcriptional activation has already been finished at an earlier point of time. In transition to asexual development only one histone acetylase (AN10238), different from the vegetative histone acetylase is expressed. Either *A. nidulans* already initiates release from genomic areas crucial for asexual development at vegetative stage or at an early stage of asexual stage. Histone release from DNA might generally be performed quickly since the organism also has to react on changing environmental conditions within a short time. Interestingly, five sexual specific genes (AN0969; AN10234; AN5330; AN6411; AN7944) and the already at vegetative stage upregulated histone acetylases are increased in expression at early sexual stage (S24).

A. nidulans initiates transcriptional activation most likely for sexual specific genomic areas. During the transcriptional initiation of developmental processes, mediated by histone acetylases, development specific secondary metabolite clusters are mostly co-activated as well.

In summary, lacking expression of light sensing proteins at S24 results in an apparent derepression of histone acetylases in darkness. Released sexual specific DNA regions are transcribed and genes required for a complete sexual cycle are expressed consequently. Basically *A. nidulans* might be prepared and initiated for sexual differentiation before S24, but most likely requires the observed transcriptional control through histone modification for a proper sexual development.

DNA release affects transcription of several genes. Among them are transcription factors, which control expression of defined genes and gene clusters. Some transcription factors are developmental regulators essential for asexual development like for instance *brlA*, *wetA* and *medA* (Clutterbuck, 1969, Boylan, *et al.*, 1987, Adams, *et al.*, 1988, Busby, *et al.*, 1996) and for sexual development like *nsdD*, *veA*, *velB* and *imeB* (Han, *et al.*, 2001, Bayram, *et al.*, 2008, Bayram, *et al.*, 2008). Numerous regulators have been described and characterized in the past. Among countless comprised transcription factors in *A. nidulans*, we could identify development specific uncharacterized transcription factors. For 24 h and 48 h stages, one bZIP asexual specific and nine Zn(2)-Cys(6) DNA binding domain transcription factors were detected. In contrast, only one Zn(2)-Cys(6) transcription factor is transcriptionally present at S48. Since they are development specific expressed, share sequence similarity to known regulatory genes or contain at least DNA binding domains, systematic deletion could reveal novel developmental regulators.

Development in *A. nidulans* depends on balanced and controlled reactive oxygen species (ROS) level. Therefore, the fungus possesses specific enzymes like superoxide dismutases (SOD), NADPH oxidases (NOX) and catalases, which trigger ROS balance during development. (Navarro, *et al.*, 1996, Kawasaki, *et al.*, 1997, Oberegger, *et al.*, 2000, Kawasaki & Aguirre, 2001, Lara-Ortiz, *et al.*, 2003, Sato, *et al.*, 2009). The genes encoding for the corresponding enzymes are specifically expressed. The putative cytosolic Cu/Zn superoxide dismutase (AN1131) is the only SOD, which is already present at early asexual development. Although Ruger-Herreros and co-workers (2011) described AN1131 as repressed by light at shorter light exposure, it is light-dependently upregulated at both stages. *A. nidulans* represses expression of AN1131 at an early asexual stage before A24 since it is most likely crucial for triggering conidiophore formation and/or maturation processes. In

contrast, *sodM* (AN0785) encodes for another SOD specifically expressed at sexual stage (S48). Since both SODs are development specific expressed, deletions would show their developmental relevance and maybe result in developmentally defective phenotypes.

4.3.2 Morphological similarity between vegetative and early sexual development reflects the genetic and metabolic processes in *A. nidulans*

Generally, direct genetic impact on metabolic level is easy to show for single genes and their gene product. This procedure becomes much harder when whole transcriptomes and metabolomes of different stages are analyzed. On the one hand both generate huge datasets and one the other hand correlations are much harder to find and interpret, since many physiological functions are still unknown in *A. nidulans*.

Anyway, for the first time transcriptomics and metabolomics were done on complete *A. nidulans* development. For both methods stage specific clusters were identified through 1D-SOM processing. The numbers of transcripts and intracellular metabolites correlate with the length of the different cycles. In contrast, more metabolites are secreted at vegetative stage compared to both late developmental stages (A48 and S96). We have two possible explanations. *A. nidulans* might be more active regarding transports, since vegetative growth takes place in submerged cultures and a direct uptake is much more effective/faster, or the fungus retains more metabolites inside to protect its mature spores. This is going to be discussed later.

Transcriptome and metabolome reveal an impressive finding. Similarity of vegetative and early sexual cultures phenotypes also arises on genetic and metabolic levels. In transcriptome, genetic expression in the transition of vegetative stage to early sexual stage is basically low. In intracellular metabolome *A. nidulans* contains numerous markers (cluster 2 in 1D SOM), which are present at both vegetative and early sexual stage. In contrast, it possesses only few markers (cluster 14 - 19) which are present in early sexual stage and the following three sexual stages. Thus, *A. nidulans* has to be very active in the transition of S24 to S48 on metabolomic level. We hypothesize that *A. nidulans* requires a delay of sexual development in order to assure proper sexual induction. This might also be an effect of a preference for asexual propagation.

4.4 Secondary metabolism during *A. nidulans* development

Secondary metabolites are of importance for many organisms to survive in their environment. Among them are toxins, antibiotics, bacteriostatics and apoptosis-inducing compounds. An active protection is best defense. Therefore, *A. nidulans* has to secret secondary metabolites. The fungus activates expression of numerous transporters homologous to known secondary metabolite transporters in other organisms at both asexual and both late sexual stages. The increased number of expressed transporters is temporally synchronic with the upregulation of secondary metabolite genes, revealing an exact coordination of secondary metabolite formation and transport. For instance, *aflT* encodes a toxin efflux pump in *A. fumigatus*, which was shown to be strictly regulated and amongst others activated in response to the mycotoxin amphotericin B (da Silva Ferreira, *et al.*, 2006, Gautam, *et al.*, 2008). It is most likely that transporters like *aflT* have multiple substrates which have to be translocated, since the number of potential ABC or MFS multidrug transporters is less than the amount of already identified foreign and self-made compounds like mycotoxins and bacteriostatics the fungus is confronted with. Most secondary metabolite genes are expressed at both asexual stages and S72. Even expression of these secondary metabolite genes is strictly coordinated with the formation and maturation of sexual structures. Expression is decreased until micro-cleistothecia formation. Thereafter, secondary metabolite pathways are activated, suggesting directed protection of the fungal proliferation units. Secondary metabolite gene expression is already increased at early asexual stage since the basis for conidiospores is already present and complete maturation is finished within a short time.

4.4.1 Early secondary metabolites in *A. nidulans* development

4.4.1.1 Discovery of linoleic and oleic acid derived *psi*-factors characteristic for the early sexual development of *A. nidulans*

A. nidulans development is closely connected to formation of *psi*-factors, combining different steps catalyzed by already described dioxygenases like PpoA and PpoC (Champe, *et al.*, 1987, Champe & el-Zayat, 1989, Mazur, *et al.*, 1990, Mazur, *et al.*, 1991, Calvo, *et al.*, 2001, Tsitsigiannis, *et al.*, 2004, Garscha, *et al.*, 2007, Andreou, *et al.*, 2009, Brodhun, *et al.*, 2009, Brodhun, *et al.*, 2010). Defined polyunsaturated fatty acids are not only basis for membrane but also for *psi*-factor formation. Required genes are already expressed at vegetative stage. Among them are the already verified fatty acid synthase (FasA, alpha subunit), a delta-9-stearic acid desaturase (SdeB) and two oleate delta-12 desaturases (An2; OdeA) (Brown, *et al.*, 1996, Wilson, *et al.*, 2004, Hoffmann, *et al.*, 2007). Obviously, *A.*

nidulans already creates a basis for *psi*-factor synthesis at vegetative stage.

The only transcriptional exception is *foxA* that is upregulated at S72 when each other gene involved in polyunsaturated fatty acid synthesis is not expressed long ago. Why *A. nidulans* increases expression of *foxA* required for beta-oxidation as recent as S72 remains speculation. Maybe ascospore formation requires another transcriptional activation to ensure the elevated membrane formation during cleistothecia and ascospore formation.

Both *odeA* and *an2* show almost similar expression pattern. Beside their vegetative upregulation, expression is still increased at early asexual and sexual development, suggesting that their presence might regulate asexual and sexual structure formation and is not only important for the decision of the development induced by light. *an2* is again present at late sexual stage when the fungus prepares for another development cycle. Deletion of *odeA* is most likely similar to the state when *odeA* is less or even not expressed. Deletion of *an2* was not generated yet and might have massive impact on *A. nidulans* development since it participates in the production of precursors essential for *psi*-factor formation.

Some findings about characteristic ratios of linoleic- and oleic acid derived *psi*-factors during development are already known and well described (Tsitsigiannis, *et al.*, 2004). Especially 8-HOE:8-HOD ratio is characteristic for induction of asexual or sexual differentiation, respectively. In asexual cultures 8-HOE:8-HOD ratio is transposed to 8-HOE. Although Tsitsigiannis and co-workers analyzed cultures grown for five days in darkness, our result is consistent with their observation. 8-HOE:8-HOD ratio is around 3-fold higher for both comparisons of A24 and S24 as well as for A48 and S48. At late sexual development S72 and S96 8-HOE:8-HOD ratio reaches asexual level, which might be consistent with the observations that *A. nidulans* secondarily forms asexual structures when sexual development is already completed. *A. nidulans* prepares most likely for another round of asexual differentiation and in a much faster way to proliferate its genetic material as a quick response to changing environmental conditions. Interestingly, even at vegetative stage 8-HOE:8-HOD ratio is characteristic for asexual stage, pointing at *A. nidulans* preference for asexual development and proliferation. This might be an additional reason for the speed of asexual development and structure formation performed in *A. nidulans*.

10-HOD accumulates in *ppoC* deletion strains defective in asexual development. (Garscha, *et al.*, 2007). In this study 10-HOD concentration is highest at early sexual stage (S24) and decreases massively in the transition of S24 to S48. Thus, 10-HOD represents a marker specific for early sexual development and might also be required for a proper ratio of asexual to sexual spores in *A. nidulans*.

Furthermore, even 8-HOD:10-HOD ratio exhibits developmental specificity in *A. nidulans*. We found 8-HOD:10-HOD ratio almost similar at A24, A48, S24 and Veg, whereas it is transposed in 8-HOD direction at S96. This is in consequence consistent with the findings of Garscha and co-workers (2007), revealing that the absence of *ppoA* results in a massive decrease of 8-HOD, whereas the amount of 10-HOD is not effected. Here *ppoA* expression is low at early sexual development (S24) and increases in the course of sexual development that causes the mentioned transposed ratio of 8-HOD:10-HOD in 8-HOD direction.

Even more interesting is the finding that the oleic acid derived 5,8-DiHOE is only present in early sexual development S24 specifically. Its concentration decreases within 24 h massively and is not detectable at late sexual development. About ten years ago Calvo and co-workers (2001) could neither detect 5,8-DiHOD nor 5,8-DiHOE in their wild type grown for 3d at 37°C in darkness or light, respectively, what might be due to the fast decreasing concentration of 5,8-DiHOE and the less sensitive UPLC/HPLC measurement devices which become more and more accurate and sensitive during the last years. At least they could detect 5,8-DiHOE in the Δ*odeA* strain that shifted towards sexual development.

In summary, we could identify novel development specific accumulations and ratios for *psi*-factors in *A. nidulans*. 8-HOE:8-HOD concentration at vegetative stage already achieves asexual level, whereas 10-HOD and 5,8-DiHOE came out as *psi*-factors specific for early sexual development. Their accumulation might be essential for *A. nidulans* to initiate sexual development.

4.4.2 Delayed secondary metabolites during *A. nidulans* development in darkness

Our studies revealed several secondary metabolites produced during both asexual and sexual development of *A. nidulans*. Each of the emericellamide species found in high contents at both asexual stages is also present in later phases of sexual development with at least 24 h delay. This could also be observed for ST and anthranilate. As described previously, *A. nidulans* also induces asexual differentiation during sexual development secondarily. The appearance of secondary metabolites with high asexual concentration at sexual stages is most likely an accompaniment and a consequence of secondary asexual differentiation. Thus, secondary metabolite like emericellamide A/C/E, ST and anthranilate might be asexual specific secondary metabolites consequently.

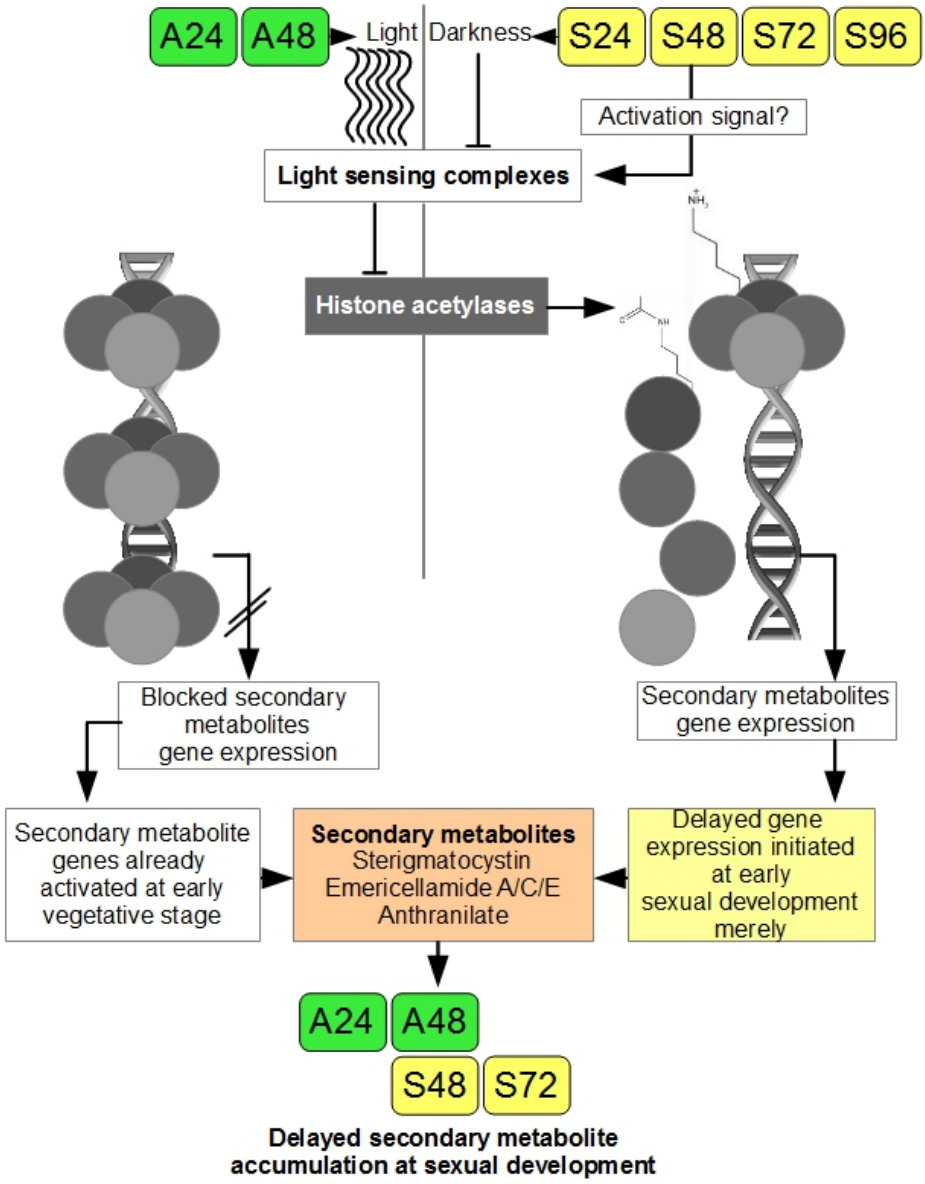


Figure 17: Derepressed histone acetylases activate delayed secondary metabolite production at early sexual differentiation of *A. nidulans* in darkness. *A. nidulans* genome comprises genes, which encode several putative light detecting complexes. Interestingly, five complexes are specifically repressed at early sexual stage. Among them are AN2581, AN3214, *nopA*, AN4113 & AN8959. Their activation might result in a derepression of GNAT-type acetyltransferases encoded by *ngn* genes partially described to be involved in bacterial-induced regulation of toxin production in *A. nidulans* (Nutzmann et al. 2011). We found an accumulated expression of five histone acetylases encoded by *ngn3*, *ngn10*, *ngn17*, *ngn28* & AN5330 at early sexual development. Acetylased histones release from DNA and transcription of secondary metabolite genes as for sterigmatocystin (ST), emericellamide species or anthranilate is initiated. Since they reveal a high concentration at early asexual point of time A24, when non of them is detectable at S24, production might already be prepared and activated at vegetative stage whereas it is merely initiated during sexual differentiation at S24 resulting in a delayed synthesis of ST, emericellamide species and anthranilate at sexual cycle. On the other hand, *A. nidulans* safes energy by repressing secondary metabolite gene expression at A24 when asexual differentiation is almost completed and these compounds are already present.

The preparation for the formation of asexual specific secondary metabolites is most likely already initiated at vegetative growth. Nutzmann and co-workers could show that GNAT-type histone acetylases encoded by *ngn* genes are involved in secondary metabolite production in

A. nidulans (Nutzmann, *et al.*, 2011). Our data reveal that several GNAT-type histone acetylases might also be involved in the delayed appearance of secondary metabolites during sexual development in darkness. Thereby, activity of these histone acetylases might be repressed by putative light sensing complexes encoded by AN2581, AN3214, *nopA*, AN4113 and AN8959 that we found upregulated at asexual development. These sensory complexes were downregulated at early sexual development of *A. nidulans*. Their downregulation leads to a derepression and thus an activation of *ngn10*, *ngn17*, *ngn28* and AN5330 at early sexual development. Acetylated histones releases from defined DNA regions required for the formation of specific secondary metabolites like emericellamide species, ST and anthranilate.

4.4.3 Late secondary metabolites in *A. nidulans* development

4.4.3.1 Transcriptionally active secondary metabolite clusters without product in *A. nidulans*

Beside the previously described defense mechanisms, *A. nidulans* comprises genes concentrated in gene clusters crucial for synthesis of numerous potential toxic compounds directed to other competitors. Some of them are silent and not expressed. Consequently, the corresponding secondary metabolite is not produced. A prominent example is orsellinic acid, which was found to be synthesized in co-cultivation with *Streptomyces hygroscopicus*. (Schroeckh, *et al.*, 2009, Sanchez, *et al.*, 2010). In contrast to the described expressional absence of *ors* genes during development, we found some of them expressed at *A. nidulans* development. Despite of the expressional activity, orsellinic acid could not be detected at the analyzed stages. Presumably it is instable or appears within a short time period.

As for orsellinic acid, monodictyphenone synthesis could also not be detected although the *mdp* gene cluster reveals high transcriptional activity. 10 of 13 *mdp* genes are specifically upregulated at late sexual phases S72 and S96, concluding monodictyphenone being a sexual specific secondary metabolite. Three *mdp* genes are not expressed during sexual development and are most likely responsible for the monodictyphenone synthesis not being performed during standard growth conditions. Among them are a regulatory protein (*mdpA*), a protein with homology to scytalone dehydratase (*mdpB*) and the polyketide synthase (*mdpG*). MdpA is a member of the monodictyphenone secondary metabolite biosynthesis gene cluster, similar to aflatoxin coactivator AflJ and O-methyltransferases. It was shown that MpdA is required for biosynthesis of monodictyphenone, a prenyl xanthone precursor (Chiang, *et al.*, 2010, Sanchez, *et al.*, 2011). MdpA most likely activates the polyketide synthase MdpG crucial for the composition of monodictyphenone. Thus, the

lacking activation of *mdpA* has the potential to block monodictyphenone synthesis although 10 *mdp* genes are already activated and wait for the missing part for a complete monodictyphenone synthesis

Extensive studies have been done on penicillin production in *A. nidulans* (MacCabe, *et al.*, 1991, Brakhage, *et al.*, 1994, Martin, *et al.*, 1994, Litzka, *et al.*, 1996, Sprote, *et al.*, 2008). Although numerous genes known to participate in penicillin production are expressed, penicillin could not be detected. This was similar to the asexual specific asperfuranone synthesis, where an increased expression of most participating genes was present, while asperfuranone was not detectable.

In summary, a quick decay might be a reason for a secondary metabolite which has not been detected. Although several genes for secondary metabolite production are synthesized, none of the previously mentioned compounds could be detected. Some might need an external signal for a defined expression pattern necessary for their synthesis as described for orsellinic acid. *A. nidulans* may express some genes of a cluster to be prepared and able for a quick answer to an external signal.

4.4.3.2 Novel insights into intra- and extracellular kinetics of secondary metabolites in *A. nidulans* development.

In contrast to the observed expressed secondary gene clusters without product, *A. nidulans* reveals also gene cluster expression with secondary metabolite production during development.

Although *apt* genes within the asperthecin cluster are reported only to be expressed in the *A. nidulans* Δ*sumo* strain (Szewczyk, *et al.*, 2008), *apt* genes expression is detectable at the analyzed stages. It correlates with the temporal appearance of asperthecin at late sexual stage. Thereby, asperthecin is localized in ascospores at late sexual development and has most likely protective properties. As a direct precursor of asperthecin, emodin is already present at S48. *A. nidulans* increases expression of *aptC* responsible for emodin/asperthecin conversion and the concentration of emodin decreases, whereas asperthecin level increases finally. This example clearly demonstrates connection between gene expression and its influence on metabolome level.

Among the sexual specific secondary metabolites are also emericellin and shamixanthone, which share synthesis genes with monodictyphenone (Chiang, *et al.*, 2010, Sanchez, *et al.*, 2011). Monodictyphenone could not be detected in this study. Interestingly, although the polyketide synthase (*mdpG*) also required for shamixanthone and emericellin

synthesis is not expressed, shamixanthone and emericellin is synthesized during sexual development. Synthesis of emericellin from variecoxanthone mediated by the upregulated *xptA* exhibits a clear temporal correlation between genetic expression and its appearance, whereas emericellin to shamixanthone conversion is not supported by a constitutive *xptC* expression. Even a steady low expression of *xptC* might be sufficient for emericellin to be converted to shamixanthone. A confirmation of this hypothesis based on the fact that emericellin concentration is similar at each analyzed point of time it could be detected (S48/S72/S96). In contrast, shamixanthone concentration is elevated from S48 to S96 when it achieves its maximum, although *xptC* expression is constant. Endocrocin or endocrocin-9-anthrone, which are precursors of asperthecin and monodictyphenone, could not be detected at the analyzed stages which implies that these compounds possess a short half-life and are presumably immediately converted into monodictyphenone, asperthecin and emodin or other still unidentified end products that might be much more effective antimicrobial substances compared to endocrocin or endocrocin-9-anthrone finally. Although their function is still elusive, they might also be protective for sexual structures since they are present at middle and late sexual development in *A. nidulans*.

In contrast to the sexual specific secondary metabolites, emericellamide A/C/E accumulate at both asexual stages, whereas they are absent from vegetative stage. Nevertheless, this is the first time emericellamide species could be isolated from sexual cultures.

Beside secondary metabolites, which remain in the intracellular region like asperthecin and emodin, *A. nidulans* also reveals development-specific secretion of several secondary metabolites. Although the fungus synthesizes three emericellamide species A, C and E, only emericellamide C is secreted. There are two explanations for the lacking presence of emericellamide A/E in the extracellular environment. Either they are generally not secreted within the course of a complete asexual cycle and appear much later as comparisons with former data suggest, or they are secreted much earlier and are more instable so that they are not detectable at the A48 extracellular metabolome. Anyhow, emericellamide C transport is constitutive. Due to the higher intracellular emericellamide C content at asexual stages, its extracellular accumulation is highest at asexual stage, respectively. Transport of emericellamide A and E must be switched off since both compounds are present in intracellular metabolome at A48. The extra- and intracellular contents of emericellamide C are relatively high.

As a precursor of aflatoxin, ST is well characterized in *A. nidulans* (Yu & Leonard, 1995, Brown, *et al.*, 1996, Bok, *et al.*, 2006). Our data show that ST production correlates with its gene expression pattern. *A. nidulans* does not accumulate ST when *stc* genes are not expressed. Its expression is present at asexual but absent at sexual 24 h stage. Accordingly, ST is produced at early asexual stage. Basal expression of *stc* gene seems to be sufficient for ST production at sexual development. Although *stc* genes are merely upregulated at S72 ST, its content is already elevated at S48. In case if *stc* genes are not marked for upregulation in the tables, this does not necessarily mean that they are not expressed. In comparison to vegetative stage, S48 *stc* genes are constitutively expressed, meaning that they are expressed as strong as at vegetative stage. Since vegetative as well as asexual cultures were grown in light *stc* genes might be expressed but their posttranscriptional modification might be different than these of sexual culture not being exposed to light. Posttranscriptional modification might be an explanation for ST accumulation at S48 in *A. nidulans*. Despite the high expression of *stc* genes at late sexual stage (S96), ST content decreases massively.

Although anthranilate is an amino acid, it is precursor and basis for the production of pigments and repellents (Wang & De Luca, 2005). As protective metabolite, UV-absorber, mold inhibitor and repellent, anthranilate has secondary metabolite function/properties and is therefore integrated in this paragraph. Its production in *A. nidulans* is light-dependent and almost restricted to asexual stage. Anthranilate secretion takes place at asexual development when its intracellular content is highest. Since *A. nidulans* has to protect against damage from UV radiation, it synthesizes anthranilate specifically at light induced asexual development A24 and A48. Anthranilate might have multiple functions in *A. nidulans*. Beside repellent and UV-absorber it is most likely an inhibitor of fungal growth additionally. Therefore, anthranilate is thought to be another protective metabolite during *A. nidulans* development in light.

4.4.3.3 Novel developmental-specific secondary metabolite cluster in *A. nidulans*

Identification of new secondary metabolites in fungi are of enormous economical interest since numerous compounds possess antibiotic properties and are very useful for the struggle against pro- and eukaryotic organisms. *A. nidulans* produces several secondary metabolites partially described and analyzed in this work. Beside the understanding of gene regulation and their direct impact on synthesis, identification of novel putative secondary metabolite gene clusters is important. Therefore, several strategies had been applied (Bok, *et al.*, 2006, Szewczyk, *et al.*, 2008, Bok, *et al.*, 2009, Chiang, *et al.*, 2010, Sanchez, *et al.*, 2011,

Yeh, *et al.*, 2012). We used transcriptomics and manual search for gene clusters that reveal on the one hand equal transcriptional regulation and on the other hand comprise genes coding for proteins, which have the potential to participate in secondary metabolite formation.

In summary, we identified three yet undescribed gene clusters with connection to secondary metabolism in *A. nidulans*. Dityrosine, which protects ascospores in yeast, might also be synthesized in *A. nidulans.* AN2606 codes for a dityrosine biosynthesis protein and is activated at late sexual development, revealing a connection to ascospore protection. AN2606 – AN2611 comprise two overlapping clusters sharing two genes AN2608 (tricarballylate dehydrogenase) and AN2609 (O-methyltransferase). Function of both genes comprising the second part of the cluster remains elusive. In contrast to the first cluster part, both are specific for asexual stage.

Hormones regulate developmental processes characteristic for all organisms. They are also present in *A. nidulans* as described for *psi*-factor synthesis. Steroids are also a group of hormones. Steroid synthesis in *A. nidulans* seems to be comprised and mediated by the AN5417 – AN5422 cluster. Beside the steroid monooxygenase a beta-lactamase is activated at both asexual and sexual stage. Steroid synthesis might be restricted to sexual stage since a FAD/FMN dehydrogenase and a protein without known domain are specifically present. Deletion of the steroid monooxygenase and/or the beta-lactamase might generate an interesting developmental phenotype answering the question whether this cluster really is responsible for the synthesis of another hormone in *A. nidulans*.

A. nidulans pigment synthesis is dilated by another cluster (AN8433 - AN8438) activated at both asexual and late sexual development. Both the AN8433 encoded luciferase-like protein required for the synthesis of bioluminescent compounds as well as the AN8435 encoded tyrosinase, known to catalyze pigment synthesis crucial for UV-protection, let presume a novel not yet identified pigment synthesis gene cluster. Late expression at both development cycles supports the assumption for this pigment protecting both conidiospores and ascospores from UV-radiation or further threats.

Interestingly, vegetative specifically expressed gene clusters could not be detected demonstrating the importance for secondary metabolism gene cluster expression and production at development.

4.5 Completed development in *A. nidulans*

4.5.1 Block in translation: Reduced amino acid formation at late development stages of *A. nidulans*

Fungal growth and development requires a lot of energy. Steady uptake and conversion is crucial for a reliable nutritional supply (Hoffmann, *et al.*, 2000, Hoffmann, *et al.*, 2001, Hinnebusch & Natarajan, 2002, Krappmann, *et al.*, 2004). In the course of development nutrient concentration decreases and the fungus suffers from nutrient limitation within a few days.

Transcriptome data demonstrate a broad downregulation of genes in several amino acid pathways at late sexual stages S72 and S96. Downregulation affects cystein/ methionine -, alanine/aspartate/glutamate -, glycine/threonine/serine-, valine/leucine/ isoleucine – and lysine – pathways. In contrast, phenylalanine/tyrosine/tryptophane-pathway downregulation is also present at late asexual stage A48, proposing the consequence of a decreased requirement for amino acids often serving as precursors for secondary metabolites like pigments protecting the fungus for instance from UV radiation. Conidiospore pigment formation is finished at A48 and the need for phenylalanine, tyrosine or tryptophane might be reduced. Downregulated genes seem to have no direct impact on phenylalanine concentration since it exhibits increased contents at each analyzed stage. Nevertheless, phenylalanine itself might have impact on regulations of the corresponding genes. Phenylalanine levels most likely increase when the requirement for pigments and other phenylalanine-derived secondary metabolites is decreased. In this case phenylalanine could inhibit expression of synthesis genes consequently.

Phenylalanine is the only amino acid that accumulates at late sexual stage. Generally transcriptomic findings agree with the amino acid accumulation. Even at late asexual stage only three amino acids reveal an elevated content, exhibiting the decrease of amino acids not being restricted to S96. Beside phenylalanine and asparagine, valine is present at A48. Asparagine provides glycosylation site for protein modification. *A. nidulans* might need asparagine for the formation of modified proteins. This behaviour is detectable for 24 h and 48 h stages of both developmental cycles, suggesting a probable requirement for proteins to be glycosylated, which might be relevant for development finally.

An additional hint for a specific transcriptional initiation at early sexual stage, beside the observed activation of numerous putative histone acetylases at S24, is the specific accumulation of methionine at S24. Its derivative S-adenosylmethionine (SAM) also serves as a methyl donor required for transcriptional control and regulation. Hints for directed histone

methylation and acetylation have never been described for the early sexual stage of *A. nidulans*.

Threonine and lysine are two central amino acids providing potential protein modification sites (Sadoul, *et al.*, 2008, Yang & Seto, 2008, Brennan & Barford, 2009). Threonine side chain OH^- residue in proteins can be phosphorylated, whereas lysine NH_3^+ side chain group in proteins can be modified in many different ways. Modification often has influence on protein and enzymatic activity finally. Threonine already accumulates at vegetative stage. Protein phosphorylation might be of central meaning not only for vegetative growth but also at both early 24 h stages. In contrast, lysine is not required for protein modification compared to threonine. But *A. nidulans* seems to need lysine at sexual differentiation for a longer time since it is still present at S48. Lysine and threonine demonstrate that protein synthesis mainly takes place at vegetative and early asexual and sexual stage. Initiation of development requires a broad protein pool for numerous pathways and regulatory processes. Thereafter, protein synthesis decreases and the fungus does not need an intensive formation of proteins anymore.

In contrast to other detected amino acids, ornithine is a non-proteinogenic amino acid which is a precursor of several polyamines like spermine, spermidine and putrescine (Weber & Miller, 1981, Lawrence & Stephen, 2003). Beside their important role in growth and differentiation control, *A. nidulans* might use them as tactical weapon against competitors in soil as well. Some of them are toxic and induce apoptosis like putrescine in neighbor organisms. That these compounds might be restricted for *A. nidulans* growth and differentiation in soil is supported by the accumulation of ornithine at early sexual stage. The fungus already accumulates ornithine at vegetative stage and might start to produce polyamines, which the fungus requires for proper induction and control of development basically.

The massive downregulation of amino acid pathways is accompanied by a broad decreased expression of translational genes. Numerous translational initiation factors and ribosomal genes for the composition of the ribosomes are affected. Thereby, downregulation is mainly detectable at a later stage of sexual development S96 when amino acid pathways are already decreased. Here, downregulation is already present at S72, suggesting a possible regulatory feedback of amino acid regulation on translational machinery.

Does *A. nidulans* induce decreased transcription of amino acid metabolism genes, less amino acid production and apoptosis since it is at the end of development cycle independent of nutrient starvation within the medium or due to a nutrient starvation state? Another

transcriptome and metabolome on YAG complex medium for the same stages would give an answer. Nutrient starvation is something the fungus is also confronted with in its environment. Therefore, the fungus developed several strategies to avoid and compensate nutrient starvation periods.

4.5.2 Programmed cell death / apoptosis in the end of *A. nidulans* development

Apoptosis is a process of programmed cell death that occurs in multicellular organisms like fungi (Horikoshi, *et al.*, 1999, Madeo, *et al.*, 1999, Wu, *et al.*, 2002, Balzan, *et al.*, 2004, Wissing, *et al.*, 2004, Semighini, *et al.*, 2006). In general, apoptosis confers advantages during an organism's life cycle.

Obviously, *A. nidulans* induces apoptosis at both asexual and sexual development. Thereby, specific apoptosis-inducing genes are expressed and each appears at only one point of time specifically, exhibiting an exactly controlled initiation of apoptosis in *A. nidulans*. Strikingly, induction of apoptotic gene expression starts around 24 h before asexual and sexual cycle is accomplished, suggesting an exact induced and temporally coordinated programmed cell death in course of an aging fungus.

Since apoptosis has impact on numerous physiological processes in organisms, it could also be responsible for the massive downregulation of amino acid metabolism and translation observed at late sexual development. However, the observed broad downregulation is not present in asexual cultures. Thus, the downregulation is most likely apart from induction of apoptosis in *A. nidulans* and an additional effect of aging.

Apoptosis is accompanied by ROS formation. However, the importance of ROS as signal is not restricted to induction of programmed cell death but spans regulation of diverse cellular processes such as defense, growth and development. Numerous enzymes like NADPH oxidase (NOX), superoxide dismutase (SOD) and catalase balance ROS levels in *A. nidulans*. Signalling for induction of development and apoptosis would be indirectional without these enzymes. We could identify four uncharacterized putative NOXs (AN0773; AN10893; AN4906; AN8683) and two putative SODs (AN1131; AN5148) that might participate in triggering apoptosis and/or development. Since apoptosis can be induced punctually and may be restricted to defined mycelia regions, it is hard to interpret a correlation between the appearance of apoptosis-inducing factors and the activation of ROS balancing enzymes. Nevertheless, induction of apoptosis is detectable and reveals an exact temporal coordination during development of *A. nidulans*.

The meaning of apoptosis for *A. nidulans* might be to escape from an area in soil nutrients had been extracted from and might not be sufficient for another developmental round. Thereby, the fungus is independent from the next generation of spores germinating in the direct neighborhood, since the dead fungal material could be used as external energy source finally. This behaviour is similar to this of female spiders sacrificing themselves for a successful start of their offspring.

4.5.3 Preparation of *A. nidulans* spores for abiotic and biotic stress

A. nidulans has to face several stressors like ROS, xenobiotics, mycotoxins and fungicides generated by different biotic and abiotic factors. ROS generation is mediated by UV radiation, inappropriate ROS balance inside an organism or ROS attacks through other organisms. The fungus comprises numerous enzymes that convert ROS either into a compound that is not relevant for ROS level or they adjust ROS balance generally. This study reveals additional uncharacterized and partially not identified ROS balancing enzymes. Beside two NOXs (AN0773; AN10893) already present at vegetative stage, *A. nidulans* shows another two NOXs (AN6477; AN8969) at cleistothecia and ascospore formation and maturation as the verified NoxA (Lara-Ortiz, *et al.*, 2003). Although NOXs are hydrogen peroxide and superoxide generating enzymes, they also participate in and are important for ROS balance in *A. nidulans*. The two vegetative expressed NOX demonstrate that ROS generation is also present at vegetative stage, suggesting hydrogen peroxide- and superoxide levels in vegetative mycelia that already might serve as initial signal for the light-dependent *A. nidulans* development.

Conversion of the very harmful superoxide is also mediated through a still uncharacterized Fe-SOD (AN5148) at vegetative stage in *A. nidulans*. Together with the verified hyphal *catB* (AN9339), *A. nidulans* possesses all enzymes for a successful ROS balancing even at vegetative stage, suggesting not only being able to react on dangerous ROS levels but also to create proper signals for induction of asexual and sexual development.

One of the still uncharacterized NOX (AN4905) expressed at late sexual development is a direct neighbor of another enzyme GstA (AN4906) known to be involved in oxidative stress response and in detoxification of dangerous xenobiotics. Both form a small transcriptional cluster and are present at late sexual stage (S72) (Fraser, *et al.*, 2002). GstA was shown to be important for resistance to diamide. Beside GstA and GstB, *A. nidulans* comprises and expresses two additional putative Gsts (AN10238; AN3299) at sexual stage. Except for late asexual (A48) and early sexual (S24) stages, *A. nidulans* always expresses two

Gsts, revealing a generated protection towards oxidative stress and xenobiotics of abiotic and/or biotic origin. Protection of maturating asexual conidiophore structures at A24 and cleistothecia formation seems to have high priority. Detoxification through Gsts in mature asexual structures or especially in widely spread conidiospores is most likely shut down since *A. nidulans* might elevate ROS concentration in conidiospores for protection towards other organisms until their germination. In contrast, *A. nidulans* follows another strategy in sexual structures. A protective oxidative burst might not be necessary for sexual proliferation since these persistence units remain and germinate with high chance in direct proximity to their relatives that already created an approved living environment.

Detoxification through Gst is not restricted to oxidative stress species but also includes environmental toxins (Nebert & Vasiliou, 2004, Hayes, *et al.*, 2005). *A. nidulans* also faces mycotoxins sent by other competitors in soil. Beside detoxification, the fungus developed another strategy to get rid of such compounds. It comprises and activates numerous transporter genes, mediating toxin secretion and causing resistance.

A. nidulans also developed strategies against predators like insects and worms. Physical injury through predators is a stress factor of biotic origin and causes loss of mycelia material as well as spores compromising fungal propagation. Bacteria incorporate endotoxins within their membrane for protection. Lipooligo- (LOS) and lipopolysaccharides (LPS) are endotoxins localized in the outer membrane of Gram-negative bacteria (Rivest, *et al.*, 2000). Interestingly, *A. nidulans* most likely possesses a similar protection mechanism and expresses two genes (AN6460; AN10601) homologous to LPS- and LOS-synthesis genes. Both homologues contain the same domains also present in the bacterial proteins. Through LOS-synthesis the fungus already generates mycelia protection at vegetative stage. Their formation is continued until both early sexual stages. In contrast, LPS-synthesis is restricted to early asexual stage. In summary, LOS- and LPS-synthesis already starts at vegetative stage and is characteristic for the regarding developmental cycle. At both late stages of development their synthesis seems to be finished. Thus, LOS and LPS are most likely absent from asexual and sexual spores.

The already discussed anthranilate is another compound that might serve as repellent for the fungus. Described as repellent in methylated form, methylated anthranilate could not be detected in this study. *A. nidulans* produces anthranilate at growth in light on the surface. There it needs effective defense from predators that might destroy mass of conidiophores and interrupt proliferation process. Anthranilate is also a precursor for pigment production. Since *A. nidulans* has to protect against damage from UV radiation, it synthesizes anthranilate

specifically at light induced asexual development. Anthranilate might have multiple functions since it is also secreted by *A. nidulans* and serves most likely as much more than for a precursor of a protective pigment.

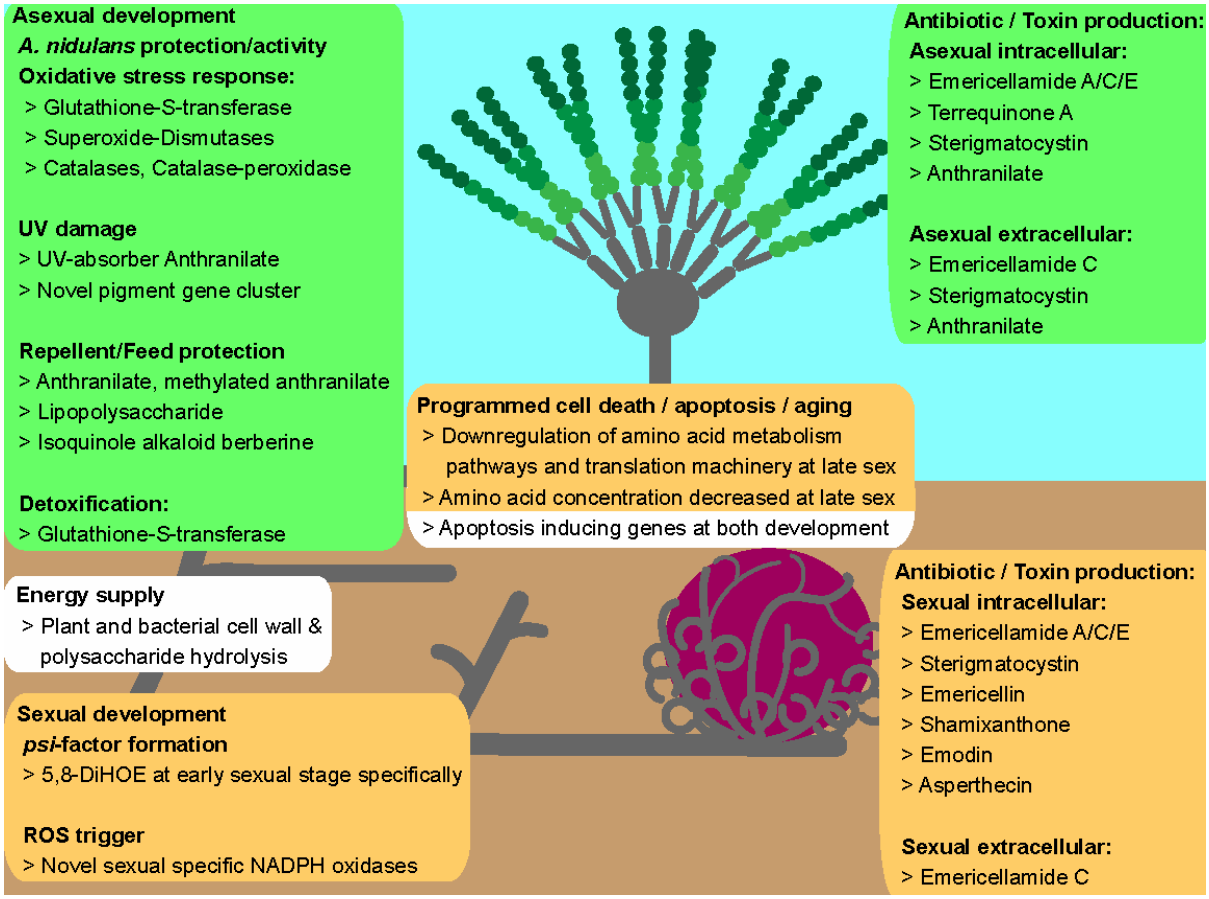


Figure 18: Model figure summarizing *A. nidulans* induced processes during asexual and sexual development in interaction with its environment. *A. nidulans* a soil-born fungus has to face many different environmental conditions and threats during development e.g. UV light that causes photooxidation and DNA damage. Pro- and eukaryotic competitors struggling for nutrients and partially attack the fungus. It induces numerous pathways at different light-dependent developmental phases to have a quick answer to many changing conditions without delay. An effective protection against DNA damage caused by UV light is the production of protective pigments e.g. the identified anthranilate. The fungus synthesizes several secondary metabolites e.g. antibiotics and mycotoxins during growth in light and in darkness to inhibit the growth of other competitors to avoid an exhaustion of nutrients within the soil and diminish the competitors which secret toxins that inhibit growth of *A. nidulans* consequently. We could identify several development specific synthesized secondary metabolites. Among them is terrequinone A as the single asexual specific compound. Emericellamide A/C/E, ST and anthranilate are present at both sexual and asexual cycle of the fungus. In contrast, emericellin, shamixanthone, emodin and asperthecin appear at sexual stages specifically. Secondary metabolites are shown regarding their temporal appearance. Surprisingly, the single secondary metabolite secreted at sexual cycle is emericellamide C. Beside emericellamide C even ST and anthranilate are translocated at asexual development. Additionally, the fungus mobilizes several factors for detoxification, feeding (repellent and oxidative stress. Reactive oxygen species (ROS) appearance is required for proper induction of development as well. Therefore expression of numerous genes for enzymes crucial for triggering ROS levels like glutathione-S-transferase, superoxide-dismutases and catalases is *A. nidulans* development specific initiated. Among them are novel, not yet identified and/or characterized ROS enzymes. Development involves synthesis of linoleic- and oleic acid-derived *psi*-factors. 5,8-DiHOE (*Psi*C1b) was shown to be specific and restricted for early sexual development of *A. nidulans*. It is most likely required for proper sexual development. The fungus induces programmed cell death and expressed several specific apoptosis genes. During aging at sexual development amino acid metabolism and translation is downregulated. For energy supply during development numerous genes mediating plant and bacteria cell wall and polysaccharide hydrolysis are activated. Green boxes = asexual specific processes, orange box = sexual specific processes, white box = processes that take place during both, asexual and sexual development

In summary, beside assurance of nutrient supply, main focus of *A. nidulans* is obviously to equip conidiospores proliferated over huge distances and persisting ascospores with several protection techniques for their survival and generation of a good basis for germination.

Generally, *A. nidulans* seems to be much more than an organism, which waits for a competitive organisms attack. The fungus developed numerous effective strategies, which point at an active confrontation with its competitors to ensure its survival even under hard environmental conditions. Finally, aggression is often best defense.

This study demonstrates *A. nidulans* contains much more cryptic and silent treasures, which remain to be identified and analyzed. Beside useful antibiotics we can extract, this fungus even teaches us several strategies to be successful in an increased threat against pathogenic organisms.

5. References

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Table of markers identified by metabolite fingerprinting (UPLC-ESI-TOF-MS analysis) (analyzed and identified by Dr. Kirstin Feussner)

ID	Metabolite marker	Extraction phase	Highest intensities	RT (min)	Detected as	Sum formula	exact mass (Da)	Data base / Ref.	Identity confirmed by comparison with	MS/MS fragments	CE (V)
1	Variecoxanthone/ Emericellin*	CHCl3_pos	Intra: S48, S72, S96	4.43	[M+H]+	$C_{25}H_{28}O_5$	408.1938	KNApSAcK	B, D, E (196, 236, 264/270, 295, 383 nm)	[M+H]+ 391.1895, 349.1436, 335.1268, 323.1275, 69.07	20
2	Shamixanthone	CHCl3_pos	Intra: S48, S72, S97	4.6 / 3.5	[M+H]+	$C_{25}H_{26}O_5$	406.1781	KNApSAcK	C (Variecoxanthone)	[M+H]+ 389.1715, 333.1121, 321.1113, 306.0918, 69.0697	20
3	Emericellamide A	CHCl3_pos	Intra: A24, A48	1.98	[M+H]+	$C_{31}H_{55}N_5O_7$	609.4101	internal DB	A[1]	[M+H]+ 592.4048, 582.4142, 539.3728, 521.3674, 450.3313, 337.2466	15
4	Emericellamide C	CHCl3_pos	Extra: A48	1.70	[M+Na]+	$C_{30}H_{53}N_5O_7$	595.3945	internal DB	F		
5	Emericellamide C	CHCl3_pos	Intra: A24, A48	1.74	[M+Na]+	$C_{30}H_{53}N_5O_7$	595.3945	internal DB	A[1]	[M+H]+ 578.3922, 568.4033, 525.3635, 507.3542, 454.3264,436.3137, 323.2325	15
6	Emericellamide E	CHCl3_pos	Intra: A24, A48	2.46	[M+Na]+	$C_{32}H_{57}N_5O_7$	623.4258	internal DB	A[1]	[M+H]+ 596.4381, 553.3926, 535.383, 482.3575, 464.3467, 351.2619	15
7	Anthranilate	MeOH_pos	Extra: A48	2.20	[M+Na]+	$C_7H_7NO_2$	137.0476	KNApSAcK	D		
8	Anthranilate	MeOH_pos	Intra: A24, A48	1.90	[M+Na]+	$C_7H_7NO_2$	138.0476	KNApSAcK	A (MassBank: KOX00006)	[M+H]+ 120.047, 92.0517, 65.0404,	20
9	Emodin	CHCl3_neg	Intra: S48, S72, S96	2.45	$[M-H]^{-[M-H]-}$	$C_{15}H_{10}O_5$	270.0528	KNApSAcK	D		
10	Asperthecin	MeOH_neg	Intra: S72, S96	4.63	$[M-H]^{-[M-H]-}$	$C_{15}H_{10}O_8$	318.0375	internal DB	E[2] (262, 286, 316, 485 nm)		
11	Terrequinone A	CHCl3_pos	Intra: A24, A48	3.10	[M+H]+	$C_{32}H_{30}N_2O_3$	490.2256	KNApSAcK	E[3] (225, 275 nm)		
14	Sterigmatocystin	CHCl3_pos	Intra: S48, S72	1.62	[M+H]+	$C_{18}H_{12}O_6$	324.0634	KNApSAcK	F		
15	Sterigmatocystin	CHCl3_pos	Extra: A48	1.7	[M+H]+	$C_{18}H_{12}O_6$	324.0634	KNApSAcK	F		

A) MS/MS fragment information from literature/data base
B) MS/MS fragment information from identical standard
C) MS/MS fragment information of related standards
D) Coelution with identical standard
E) Exact mass measurement and UV/VIS spectra
F) Exact mass measurement only

Metabolite data bases:
KEGG (http://www.genome.jp/kegg/ligand.html)
Lipid maps (http://www.lipidmaps.org/)
MassBank (http://www.massbank.jp/)

Continued table of markers identified by metabolite fingerprinting (analyzed and identified by Dr. Kirstin Feussner)

ID	Metabolite marker	Extraction phase	Highest intensities	RT (min)	Detected as	Sum formula	exact mass (Da)	Data base / Ref.	Identity confirmed by comparison with	MS/MS fragments	CE (V)
16	Lysine	MeOH_pos	Intra: A24, S24, S48	0.948	[M+H]+	$C_6H_{14}N_2O_2$	146.1058	KEGG	A (MassBank: KO003283)	[M+H]+ 130.085, 84.0809	10
17	Aspartate	MeOH_pos	Intra: A24, S48, S72	0.443	[M+H]+	$C_4H_7NO_4$	133.0391	KEGG	A (MassBank: KOX00014)	[M+H]+ 88.041, 74.0239, 70.0291	15
18	Glutamine	CHCl3_pos	Intra: A24, S48, S72	0.4119	[M+H]+	$C_5H_{10}N_2O_3$	146.0696	KEGG	A (MassBank: KOX00733)	[M+H]+ 84.0446, 130.0471	10
19	Valine	MeOH_pos	Intra: A48	0.955	[M+H]+	$C_5H_{11}NO_2$	117.0806	KEGG	A (MassBank: PB000388)	[M+H]+ 72.081	10
20	Methionine	MeOH_pos	Intra: S24	0.4908	[M+H]+	$C_5H_{11}NO_2S$	149.05332	KEGG	A (MassBank: KOX00406)	[M+H]+ 133.0322, 104.0524, 102.054, 87.0265	10
21	Glutamate	MeOH_pos	Intra: Veg, A24	0.4187	[M+H]+	$C_5H_9NO_4$	147.0547	KEGG	A (MassBank: KOX00270)	[M+H]+ 130.0509, 102.0543, 84.0456,	10
22	Ornithine	MeOH_pos	Intra: Veg, S24	0.3591	[M+H]+	$C_5H_{12}N_2O_2$	132.09112	KEGG	A (MassBank: KO003632)	[M+H]+ 116.0739	10
23	Phenylalanine	MeOH_pos	Intra: All	0.615	[M+H]+	$C_9H_{11}NO_2$	165.0803	KEGG	A (MassBank: PB000408)	[M+H]+ 120.7080, 103.0539	10
24	Proline	MeOH_pos	Intra: A24, S24, S48	0.437	[M+H]+	$C_5H_9NO_2$	115.0645	KEGG	A (MassBank: PB000450)	[M+H]+ 70.0655	10
25	Threonine	MeOH_pos	Intra: Veg, A24, S24	0.4358	[M+H]+	$C_4H_9NO_3$	119.0578	KEGG	A (MassBank: PB000404)	[M+H]+ 102.0559, 84.0584, 74.0602	10
26	Asparagine	MeOH_pos	Intra: A24, A48, S24, S48	0.448	[M+H]+	$C_4H_8N_2O_3$	132.0539	KEGG	F		
27	Alanine	MeOH_pos	Intra: Veg, S24	0.4445	[M+H]+	$C_3H_7NO_2$	89.0477	KEGG	F		
28	Histidine	MeOH_pos	Intra: A24, S48, S72	0.3808	[M+H]+	$C_6H_9N_3O_2$	155.0706	KEGG	F		

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