

**Molecular characterization and evolution of
indole-3-acetic acid (IAA) biosynthesis pathway
in the filamentous fungus *Neurospora crassa***



Dissertation

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Every challenging works need self-efforts as well as guidance and support of loved ones especially those who are very close to our heart.

My humble effort I dedicate to my loving:

Parents and Brother

Whose affection, love, encouragement and prays of day and night made me able to get such success and honor. End of the day who always reminded me doesn't matter what happen we are family and they love me.

Moumita

For her love, her patience and her faith in me even when I lost on myself. Because she always understood.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
µg	Microgram
µl	Microliter
aa	Amino acids
ALTER	Alignment transformation Environment
BLAST	Basic Local Alignment Search Tool
DD	Double distilled
[DiOC ₆ (3)]	3,3' dihexylocarbocyanine iodine
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FGSC	Fungal Genetics Stock Center
Fwd/fwd	Forward
H ₂ O	Water
HCl	Hydrochloric acid
<i>hph</i>	Hygromycin phosphotransferase
HPLC	High Performance Liquid Chromatography
IAA	Indole-3-acetic acid
IAN	Indole-3-acetonitrile
IAM	Indole-3-acetamide
ILA	Indole-3-lactic acid
IPA	Indole-3-pyruvic acid
IPD/ <i>ipd</i>	Indole-3-pyruvate decarboxylase
IAD/ <i>iad</i>	Indole-3-acetaldehyde dehydrogenase
I-TASSER	Iterative Threading Assembly Refinement

KCl	Potassium chloride
KH ₂ PO ₄	Orthophosphate potassium dihydrogen
KO	Knock-out
l	liter
M	Mole/Molar
mA	Milliamperere
MEGA	Molecular Evolutionary Genetics Analysis
mg	Milligram
MgCl ₂	Magnesium chloride
MgSO ₄	Magnesium sulfate
min	Minute
mM	Millimolar
mm	Millimeter
mRNA	Messenger ribonucleic acid
<i>N. crassa</i>	<i>Neurospora crassa</i>
Na ₂ HPO ₄	Potassium dihydrogen orthophosphate
NaCl	Sodium chloride
NaHCO ₃	Sodium hydrogen carbonate
NaOH	Sodium hydroxide
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Protein Data Bank
Rev/rev	Reverse
TAM	Tryptamine
TAM1/ <i>tam1</i>	Tryptophan amino transferase
TLC	Thin Layer Chromatography
T _m	Melting temperature

TOL	Indole-3-ethanol
Trp/trp	Tryptotophan
UTR	Untranslated region
VMM	Vogel's Minimal Medium
v/v	volume/volume
w/v	weight/volume
× g	x-fold gravity
YASARA	Yet Another Scientific Artificial Reality Application

ABSTRACT

Plants, bacteria and some fungi are known to produce indole-3-acetic acid (IAA) employing various pathways. Among these pathways, the indole-3-pyruvic acid pathway (IPA) is the best studied in green plants and plant associated beneficial microbes. While IAA production circuitry in plants has been studied for decades, little is known regarding the IAA biosynthesis pathway in fungal species. This thesis presents the first data about IAA producing genes and its biosynthesis pathway in a non-pathogenic fungus, *Neurospora crassa*. For this purpose, computational approach was taken to determine the genes and outlined the IAA production circuitry in *N. crassa*. Then these computational data were validated with experimental evidences. In this thesis all the homologous genes were described that are present in IPA pathway of IAA production in *N. crassa*. High-performance liquid chromatography and thin-layer chromatography unambiguously identified IAA, indole-3-lactic acid (ILA) and tryptophol (TOL) from cultures supplemented with tryptophan. Deletion of the *ipd* gene product encoding indole-3-pyruvate decarboxylase enzyme that converts IPA to indole-3-acetaldehyde (IAAld) results in accumulation of higher level of ILA in the culture medium. Double knock-out strain ($\Delta iad1\Delta iad3$) for the enzyme that converts IAAld to IAA shows many fold decrease in IAA production compared to the wild type strain. The $\Delta iad1\Delta iad3$ strain also has slower conidiation and produces much less conidiospore than the wild type strain. It was also shown that *N. crassa* mutants for the IAA biosynthetic genes have an effect on morphology of growing hyphae too.

Zusammenfassung

Pflanzen, Bakterien und einige Pilze sind in der Lage Indol-Essigsäure (IAA) über diverse Synthesewege zu produzieren. Unter den bekannten Synthesewegen ist der über Indol-3-Pyruvat (IPA) der am besten untersucht in grünen Pflanzen und in pflanzensymbiotischen Mikroben. Obwohl die IAA-Produktion in Pflanzen seit Jahrzehnten untersucht wird, gibt es bisher wenig Informationen über die IAA-Biosynthese in Pilzen.

Die vorliegende Arbeit behandelt zum ersten Mal die IAA-Biosynthese und die in diesem Biosyntheseprozess beteiligten Gene des nicht phytopathogenen Pilzes *Neurospora crassa*. Zunächst wurden putative IAA-Biosynthesegene in dem *N. crassa* Genom mittels bioinformatischen Analysen identifiziert und in einem putativen Biosynthesenetzwerk zusammengefasst. Anschließend wurde das Netzwerk experimentell geprüft.

In der vorliegenden Arbeit wurde die vollständige IAA-Biosynthese über den IPA-Weg in *N. crassa* beschrieben. Mittels Hochleistungs-Flüssigkeitschromatographie und Dünnschicht Chromatographie konnten IAA, Indol-Laktat (ILA) und Tryptophol (TOL) in den mit Tryptophan supplementierten Kulturmedien nachgewiesen werden. Die Deletion des für das Enzym Indol-3-Pyruvat-Decarboxylase (*ipd*) kodierenden Gens resultiert in der Akkumulation von einer hohen Konzentration ILA im Kulturmedium. In Abwesenheit der Indol-Pyruvate-Decarboxylase wird das instabile IPA nicht in das Produkt Indol-3-Acetaldehyd (IAAld) umgewandelt und wird schließlich spontan in ILA konvertiert. Gleichzeitige Deletion der beiden Gene *iad1* und *iad3* führt in dem Doppel-*knock-out* Stamm ($\Delta iad1 \Delta iad3$) zur mehrfachen Reduktion der IAA-Produktion im Vergleich zu dem Wildtyp. Die Genprodukte der beiden Gene *iad1* und *3* konvertieren IAAld zu dem Endprodukt IAA. Der $\Delta iad1 \Delta iad3$ Stamm zeigt eine verlangsamte Konidienbildung und er produziert deutlich weniger Konidiosporen als der Wildtyp. Darüber hinaus konnte gezeigt werden, dass die Deletion verschiedener IAA-Biosynthesegene eine Auswirkung auf die Morphologie des Hyphenwachstums hat.

1 INTRODUCTION:

1.1 Historical Background and Auxin Discovery

In 1880 Charles Darwin explored a specific response in Canary grass (*Phalaris canariensis*) and proposed that a transmissible substance that is produced in the tip of the plants is responsible for inducing the curvature (DARWIN 1880). This observation and proposition further lead to the discovery of the first plant hormone, now known as auxin. In support of this discovery in 1894 Rothert has presented evidences that light sensitivity near the tip of maize coleoptiles is the most (ROTHERT 1894). That is how Darwin's 'transmissible substance' started to accumulate further and lead into more extensive studies in the field. Later independent researches by Cholodny and Went has shown that differential growth associated phototropic curvature is caused by light-mediated lateral distribution of a plant growth hormone (WENT 1926; CHOLODNY 1927). Sooner this growth substance was also identified in human urine by Kogl and Haagen-Smit, who named the hormone "auxin" (KOGI 1931) which was derived from the Greek verb *auxein*, meaning "to grow." Thus the concept of "auxin" was explained as a substance that has the property to regulate the growth and localized in plant tissues. In 1934 the structure of this substance was first elucidated as the indole derivative indole-3-acetic acid (IAA). Since then another three naturally occurring auxins (SIMON and PETRASEK 2011) (Fig. 1) were identified: indole-3-butyric acid (IBA), 2-phenylacetic acid (PAA) and 4-chloroindole-3-acetic acid (4-CI-IAA). On the molecular level all the substances which are composed of an aromatic ring and a carboxylic acid group and resemble the structure of indole-3-acetic acid (IAA) are classified as auxin (TAIZ 1998). It is important to mention that among these naturally occurring auxins, indole-3-acetic acid (IAA) is the most abundant as well as important member of the auxin family, and responsible for the major auxin effects.

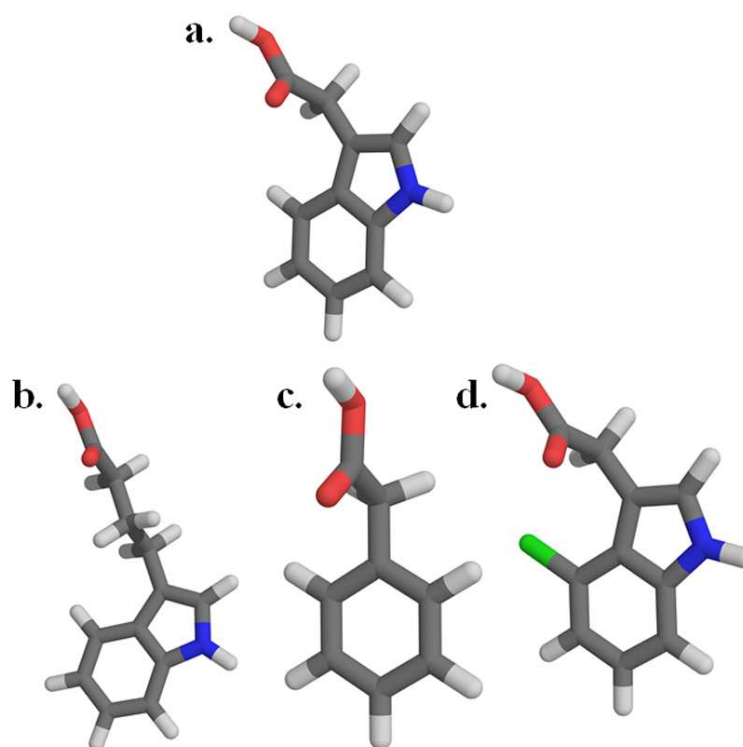


Fig. 1: Natural auxins. (a) indole-3-acetic acid (IAA), (b) indole-3-butyric acid (IBA), (c) 2-phenylacetic acid (PAA) and (d) 4-chloroindole-3-acetic acid (4-CI-IAA).

Besides these there exist different synthetic auxins, which are compounds with a good amount of auxin activity. Synthetic auxins include the following compounds: α -naphthalene acetic acid (α -NAA), 2-methoxy-3,6-dichlorobenzoic acid, 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and 4-amino-3,5,6-trichloropicolinic acid (tordon or picloram) (SIMON and PETRASEK 2011). These synthetic auxins are mainly used for commercial purposes as herbicide, root inducing agents, artificially controlled growth in plants and agronomy and in laboratory uses. After the discovery of the first plant growth hormone subsequent discovery of several other molecules and compounds that affect the plant growth and development were made (BROSA 1999). Based on their molecular and chemical properties these compounds were categorized in five different classes of phyto hormones: auxins, abscisic acid, cytokinins, ethylene and gibberellins. In this context it is important to mention that similarity between plant and animal hormone virtually ends with the term “hormone” itself while considering their presence differentially in plant and animal. By the classical definition hormones are supposed to be small organic chemical compounds which act on a distant organ from their origin of synthesis at very low concentration. Plant hormones defy these arguments in all the ways possible. Many plant

hormones act on their origin of biosynthesis and in fact this is crucial for their overall function. Further discoveries of brassinosteroids, jasmonic acid (JA), polyamines and salicylic acid (SA) (CREELMAN and MULLET 1997; BROSÁ 1999; GHOSH 2000; MCDOWELL and DANGL 2000) with hormonal properties in plant even diversify the general categories of existing five classes of phytohormones mentioned above. In spite of these differences there are similarities in chemical structure and properties among these molecules.

Generally, indole-3-acetic acid (IAA) is considered as a main hormone in green plants and thus related studies regarding it is mainly plant oriented. Research fields which includes IAA biosynthesis in microorganisms are mostly an extrapolation of plant oriented IAA studies and/or conclusions are mostly derived from the same. In this thesis a systemic study has been conducted where knowledge from plant and microorganism derived IAA biosynthesis has been scrutinized and used to build, propose and prove indole-3-acetic acid production pathway in *Neurospora crassa* with experimental evidences. For better understanding and elaboration of relationship between auxin and plant/microorganism next few chapters will be covered first with general introduction about different aspects of IAA in green plants and other microorganisms.

1.2 Auxin metabolism in plants and bacteria

Auxin, when discussed as a plant hormone is predominantly represented by indole-3-acetic acid (IAA). Although centuries back IAA had been identified as the first plant hormone, still the biosynthetic pathway at the genetic level has remained unclear and elusive (MANO and NEMOTO 2012). For green plants there exist two major proposed IAA biosynthesis pathways: the tryptophan (Trp)-dependent and Trp-independent pathways.

1.2.1 Tryptophan dependent IAA biosynthesis pathways in plants and bacteria

IAA biosynthesis pathways in plants and bacteria are highly similar with some difference in produced intermediates. It has been shown that both tryptophan (Trp)-dependent and Trp-independent IAA biosynthesis pathways exist in plants as well as in microbes (SITBON *et al.* 2000; SPAEPEN *et al.* 2007; MANO and NEMOTO 2012). The intermediate stages and genes involved in Trp-independent IAA biosynthesis pathways are still unclear consequently most of the studies are

focused on Trp-dependent pathways resulting limited knowledge about biochemical processes involved in Trp-independent IAA production in plants and microbes (OUYANG *et al.* 2000; ZHANG *et al.* 2008). The amino acid tryptophan (Trp) is the precursor molecule for many secondary metabolites with diversified biological effects in both plants and animals, and is considered the major source of IAA bio-synthesis in plants (NORMANLY 2010). Within the tryptophan dependent pathway there are four proposed pathways for auxin biosynthesis in plants (Fig. 2): (i) the indole-3-pyruvic acid (IPA) pathway, (ii) the indole-3-acetamide (IAM) pathway, (iii) the tryptamine (TAM) pathway, and (iv) the indole-3-acetaldoxime (IAOX) pathway, which is also known as the CYP79B2/B3 pathway. In another recent study YUC pathway has been differentiated from the IPA pathway and demonstrated as an independent pathway. (ZHAO 2010; MASHIGUCHI *et al.* 2011; MANO and NEMOTO 2012). Additional to these two other pathways has been demonstrated to be functional in bacteria namely, the indole-3-acetonitrile (IAN) and the Trp side chain oxidase pathway (CARRENO-LOPEZ *et al.* 2000; SPAEPEN *et al.* 2007).

1.2.1.1 The indole-3-pyruvic acid (IPA) pathway

The IPA pathway for IAA biosynthesis is divided into three major steps. First, tryptophan is deaminated into indole-3-pyruvic acid (IPA) by the enzyme *TAA1* (*TRYPTOPHAN AMINOTRANSFERASE of ARABIDOPSIS 1*) gene. In fact there are four genes closely related to *TAA1* in the *Arabidopsis* genome namely *TARI–TAR4* (*TRYPTOPHAN AMINOTRANSFERASE RELATED 1 to 4*). Next step, conversion of IPA into IAD in green plants and microorganisms is completely different. Even it is unknown if IPA is converted into IAD in plants at all, as neither the gene nor the enzyme has yet been detected so far (MANO and NEMOTO 2012). In the bacterial IPA pathway, first the precursor tryptophan is converted to IPA by tryptophan aminotransferase, and then IPA is converted to indole-3-acetaldehyde (IAD) by enzymatic reaction of indole-3-pyruvate decarboxylase. IAA is produced after oxidation of IAD by indole-3-acetaldehyde oxidase (or aldehyde dehydrogenase) (TSAVKELOVA *et al.* 2012). On the other hand in *Arabidopsis* the deamination of tryptophan to IPA by *TAA1* remains the same in the YUC pathway and then IPA is directly converted to IAA by the enzymatic reaction of YUC (discussed in detail later), also known as flavin monooxygenase (MASHIGUCHI *et al.* 2011). The synthesis of IAA via IPA pathways, implicated by indole-3-lactic acid (ILA), tryptophol (TOL) and indole-3-acetaldehyde

(IAD/IAAld), compounds which are metabolized in the course of IAA biosynthesis has been reported to occur in plants and bacteria.

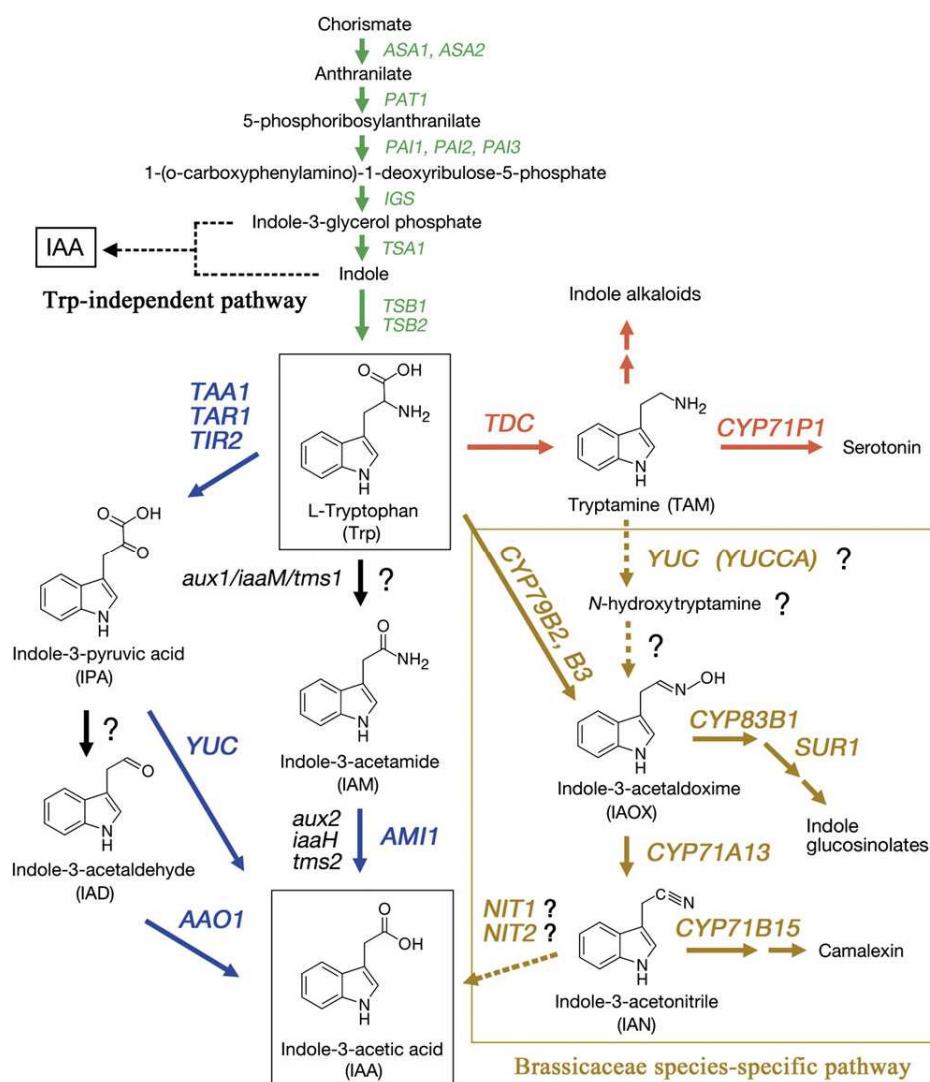


Fig. 2: Proposed IAA biosynthesis pathways in plants. Green arrows are indicative for tryptophan synthetic pathway in the chloroplast. Tryptophan-independent IAA biosynthetic pathway has been shown with dashed black arrow. Blue arrows are for known genes and their enzymatic function involved in steps in the tryptophan-dependent IAA biosynthetic pathway. Black solid arrows with question marks indicate steps where the gene(s) and enzymatic function(s) are unknown. Red arrows are for the indole alkaloid and serotonin biosynthetic pathway. Mustard-coloured arrows for the Brassicaceae species-specific pathway where dashed arrows indicate steps for which the gene and enzymatic function(s) are elusive. Letters in italics are representation for genes involved in the conversion process where upper case letters are for plants and lower case letters are for bacterial genes. Figure is adopted from (MANO and NEMOTO 2012).

1.2.1.2 The indole-3-acetamide (IAM) pathway

The IAA biosynthetic pathway via IAM was first considered to be a microorganism-specific pathway because no homolog for the reaction step conversion of Trp to IAM has been detected in plants. This pathway had been identified during study of “hairy roots” in plant pathogen *Agrobacterium rhizogenes* that harbors a large root-inducing (Ri) plasmid and causes hairy-root disease, which is characterized by root proliferation from the infected tissue (MOORE *et al.* 1979; WHITE and NESTER 1980). In this pathway tryptophan is first converted to IAM by the enzymatic reaction of tryptophan-2-monooxygenase (encoded by the *aux1/iaaM/tms1* gene) (YAMADA *et al.* 1985; CAMILLERI and JOUANIN 1991). In the next step IAM is converted to IAA by indole-3-acetamide hydrolase encoded by *aux2/iaaH/tms2* gene (YAMADA *et al.* 1985; CAMILLERI and JOUANIN 1991; GAUDIN *et al.* 1993). Though a functional IAM pathway has not been detected in green plants but the compound IAM has been detected in many plants including the *Arabidopsis* (POLLMANN *et al.* 2009), maize, rice (SUGAWARA *et al.* 2009), tobacco (LEMCKE *et al.* 2000), squash seedlings (RAJAGOPAL *et al.* 1994). IAM is converted to IAA by the enzymatic reaction of indole-3-acetamide hydrolase, activity of this enzyme has been detected in rice (KAWAGUCHI *et al.* 1991). Study has confirmed that cell-free extracts from different plant species, including *Arabidopsis*, tobacco, potato, cauliflower, maize, tomato, are capable of converting Trp into IAM and followed by conversion of IAM to IAA (POLLMANN *et al.* 2009). These studies suggest that IAM is an endogenous compound and an IAM-dependent pathway for IAA biosynthesis in plant is more likely to be functional.

1.2.1.3 The tryptamine (TAM) pathway

Tryptophan decarboxylase (TDC) is a cytosolic enzyme from the gene product of *TDC* that converts Trp to tryptamine. Being a member of a well-studied carboxylase family enzyme TDC is well characterized at the molecular and functionally in biochemical level. Phylogenetic analysis suggests that the TDC protein is widespread in the plant kingdom (MANO and NEMOTO 2012). Tryptamine has been suggested as the intermediate compound for serotonin biosynthesis from Trp by enzymatic reaction of tryptophan decarboxylase. Transgenic plants overexpressing the *TDC* gene has been shown to accumulate very high levels of tryptamine but with unchanged levels of

IAA (SONGSTAD *et al.* 1990). It has also been shown that *YUCCA* gene that encodes flavin monooxygenase-like enzyme oxidizes TAM to N-hydroxytryptamine which is presumably the linking compound between TAM and IAOX pathway (MANO and NEMOTO 2012), has been found in *A. thaliana* (ZUTHER *et al.* 2008).

1.2.1.4 The indole-3-acetaldoxime (IAOX) pathway

IAOX is synthesized from Trp by CYP79B2 and CYP79B3, two homologous cytochrome P450 enzymes. Both of these enzymes are predicted to be targeted to the chloroplast (HULL and CELENZA 2000; HULL *et al.* 2000). The *CYP79B* gene family has so far only been identified in Brassicaceae family (BAK *et al.* 1998). Phylogenetic analysis and biochemical evidences suggests that the CYP79B protein family is restricted to only Brassicaceae species and also IAOX dependent IAA biosynthesis is not a common but rather a species-specific pathway in plants (SUGAWARA *et al.* 2009). It was reported that CYP79B2 enzyme is induced upon pathogen infection (HULL *et al.* 2000), and is expressed in flowers, leaves, stem, and roots, with the highest expression in roots (MIKKELSEN *et al.* 2000). The main function of CYP79B3 has been demonstrated is to be involved in indole glucosinolate biosynthesis (GLAWISCHNIG 2007). Double knock-out *Arabidopsis* strain for *CYP79B2* and *CYP79B3* genes do not have exhibit any detectable amount of either indole glucosinolates or camalexin, and also have reduced levels of Indole-3-acetonitrile (IAN) (ZHAO *et al.* 2002; GLAWISCHNIG *et al.* 2004), but the IAA content of the knock-out strain was found to be almost the same as in the wild-type strain (ZHAO *et al.* 2002). On the other hand, transgenic *Arabidopsis* that is overexpressing the *CYP79B2* gene have significantly high levels of indole glucosinolates and IAN (MIKKELSEN *et al.* 2000; ZHAO *et al.* 2002), but the same strain shows a normal level of IAA (ZHAO *et al.* 2002). These results indicate that CYP79B is specifically involved in indole glucosinolate and camalexin biosynthesis (MIKKELSEN *et al.* 2000).

1.2.1.5 The TAA1–YUC pathway reported to be “the main auxin biosynthesis pathway in Arabidopsis”

Previous genetic and enzymatic studies done on *Arabidopsis thaliana* demonstrated the enzymes TAA and YUCCA (YUC) flavin monooxygenase-like proteins are involved in IAA biosynthesis but are present in two independent pathways. Gene based metabolite analysis evidence in a recent research showed that TAA and YUC families both function in the same auxin biosynthetic pathway in *Arabidopsis* (MASHIGUCHI *et al.* 2011). The study shows that TAA family converts tryptophan to IPA and the YUC family converts the available IPA to IAA in *A. thaliana*. This finding is important from the perspective that so far no genes have been identified for the conversion of IPA to IAD in green plants although evidence is present for a functional IPA pathway in the green plants (MANO and NEMOTO 2012). The above mentioned pathway does not include IAD as an intermediate compound however IAD is known to be present across the plant kingdom (MANO and NEMOTO 2012) as an intermediate compound of the IAA biosynthesis. So far this finding is restricted to *A. thaliana* only, but further studies are required to deduce whether this pathway is common the in plant kingdom.

1.2.2 Tryptophan independent auxin biosynthesis pathway

In spite of existing numerous pathways tryptophan dependent IAA production has been challenged by recent studies especially studies in tryptophan biosynthetic mutants of *Arabidopsis* and maize (WRIGHT *et al.* 1991; NORMANLY *et al.* 1993). Although a Trp-independent IAA biosynthetic pathway have been proposed but the enzymes, actions and regulations are largely unknown. It has been demonstrated that IAA *de novo* biosynthesis occurs from a precursor that is an intermediate of tryptophan biosynthesis pathway itself. This IAA biosynthetic pathway from the precursor other than tryptophan is considered as the tryptophan independent pathway (OUYANG *et al.* 2000). Tryptophan biosynthesis pathway is similar in both plants and microorganisms, in which chorismate is first converted to anthranilate by the enzyme anthranilate synthase α -subunit (ASA) and anthranilate synthase β -subunit (ASB). Then 1-(O-carboxyphenylamino)-1-deoxyribulose-5-phosphate (CdRP) is synthesized from anthranilate via 5-phosphoribosylanthranilate (PRA) by the enzymatic reactions of phosphoribosylanthranilate

transferase (PAT) and phosphoribosylanthranilate isomerase respectively (PAI). Then CdRP is converted to indole-3-glycerol phosphate (IGP) by indole-3-glycerol phosphate synthase (IGS). IGP is further converted to tryptophan by producing indole as an intermediate compound by the enzyme tryptophan synthase α -subunit (TSA) and tryptophan synthase β -subunit (TSB) respective in two subsequent reactions (RADWANSKI and LAST 1995; OUYANG *et al.* 2000; MANO and NEMOTO 2012). In tryptophan independent IAA biosynthesis, indole-3-glycerol phosphate (IGP) and/or indole is proposed to be the precursor, but very little is known about the biochemical pathway and regulation (Fig. 2) (MANO and NEMOTO 2012). From this branch point IAA is synthesized tryptophan-independently. However most of the experiments regarding tryptophan independent IAA biosynthesis were done and enzymes were identified in *Arabidopsis* (OUYANG *et al.* 2000; ZHANG *et al.* 2008).

1.3 IAA biosynthesis pathways in fungi

The relationship between auxin and fungi has been investigated long back almost since the discovery of this phytohormone in higher plants. But little got attention on auxins in fungi by the researchers as that time auxin became a famous compound for its versatile functions especially on green plants. Later on, the topic fungi and auxin were discussed together in the context of auxin production by phytopathogenic fungi (ALLEN 1954). Not only plant-interacting fungi but also many other fungal species produce auxin which suggests that IAA have an endogenous role in fungi (GRUEN 1959; ULRICH 1960). But majority of the studies on fungal IAA production with detailed auxin producing genetic architectures have been conducted in pathogenic and symbiotic fungi (CHANCLUD and MOREL 2016).

Fungi can produce functional auxins, which can act as an active plant growth promoter. Nielsen reported that the substance which accumulates on agar plate after growth of *Rhizopus suinus* and *Absidia ramosa* has the ability to promote growth of *Avenea* coleoptiles (NIELSEN 1928). There is a detailed list of auxin producing fungal species and detection procedure published (GRUEN 1959). Although Trp has been considered as a major IAA precursor, it has also been shown that Trp-independent IAA biosynthesis exists in many yeast species (RAO *et al.* 2010; SUN *et al.* 2014) but least is known about the biosynthesis process. IAA is a well-known plant hormone that promotes and regulates plant growth but a negative correlation between fungal growth and auxin

production has been reported in several species (GRUEN 1959; NAKAMURA *et al.* 1978). By contrast, auxin treatment promotes the growth of filamentous forms of *Saccharomyces cerevisiae* and *S. ellipsoideus* and promoted the invasion property of the yeast, which supports the role of IAA as a growth enhancer and signaling molecule regulating yeast cellular elongation and sporulation (YANAGISHIMA 1965; KAMISAKA 1967; PRUSTY *et al.* 2004). Similarly, IAA also induces hyphal growth in the human pathogen *Candida albicans* (RAO *et al.* 2010). In *C. albicans*, growth and cell density triggers dimorphism and this morphological change has been associated with virulence traits like macrophage evasion, tissue invasion, and biofilm formation (SUDBERY 2011; VEDIYAPPAN *et al.* 2013). IAA also promotes spore germination of the filamentous fungus *Neurospora crassa* at an optimal concentration of 10 μM (NAKAMURA *et al.* 1978) in contrast when tested on the tomato pathogen *Fusarium oxysporum lycopersici*, IAA reduced spore germination (SHARAF and FARRAG 2004). As seen in *N. crassa* the effect of auxin on growth is similarly concentration dependent in the pea pathogen *Fusarium delphinoides* too where IAA in low concentration increases fungal growth, whereas high concentration decreases the growth (KULKARNI *et al.* 2013). Thus, IAA might act as a general signaling molecule in fungi but its effects on fungal physiology strongly differs from one species to another and is concentration dependent. The topic of IAA biosynthesis in fungi becomes more complex when it comes to the role of IAA in fungi. Although the pathway for IAA production in fungi is poorly understood it is known that tryptophan supplementation promotes auxin production. This tryptophan dependent auxin production has been shown in many different fungi (BRIAN 1957).

The most studied plant metabolic route for IAA biosynthesis through indole-3-pyruvic acid (IPA pathway) has been conclusively demonstrated only in the smut fungus *Ustilago maydis* (BASSE *et al.* 1996; REINEKE *et al.* 2008). Several genes have been identified for this pathway in the ectomycorrhizal fungus *Tricholoma vaccinum* (KRAUSE *et al.* 2015). *U. maydis* and *T. vaccinum* so far are the only fungal species where IAA metabolic genes from the IPA pathway have been isolated and functionally characterized. In addition a few genes of the same pathway have been reported to be present in the root endophyte *Piriformospora indica* (HILBERT *et al.* 2012) and in some species of *Rhizoctonia* (FURUKAWA 1996). In these studies, two indole-3-acetaldehyde dehydrogenase genes (IAD1, IAD2) were identified in *U. maydis*, and a *Δiad1/Δiad2* double knock-out was shown to be disabled for conversion of both indole-3-acetaldehyde and tryptamine to IAA. Deletion of two aromatic amino acid aminotransferases (TAM1 and TAM2) in the same

species with a *Diad1/Diad2* double knock-out background resulted in a further decrease in overall IAA production (REINEKE *et al.* 2008). In recent years it has been shown that *P. indica* can synthesise IAA and indole-3-lactic acid (ILA) through the intermediate indole-3-pyruvic acid (IPA) via the IPA pathway (HILBERT *et al.* 2012). *Rhizoctonia* has been shown to produce TOL, an indolic compound that is produced as a side product of IAA biosynthesis through IPA pathway. *Colletotrichum acutatum* that causes lime anthracnose and postbloom fruit drop of citrus has been reported to produce IAA along with other indolic compounds such as indole-3-acetamide (IAM), indole-3-pyruvic acid (IPA), indole-3-lactic acid (ILA), tryptophol (TOL) and indole-3-acetaldehyde (IAD) upon Trp supplementation in the media (CHUNG *et al.* 2003). These intermediate compounds encompasses IAM and IPA pathway both.

1.4 Evolutionary patterns in auxin functionality

1.4.1 Parallel evolution of IAA along with organismal evolution

Every green plant examined so far was found to be producing auxin (IAA). This suggests that there must be an evolutionary conserved core mechanism for auxin production and also there is a constraint on its functionality. In recent years researches on IAA action are almost completely focused on the seed plants, but documented knowledge about IAA action also exists regarding liverworts, algae, and mosses (MARAVOLO 1980; BRADLEY 1991; CHRISTIANSON 1999). The importance of studying the evolutionary patterns of auxin action can be explained by giving a comparison of hormonal regulation between angiosperm and lower plants. A study has found that during developmental stage angiosperms employ a complex signal transduction network composed of multiple cross talking and somewhat redundant pathways of IAA regulation (BENNETT *et al.* 1996). In contrast, lower plants employ rather simpler mechanisms of IAA regulation (SZTEIN *et al.* 2000). This indicates that IAA is even involved in the basic fundamental processes in plant development like morphogenesis. When organisms evolved further and the systems became more complex, the overall auxin production, action and regulation also became complex and interconnected (COOKE *et al.* 2002a).

It is important to ask the question of why to consider and study evolutionary pattern of auxin at all. A more logistic justification for this question lies in the potential of evolutionary

approaches that can be taken by a molecular biologist studying auxin action as modern experimental biology demands evidences from the study at molecular level from every experiment that has been conducted. As mentioned above that along with the organismal evolution auxin production, action and signal transduction not only evolved but became complex too. Which means the total auxin action became a cumulative output of involvement of many genes and their regulatory network/s. In auxin biosynthesis alone there are multiple pathways involving multiple genes and the number of genes go up when someone consider IAA production as a general fact in green plants, bacteria and fungi. Now prediction and functional characterization of any less-characterized IAA producing gene from any organism can be solved by studying the molecular evolution of genes/gene family closely related to that particular gene. . It has been properly argued, that understanding about molecular evolution of different members of any gene family would give more relevant insights about functional prediction of a less-characterized member of the same gene family (EISEN 1998). In the context of IAA synthesis and regulation knowledge about molecular evolution would greatly help in understanding the differential roles of homologous enzymes with overlapping functionality that acting on same or bypassing regulatory pathways.

1.4.2 Why to consider evolutionary consequences of IAA in general?

IAA is the most abundant and common phytohormone of the auxin class regulating various physiological aspects of plant growth and development. Keeping the importance of IAA in mind, modern research is underway to explore the potential for IAA-producing microorganism for promoting plant growth and protection for sustainable and efficient agriculture. Phylogenetic analysis suggest that IAA biosynthesis evolved independently in bacteria, algae, fungi, and plants (GOPINATHAN and RAMAN 1992; JAMESON 2000; SPAEPEN *et al.* 2007; REINEKE *et al.* 2008; LIMTONG and KOOWADJANAKUL 2012). This evidence for the convergent evolution of IAA production leads to the hypothesis that natural selection might have chosen IAA as a physiological code for interspecies communication in plants and these microorganisms. Some studies show that IAA not only regulates the physiological response but also modulates the gene expression in these microorganisms (PRUSTY *et al.* 2004; YUAN *et al.* 2008). IAA can serve as a major interacting molecules between IAA-producing organisms. Several supporting studies have shown that auxin acts as an effector molecule between IAA producing bacteria and plants, and bacteria-bacteria

interactions (BASSE *et al.* 1996; LAMBRECHT *et al.* 2000; SPAEPEN *et al.* 2007). Although IAA biosynthesis pathways and its role in fungal ecology is not well understood (BASSE *et al.* 1996; REINEKE *et al.* 2008; RAO *et al.* 2010), it has been shown that there is a difference in gene signaling in host plants upon infection by phytopathogenic IAA producing fungi (TANAKA *et al.* 2011; TSAVKELOVA *et al.* 2012). The genetic mechanisms of auxin biosynthesis and regulation are well studied in phytopathogenic bacteria such as *Pseudomonas*, *Agrobacterium*, *Rhizobium*, *Bradyrhizobium*, and *Azospirillum* (COSTACURTA and VANDERLEYDEN 1995). It has been shown that the pathogenic bacteria *Pseudomonas* and *Agrobacterium* produce IAA via the IAM pathway (SURICO 1992). The respective genes are localized on plasmids. However, these bacteria also possess the IPA pathway where the genes are chromosomally encoded. These facts suggest that an evolutionary conserved mechanism for IAA production is sustained even in bacteria, but depending upon the situation and needs, an organism can switch between these pathways. All these evidences and arguments finally lead to the hypothesis that there must be a well conserved evolutionary core mechanism for auxin biosynthesis which further has multimodal roles in interspecies communication and their ecology (FU *et al.* 2015).

1.5 *Neurospora crassa* as a model organism

Model organism by definition is a non-human species that has been/is extensively studied to understand its biological aspects in expectation that acquired knowledge from the model can be implemented to understand similar or more complex biological systems (FIELDS and JOHNSTON 2005). Choosing any model organism is firstly based on its taxonomical classification that validate its need and importance to study such organism. *N. crassa* is a fungus which belongs to the phylum Ascomycota and family Sordariaceae. In 1843, some bakeries in France were infested by a mold that produced powdery orange spores. This mold was named *Monilia sitophila* and was commonly found on bread or other carbohydrate- rich foodstuffs. It was also observed as the first species to colonize on burnt vegetation. Later the fungus was reclassified into the new genus *Neurospora*. The genus name meaning the “nerve spore” refers to its characteristic growth and spore formation. The early work of Shear and Dodge described three species of *Neurospora* namely, *N. crassa*, *N. sitophila*, and *N. tetrasperma* (pseudohomothallic) (SHEAR 1927). Today, using the modern technology and sophisticated method it was identified that there are at least 43 species under the genus *Neurospora*, of which 28 are homothallic, 13 are heterothallic and 2 are pseudo-homothallic (CATCHESIDE 2000; NYGREN *et al.* 2011).

The filamentous fungus *N. crassa* was adopted as an experimental organism by Dodge (SHEAR 1927) in the late 1920s. 10 years later it was employed by Beadle and Tatum for their famous historic “one gene–one protein” hypothesis which practically linked biochemistry and genetics for the first time (DAVIS 1970). *Neurospora crassa* has impact on our understanding of genetic, biochemical, molecular and molecular-biochemical cross talking mechanisms in microbes and eukaryotes. Starting from Mendelian genetics, photobiology, circadian rhythms, DNA repair and recombination, population biology, morphogenesis, the gene–enzyme relationship, plant cell wall degradation, mitochondrial import to DNA methylation, and other epigenetic processes (BORKOVICH *et al.* 2004) *Neurospora* has always been there to understand these complex systems and broaden modern day scientific knowledge. Major findings and understandings about basic biology using *Neurospora crassa* are given in table 1.

Neurospora has both a sexual and an asexual life cycle and it possesses two mating types (“A” and “a”), which are morphologically identical. The vegetative cycle of the organism is initiated when either an ascospore or a conidium germinates, which further gives rise to

multinucleate cells that form branched filaments called hyphae. The hyphal branches grow rapidly (>5 mm/h at 37°C) to form a mycelium. Once the mycelium is well formed, aerial hyphae (conidiophores) begins to develop which further produce myriads of orange macroconidia that are typical characteristic of the *N. crassa*. There is another type of spore namely microconidia. By the name it is quite clear that microconidia are relatively smaller than the macroconidia. Production of microconidia is less in number as compare to the macroconidia, produced from mycelium and uninucleate in nature. The microconidia can function either as spermatia (the male gametes) or as asexual reproductive structures or both. Microconidia is probably exclusively involved in fertilization of protoperithecia (MAHESHWARI 1999).

Macroconidia, which often contain several nuclei can either form a new mycelium or fertilize another strain with the opposite mating type. Typically if the organism is growing on nutrients-limiting (nitrogen limiting) media *N. crassa* activates its sexual phase by producing nascent fruiting bodies known as protoperithecia (WESTERGAARD and MITCHELL 1947). These protoperithecia have a specialized hyphal structure called trichogyne. When the trichogyne contacts the tissue of the opposite mating type, a heterokaryon can form and a male nucleus is transported back to the protoperithecium. Subsequently this protoperithecium matures and become a perithecium producing asci, which contain eight haploid meiotic spores (Fig. 3). Strains of either mating type can act as male or female.

The *N. crassa* genomic sequence was published in 2003 consists of seven chromosomes also known as linkage groups (I-VII) with approximately 10000 predicted protein coding genes (GALAGAN *et al.* 2003). It was found that ~8% of the whole genome consists of repetitive DNA (KRUMLAUF and MARZLUF 1980) and ~180 copies of the ~9.3 kb recombinant DNA (rDNA) (BUTLER and METZENBERG 1989). Most of the repetitive DNA consists of inactivated transposable elements.

Table 1: *Neurospora* history. Major findings and events.

Year	Event	References
1843	First document regarding <i>Neurospora</i> contaminated bakery material in Paris	(PAYEN 1843)
1927	Naming the genus, describing the species is done. Genetic and cytological studies were initiated by Bernard O. Dodge.	(SHEAR 1927)
1939	First and second division segregation in the ascus, with crossing over at the four-chromatid stage.	(STURTEVANT 1939)
1941	"One gene one enzyme hypothesis", first biochemical mutants of <i>Neurospora</i> by George Beadle and Edward Tatum.	(BEADLE and TATUM 1941)
1945	Barbara McClintock described: i) the seven chromosomes ii) meiosis and post-meiotic mitoses iii) a translocation.	(MCCLINTOCK 1945)
1953	Heterokaryon-incompatibility (het) genes are identified.	(GARNJOBST and WILSON 1956)
1955	First definitive proof of gene conversion was shown by Mary Mitchell by using ascus.	(MITCHELL 1955)
1960-1962	The Fungal Genetics Stock Center (FGSC) is established. First <i>Neurospora</i> Information Conference.	http://www.fgsc.net/fgn/nn1/nn1tofc.html
1965	The existence and role of coordinate control or unlinked genes in the same biosynthetic pathway and cross-pathway control of amino-acid synthesis is discovered and described.	(GROSS 1965)
1968	Systematic sampling of wild <i>Neurospora</i> populations from different geographic location begins.	(PERKINS <i>et al.</i> 1976)
1970	Microelectrode techniques demonstrated, a proton pump in the cell membrane.	(SLAYMAN and GRADMANN 1975)
1972	Mutants are found to have altered period length of circadian conidiation.	(FELDMAN and HOYLE 1973)
1976-1979	Spore-killer factors are discovered in natural populations.	(TURNER and PERKINS 1979)
1982	Physical and genetic maps of the mitochondrial genome are first published.	http://www.fgsc.net/nn29/29collins.pdf
1986	" <i>Neurospora</i> Information Conference" became the "Fungal Genetics Conference" and the " <i>Neurospora</i> Newsletter" was renamed to the "Fungal Genetics Newsletter."	http://www.fgsc.net/fgn33/toc33.htm
1988	Mating-type genes are shown to be present as single copies. Those genes were cloned, and sequenced. As ' <i>matA</i> ' and ' <i>mata</i> '	(GLASS <i>et al.</i> 1988)

	genes are non-homologous, they are called idiomorphs rather than alleles.	
1994	Circadian clock gene <i>frequency</i> is shown to be involved in a negative feedback loop.	(ARONSON <i>et al.</i> 1994)
1996	Genes present in unpaired DNA segments are shown to be inactivated during meiosis.	(PERKINS 1997)
1997-1999	Vegetative-phase gene silencing requires an RNA-dependent RNA polymerase.	(COGONI and MACINO 1999)
1998-2000	Genome sequencing of linkage groups II and V was initiated in Germany later on the Whitehead Institute receives a National Science Foundation (NSF) grant to sequence the entire genome <i>Neurospora crassa</i> .	(SCHULTE <i>et al.</i> 2002)
2001	DNA methylation is shown to be mediated by a functional histone H3 methyltransferase.	(TAMARU and SELKER 2001)
2002	Genome sequence version 3 is released in February 2002.	
2003	24 th April 2003 issue of Nature reported the completely sequenced genome of <i>N. crassa</i> .	(GALAGAN <i>et al.</i> 2003)
2006	A high-throughput gene knockout procedure using homologous recombination by <i>hph</i> cassette exchange.	(COLOT <i>et al.</i> 2006)

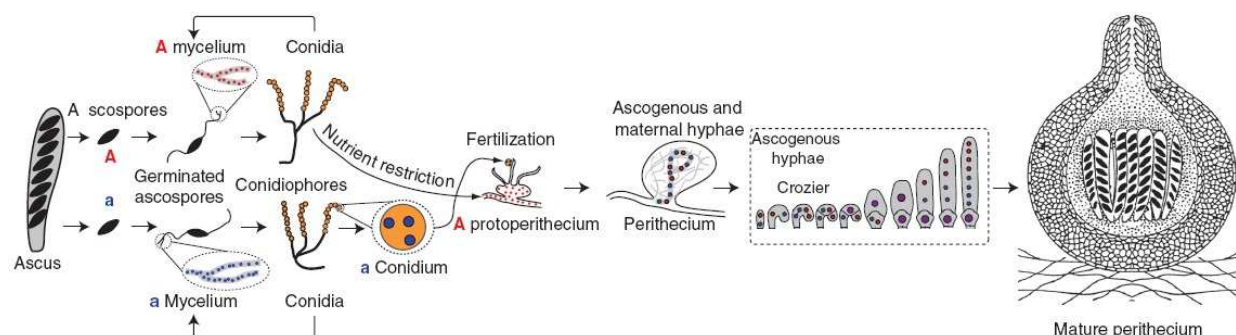


Fig. 3: *N. crassa* life cycle. *N. crassa* has sexual spores (ascospores) with mating type A (red) and mating type a (blue) in 1:1 ratio. Ascospores and vegetative spores (conidia) germinate and form mycelia. Mycelia gradually give rise to asexual fruiting bodies (conidiophores). Conidiophores form conidia, which are typically multinucleate. Upon nitrogen starvation mycelia of either mating type form specialized female structures, protoperithecia. Contact with vegetative tissue (conidium) of the opposite mating type which serves as the “male” counterpart fertilizes and initiates development of fruiting bodies (perithecia). After fertilization, nuclei from both male and female body coexist in the same cytoplasm, undergo mitoses and eventually become organized into a dikaryotic tissue. Within this tissue each cell has one nucleus of each mating type (A and a). The nuclei then start to undergo a series of synchronous mitoses until the tip of the containing hyphal cell bends to form a hook-shaped structure called a crozier. Haploid nuclei fuse together followed by meiosis and a mitotic division such a way that one crozier gives rise to one complete ascus containing eight ascospores. Image adopted from (ARAMAYO and SELKER 2013)

1.6 Aims of this thesis

In this study for the first time IAA producing genes and its biosynthesis pathway have been investigated in the non-pathogenic fungi *N. crassa*. This study is relevant mainly because of two important factors:

- (1) It helps to understand the basic mechanism of IAA production in any fungus irrespective of its pathogenicity factor, and
- (2) It suggests IAA and its biosynthesis pathway to be well conserved and well maintained throughout the evolution.

Although auxin biosynthesis in fungi had been reported many decades ago (GRUEN 1959) but auxin biosynthesis pathways has been conclusively demonstrated only in few fungi. In one of our recent study on auxin biosynthesis in *Neurospora crassa* few IAA producing genes has been proposed. The same study has shown to have differential expression level of those genes upon modulation of another non-IAA producing gene and has shown overall difference in produced IAA level (KOLLATH-LEISS *et al.* 2014). So far those proposed IAA-producing genes are not supported by any experimental evidences and roles of those predicted genes in IAA production has not been studied. In my thesis I reconsidered those predicted genes and aimed to find more other genes from all the plausible IAA biosynthesis pathways, functionally characterized them and propose a functional auxin biosynthesis network in *Neurospora crassa*. For conducting this study, a systemic approach was taken to determine the plausible genes and outline the IAA production circuitry in *N. crassa*. First, genes that are involved in the IAA biosynthesis pathways have been identified and tested computationally. Second, the data were validated with experimental support and evidences. The experimental goal was to create knock-out strains with different IAA-producing gene combination and study their morphology and indolic compounds including IAA production pattern. This systemic approach was undertaken to identify the candidate genes involved in the IAA biosynthesis circuitry in *N. crassa* and to determine their function(s) for the same.

2 MATERIALS

2.1 General consumable materials

General materials were purchased from Sarstedt, Germany; if not otherwise mentioned.

13 ml tubes (101 × 16.5 mm); 50 ml tubes; Cover-slips (24 × 40 mm): Carl-Roth; **Microscope Slides (76 × 26 mm):** Carl-Roth; **Micro tubes (1.5 ml); Micro tubes (2.0 ml); PCR tubes (50 µl); Petri dishes; pH indicator strips:** Macherey-Nagel; **TLC Silica gel 60 F₂₅₄:** Merck;

2.2 General Equipment

Allegra™ 21R Centrifuge: Beckman Coulter™; **Allegra™ X-30R Centrifuge:** Beckman Coulter™; **Gel Documentation platform:** PHASE;

HPLC 10A model: Shimadzu, **Specification:**

Auto sampler: SIL-10AD VP

Column oven: CTO-10AS VP

Controller: SCL-10A VP

Guard Cartridge Holder with Cartridge: Phenomenex AQ-C18 (4.0 × 3.0)

Fluorescence detector: RF-10A XL

Pumpe A: LC-10AT VP

Pumpe B: LC-10AD VP

HPLC Column, NUCLEODUR® 100-5 C18 ec, 250mm length × 4mm ID (internal diameter):

Macherey-Nagel; **Incubator Shaker:** Infors HT Minitron; **NANODROP 2000**

Spectrophotometer: Thermo Scientific; **Programmable Thermal Controller, PTC-100™:** MJ

Research, Inc.; **PTC-150 Mini Cycler™:** MJ Research; **UVIKON 930 spectrophotometer:**

Kontron.

2.3 Chemicals

If not otherwise mentioned, chemicals were purchased from Carl Roth GmbH, Germany.

3-(2-Hydroxyethyl)indole/tryptophol (TOL/C₁₀H₁₁NO): Sigma-Aldrich; **Acetic acid (CH₃COOH)**; **Acetonitrile (C₂H₃N)**, HPLC Gradient grade; **Agar**; **Agarose**; **Ammonium iron(II) sulfate hexahydrate ([Fe(NH₄)₂(SO₄)₂)]**: Sigma-Aldrich; **Ammonium nitrate (NH₄NO₃)**; **Biotin**; **Boric acid (H₃BO₃)**: Merck; **Calcium chloride (CaCl₂)**; **Citric acid (C₆H₈O₇)**; **Chloroform (CHCl₃)**; **Copper(II) Sulfate (CuSO₄, 5H₂O)**; **Dimethylaminobenzaldehyde (DABA)**; **Ethanol (C₂H₆O)**, HPLC grade; **Ethyl Acetate (C₄H₈O₂)**, HPLC grade; **Ethylenediaminetetraacetic acid Disodium Salt Dihydrate (EDTA, Disodium Salt, Dihydrate)**; **Ferric chloride (FeCl₃)**: Merck; **Fructose (C₆H₁₂O₆)**; **Glucose (C₆H₁₂O₆)**; **Histidine (His/C₆H₉N₃O₂)**: Sigma-Aldrich; **Hydrochloric acid (HCl)**; **Indole-3-acetamide (IAM/C₁₀H₁₀N₂O)**: Sigma-Aldrich; **Indole-3-acetic acid (IAA/C₁₀H₉NO₂)**: Sigma-Aldrich; **Indole-3-acetonitrile (IAN/C₁₀H₈N₂)**: Sigma-Aldrich; **indole-3-lactate (ILA/C₁₁H₁₁NO₃)**: Sigma-Aldrich; **Indole-3-pyruvic acid (IPA/C₁₁H₉NO₃)**: Sigma-Aldrich; **L-Tryptophan (L-Trp/C₁₁H₁₂N₂O₂)**: Sigma-Aldrich; **Magnesium sulfate (MgSO₄)**; **Manganese Sulfate (MnSO₄)**: Merck; **Methanol (CH₃OH)**; **Monopotassium phosphate (KH₂PO₄)**; **peqGOLD TriFast**: peqlab; **Potassium nitrate (KNO₃)**: Merck; **Sodium molybdate, (Na₂MoO₄)**; **Sodium chloride (NaCl)**: Merck; **Sorbitol (C₆H₁₄O₆)**; **Sorbose (C₆H₁₂O₆)**; **Sucrose (C₁₂H₂₂O₁₁)**; **Sulfuric acid (H₂SO₄)**; **Trisodium citrate (Na₃C₆H₅O₇)**; **Tween 20**; **Zinc sulfate (ZnSO₄)**: Merck; **Tryptamine (TAM/ C₁₀H₁₂N₂)**: Sigma-Aldrich;

2.4 Fluorescent dyes

SynaptoRed™: Sigma-Aldrich; **Fluorescent Brightner 28 (Calcufluor)**: Sigma-Aldrich; **3,3'-Dihexyloxycarbocyanine Iodide [DiOC6(3)]**: Sigma-Aldrich;

2.5 Buffers and Solutions

Tris HCl	121.14 g Tris is dissolved in distilled water with total volume of 1000ml (pH 8.0)
0.5 M EDTA/250 ml	46.53 g EDTA (Disodium Salt, Dihydrate) is dissolved in distilled water with total volume of 250ml (pH 8.0)
TAE buffer	40mM Tris, 20 mM acetic acid, 1 mM EDTA (pH 8.0)
TE buffer	1 mM Tris (pH 8.0), 0.1 mM EDTA
DNA lysis buffer/100ml	0.12 g Tris, 37mg EDTA, 1.16 g NaCl, 10ml 20% SDS (pH 8.0)
8M Ammonium acetate/100ml	61.664 g Ammonium acetate is dissolved in distilled water with total volume of 100ml
Trace Element Solution	5g Citric acid, 5 g ZnSO ₄ , 1 g Fe(NH ₄) ₂ (SO ₄) ₂ , 0.25 g CuSO ₄ , 0.05 g MnSO ₄ , 0.05 g H ₃ BO ₃ , 0.05 g Na ₂ MoO ₄ , for total volume of 100 ml. Store by adding 2 ml chloroform top on the solution at -20°C

2.6 Chemical reagents

Ehmann reagent	van Urk Reagent : Salkowski-Reagent in 1 : 3
Salkowski-Reagent	1.015g FeCl ₃ · 6H ₂ O dissolved in 250 ml H ₂ O (dist) and add 150 ml concentrated H ₂ SO ₄ . (Caution: Always put acid in water. As this is an exothermic reaction so always set the reaction on ice.)
TLC running buffer	Chloroform : Methanol : Water in 84 : 14 : 1
Van Urk reagent	1g Dimethylaminobenzaldehyde (DABA) is dissolved in 50 ml concentrated HCL and add 50 ml absolute ethanol. (Note: This reagent is stable for several months at room temperature when stored in a brown glass bottle.)

2.7 *Neurospora* Culture media

Sorbose/glucose/fructose medium (SGF) (20×) solution	20 g Sorbose, 0.5 g Glucose, 0.5 g Fructose for total volume of 100 ml
TOP-Agar	For each 100 ml of total media, 2 ml 50× Vogel's salts, 2 g Agarose volume is made up to 95 ml. Autoclaved @ 121°C for 20 minutes then added 5 ml SGF
Vogel's salts (50×) (VSL)	150 g Na ₃ citrate, 250 g KH ₂ PO ₄ , 100 g NH ₄ NO ₃ , 10 g MgSO ₄ , 5 g CaCl ₂ , 5 ml Trace Element Solution, 25 mg Biotin for total volume of 1000 ml. Store by adding 2 ml chloroform top on the solution
Vogel's Minimal Medium (VMM)	2 ml 50× Vogel's salts, 2 g Sucrose, (2 g Agar for solid media) for total volume of 100 ml
Westergaard solution (4×)	4 g KNO ₃ , 4 g KH ₂ PO ₄ , 4.1 g MgSO ₄ , 0.8 g CaCl ₂ , 0.4 g NaCl 400 µl Trace Element Solution, 10 mg Biotin for total volume of 1000 ml. Store by adding 2 ml chloroform top on the solution
Westergaard crossing media	25 ml 4× Westgaard crossing solution, 2 g Sucrose, adjust pH 6.5, (2 g Agar for solid media) for total volume of 100 ml

2.8 Detailed specification of purchased *Neurospora crassa* strains

Serial	FGSC Number	Genotype/ Locus	Classical enzyme names of the genes those are knocked out	<i>N. crassa</i> specific enzyme names for the genes those are knocked out	Mating Type	Linkage Group	Reference
1.	2489	<i>N. crassa</i> 74-OR23-1VA	-		A	-	Mylyk et al 1974. N.N. 21:24
2.	4200	<i>N. crassa</i>	-		a	-	Kafer & Fraser 1979 Mol Gen Genet 169:117-127
3.	12555	NCU06112	<i>tdc2</i> Or <i>trpdc2</i>	<i>gdc3</i>	a	VII	Colot HV et al. ProcNatlAcadSci U S A. 2006 103:10352-7
4.	12556	NCU06112	<i>tdc2</i> Or <i>trpdc2</i>	<i>gdc3</i>	A	VII	Colot HV et al. ProcNatlAcadSci U S A. 2006 103:10352-7
5.	12919	NCU00378	<i>iad3</i>	<i>ahd2</i>	a	III	Colot HV et al. ProcNatlAcadSci U S A. 2006 103:10352-7
6.	12921	NCU03415	<i>iad1</i>	<i>cbs3</i>	a	II	Colot HV et al. ProcNatlAcadSci U S A. 2006 103:10352-7
7.	12922	NCU03415	<i>iad1</i>	<i>cbs3</i>	A	II	Colot HV et al. ProcNatlAcadSci U S A. 2006 103:10352-7
8.	14354	NCU09648	<i>iad2</i>	<i>ahd3</i>	A	IV	Colot HV et al. ProcNatlAcadSci U S A. 2006 103:10352-7
9.	16277	NCU04092	<i>ami1</i>	<i>naa2</i>	A	VI	Colot HV et al. ProcNatlAcadSci U S A. 2006 103:10352-7
10.	18065	NCU02193	<i>ipd</i>	<i>cfp</i>	a	VII	Colot HV et al. ProcNatlAcadSci U S A. 2006 103:10352-7
11.	18066	NCU02193	<i>ipd</i>	<i>cfp</i>	A	VII	Colot HV et al. ProcNatlAcadSci U S A. 2006 103:10352-7
12.	18224	NCU08275	<i>tdc1</i> Or <i>trpdc1</i>	<i>aad1</i>	a	IV	Colot HV et al. ProcNatlAcadSci U S A. 2006 103:10352-7
13.	18225	NCU08275	<i>tdc1</i> Or <i>trpdc1</i>	<i>aad1</i>	A	IV	Colot HV et al. ProcNatlAcadSci U S A. 2006 103:10352-7
14.	20239	NCU01106	<i>iaam</i>	<i>lao2</i>	a	V	Colot HV et al. ProcNatlAcadSci U S A. 2006 103:10352-7
15.	21462	NCU03755	<i>yuc</i>	<i>mox2</i>	a	V	Colot HV et al. ProcNatlAcadSci U S A. 2006 103:10352-7

Serial	FGSC Number	Genotype/Locus	Classical enzyme names of the genes those are knocked out	<i>N. crassa</i> specific enzyme names for the genes those are knocked out	Mating Type	Linkage Group	Reference
16.	21463	NCU03755	<i>yuc</i>	<i>mox2</i>	A	V	Colot HV et al. ProcNatlAcadSci U S A. 2006 103:10352-7
17.	22136	NCU09116	<i>tam1</i>	<i>aro8</i>	A	I	Colot HV et al. ProcNatlAcadSci U S A. 2006 103:10352-7

2.9 General computer programs

Microsoft Excel 2007 and 2013: Microsoft; Microsoft Word 2007 and 2013:

Microsoft; Microsoft PowerPoint 2007 and 2013: Microsoft; Photoshop CS: Adobe;

Windows 7 Professional: Microsoft. GelDocImager: PHASE GmbH;

2.10 Scientific tools

Clone Manager: Clone Manager Professional; GeneDock; Geneious R7: Biomatters Ltd.; Olympus

Soft Imaging Solutions: Olympus; PyMol: Open source, distributed by distributed by Schrödinger;

YASARA View: www.YASARA.org; MEGA; MrBayes

2.11 Databases and servers

Name of Database/Server	Link
ALTER	http://sing.ei.uvigo.es/ALTER/
BioLiP	http://zhanglab.ccmb.med.umich.edu/BioLiP/
BLAST	http://blast.ncbi.nlm.nih.gov/
BLAST2GO	https://www.blast2go.com/
BROAD INSTITUTE	http://www.broadinstitute.org/
COFACTOR	http://zhanglab.ccmb.med.umich.edu/COFACTOR/
FungiDB	http://fungidb.org/fungidb/
Fungal Genetics Stock Center	http://www.fgsc.net/
I-TASSER	http://zhanglab.ccmb.med.umich.edu/I-TASSER/
MUSCLE	http://www.ebi.ac.uk/Tools/msa/muscle/
NCBI	http://www.ncbi.nlm.nih.gov/
PDB	http://www.rcsb.org/pdb/home/home.do
PDBsum	https://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum/GetPage.pl?pdbcode=index.html
Pfam	http://pfam.xfam.org/
PROCHECK	http://www.ebi.ac.uk/thornton-srv/software/PROCHECK/
PubChem	https://pubchem.ncbi.nlm.nih.gov/
SWISS MODEL	http://swissmodel.expasy.org/

3 METHODS

3.1 Methods of computational studies

3.1.1 Sequence retrieval and alignments

Homologous protein sequences of the enzymes present in IAA producing pathways from different fungal species were obtained by BLAST (ALTSCHUL *et al.* 1990) analysis. Targeted enzymes were tryptophan aminotransferase (TAM1), indole-3-pyruvic acid decarboxylase (IPD), indole-3-acetaldehyde (IAld) dehydrogenase (IAD), flavin monooxygenase (YUC), indole-3-acetamide (IAAM), amidohydrolase (AMI1) and NCU00589 (putative IAA transporter). First *Neurospora crassa* protein sequences for the above mentioned enzymes and transporter were determined using Broad Institute database (<http://www.broadinstitute.org>) and FungiDB (<http://fungidb.org/fungidb/>). Then homologous sequences in other species were checked and retrieved by bidirectional BLAST using both Broad Institute database and National Centre for Biotechnology Information (NCBI) (WHEELER *et al.* 2006; WHEELER *et al.* 2007). Data on these sequences in different species were documented as per the NCBI and Broad Institute database annotation. Same bidirectional BLAST analysis was used by using Blast2GO tools (CONESA *et al.* 2005) to find annotated name for the corresponding protein sequences in *N. crassa*. In the Broad Institute database, TAM1, IPD, IAD1, YUC, IAAM, AMI1 and NCU00589 are annotated as “aromatic aminotransferase Aro8”, “pyruvate decarboxylase”, “aldehyde dehydrogenase”, “Flavin dependent monooxygenase”, “L-amino acid oxidase”, “N-acylethanolamine amidohydrolase” and “auxin Efflux Carrier superfamily” respectively. Only full length sequences of above mentioned proteins from different fungal species were considered for all the analysis performed further. Sequences were aligned using MUltiple Sequence Comparison by Log-Expectation (MUSCLE) (EDGAR 2004a; EDGAR 2004b) and the alignments were visualized, edited, and presented using GeneDoc (NICHOLAS 1997). It was found that same genes are annotated differently in different databases. For that reason common enzyme names are used for their corresponding genes. A detailed gene identity with their annotations are given in strain specification in section 2.8.

3.1.2 Pfam analysis of the selected protein sequences

After retrieving the selected protein sequences from different databases only *Neurospora crassa* protein sequences were used for Pfam (SONNHAMMER *et al.* 1997) analysis to find the protein classification. In Pfam database it was found that TAM1, IPD, IAD1, YUC, IAAM, AMI1 and NCU00589 are designated and described as “Aminotransferase class I and II” domain, “Thiamine pyrophosphate enzyme” domain, “Aldehyde dehydrogenase” family, “Flavin-binding monooxygenase-like” family, “Flavin containing amine oxidoreductase” domain, “amidase” family and “Membrane transport protein” family respectively.

3.1.3 Construction of phylogenetic trees

For construction of phylogenetic trees only full length protein sequences from different species were considered to avoid “noise” and misinterpretation of the result that could happen just due to unavailability of the full length sequence. Sequences were first aligned and tested in MEGA (TAMURA *et al.* 2011) to generate best protein model by Maximum Likelihood. Then on the basis of scoring best model was chosen for further tree construction. The Bayesian approach was implemented to construct Bayesian phylogenetic trees for all the proteins analyzed here. The tree constructions were consisted of 5 runs, 7500,000 generations with 25% burn-in-period. For this Bayesian approach MrBayes 3.2 program (RONQUIST *et al.* 2012) was used. Constructed trees were imported in Geneious work-platform to visualize, edit and represent graphically.

Note: Before generating the final trees in the MrBayes, one round of “mock” tree construction was done in MEGA with Maximum Likelihood to check for the overall tree structure and any discrepancies if there are any due to sequence error.

3.1.4 Structure analysis of individual enzymes

For structural analysis, two independent methods were implemented to establish the consistency and reliability of each method as well as the structure. One of them was a hierarchical method using multiple threading approach in the I-TASSER (<http://zhanglab.ccmb.med.umich.edu/I-TASSER/>) online-server (ZHANG 2008) and the other one was "SWISS-MODEL Repository portal" (<http://www.expasy.org/>) for homology modeling (GUEX and PEITSCH 1997). For the hierarchical method, corresponding enzyme sequences from *N. crassa* were submitted to the I-TASSER server and the best model was chosen on the basis of C-scores. To choose a proper

homologous structural template, individual enzymes were analyzed using BLAST against the PDB data base (www.rcsb.org) prior to the homology modeling. Suitable models were chosen for homology modeling based on the BLAST scores. Then cofactor binding properties were predicted and cross-checked in the predicted models by assessing the "structure-based function prediction" using the COFACTOR online server (<http://zhanglab.ccmb.med.umich.edu/COFACTOR/>) (ROY *et al.* 2012) for all the enzymes evaluated in this study. COFACTOR makes predictions about the ligand and its binding sites on the structures. The reliability of ligand binding is defined by the BS-score. A score >1 reflects significant ligand binding in the structure. Ligand-binding sites present on both *Neurospora* enzymes and template structures (those used for homology modeling) were identified by aligning with known proteins. Finally, enzyme structures those were predicted from I-TASSER were aligned with template structures from the PDB. This structural alignment helps to obtain information about key residues inside enzymatic catalytic sites. As an integral part of the multiple threading a model based on functional assessment is also derived from protein function database BioLiP (<http://zhanglab.ccmb.med.umich.edu/BioLiP/>). For structure analysis of the auxin carrier from *N. crassa* it was not possible to use any known auxin transporter as a template structure. For that reason rather a comparative approach was taken where auxin carriers from both *N. crassa* and *Arabidopsis thaliana* were independently subjected for multiple threading modeling in I-TASSER. After that both of the structures were evaluated independently to find a generalized pattern in the predicted auxin carriers.

3.1.5 Searching for ligand binding site(s) at the sequence level

For every enzyme to be functional, a specific ligand has to bind to the catalytic site/s of the enzyme. For a class of enzymes the ligands are also same. Information about ligand binding sites of the similar class/group of enzymes can be found in the published literatures. In this study an intensive literature search has been done to find out the key amino acid residues that bind to the specific group of ligands. First the key residues were identified in *Neurospora* protein sequence and then a multiple sequence alignment has been performed to find the degree of conservation of those amino acid residues in other fungi.

3.1.6 Structure optimization & validation

All the predicted structures generated by I-TASSER or SWISS-MODEL were evaluated in the PDBsum database using PROCHECK analysis (LASKOWSKI *et al.* 1993). The amino acid distribution from Ramachandran plots of I-TASSER, and SWISS-Model predicted structures were obtained and analyzed. As two independent approaches (multiple threading and homology modeling) were used for structure prediction, the consistency and reliability of the models were further checked by structural alignment within the predicted models themselves.

3.1.7 Molecular visualization

All the molecular visualization and editing was done using PyMol (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC.) and YASARA (<http://www.yasara.org/>). Images were processed using POV-Ray (<http://www.povray.org/>) (BUCK).

3.1.8 Structure alignment & marking the key amino acid residues

Structural alignments were done between template structures where the position of key amino acid residues are known and predicted structure of *Neurospora* enzymes. Visualization, editing and graphical representation was done using either in PyMol or in YASARA. A comprehensive graphical illustration about the whole computational approach has been explained in Fig. 4.

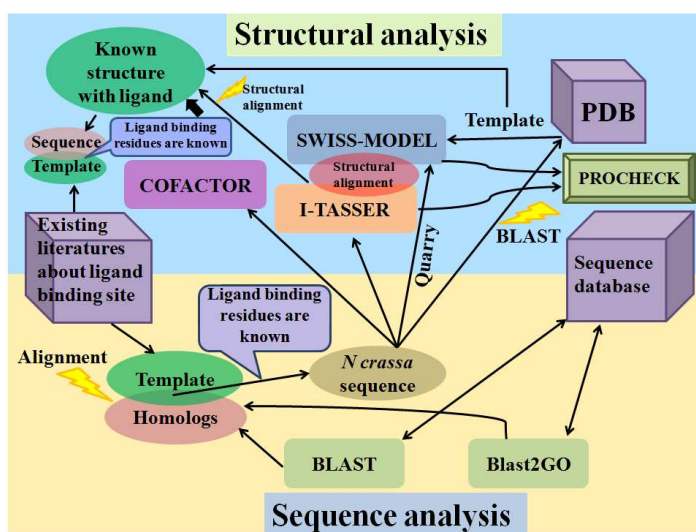


Fig. 4: Schematic representation of methods on how computational approach was taken to find the candidate gene/protein involved in auxin biosynthesis pathway in *Neurospora crassa*.

3.2 Methods of experimental studies

3.2.1 General methods of culturing, crossing & growing *N. crassa*

3.2.1.1 Culturing of *Neurospora crassa*

All the experiments were performed with the fungal species *Neurospora crassa*. Depending on the experiments fungi were cultured either in liquid or solid medium at temperature 25°C or 28°C as per experimental need. Solid agar medium in slant-glass tubes containing VMM-sucrose with cotton plugs were used for maintaining the stock or storing the strains for further use. In case of stocking or storing, fungi were kept in -20°C after optimal amount of growth in the glass tubes. A list of fungal strains those were studied is given in section 2.8.

3.2.1.2 Crossing

A wide range of different crosses were set among different strains depending on their compatible mating types. For this purpose wild type as well as single and double knock-out strains were used. All the crossings were set on “Westergaard crossing medium”. To set a cross two different approaches were taken (i) strains with different mating types were placed ~180° apart from each other (Fig. 5) and (ii) one strain was cultured on plate containing “Westgaard crossing medium” and another strain with different mating type was cultured in a tube containing “minimal medium.” When both of the strains started to produce conidia spores then spores from the tube were flushed top on the plate where another strain is already growing (Fig. 6) and allowed to grow in 25°C. After ~7-8 days when the fungi start to eject the ascospores, lid of the petri dishes are changed and put new lid to avoid the extra amount of moistures and collect the ascospores on the new lid. Finally ~14 days after setting of cross the ascospores were collected for further analysis. Before final collection of the ascospores the perithecia were checked for any anomalies in spore formation. Crosses were also set in presence of tryptophan (400mg/L or 1.96 mM concentration) to check if there is any changes on crossing patterns or ascospore formation. For that purpose tryptophan was dissolved in water and stored as stock solution and added to the molten “Westergaard crossing media” at right quantity before pouring into the plates.

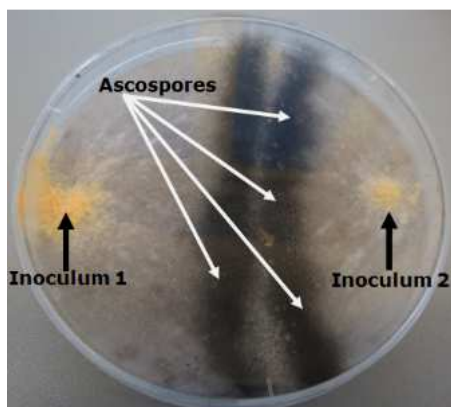


Fig. 5: Example of procedure of setting crosses. Two fungal inoculums with different matting types are placed on agar media in a petri dish. Over time ascospores are accumulated on the lid of the petri dish. Generally ascospores are formed across the line where two strains meet.

Single knock-out strains were purchased from the Fungal Genetics Stock Center (FGSC; Kansas City, MO, USA) (<http://www.fgsc.net/>). The single knock-out strains were created using *hph* cassette exchange through homologous recombination technique (COLOT *et al.* 2006). A schematic of the technique is given in Fig. 7. 5' and 3' flank DNA regions of the targeted gene (blue color) are amplified separately from the genomic DNA with primer set 5f+5r and 3f+3r. Primers 5r and 3f are having MmeI sites (M) and 5' tails homologous to the *hph* cassette (green thin lines), where 5f and 3r primers are having 5' tails homologous to the vector (yellow thin lines).

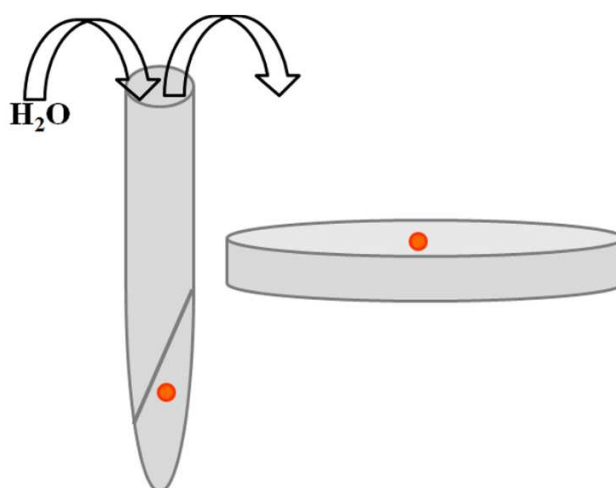


Fig. 6: A schematic diagram of reciprocal cross by flushing the conidia spore from a slant onto the fungi growing in a plate containing “Westgaard crossing media”. Fungal strains with different matting types are represented with orange dots.

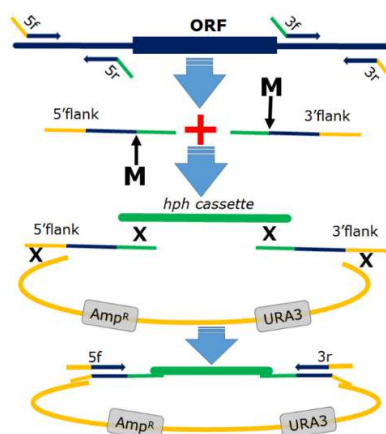


Fig. 7: Schematic representation of the technique to knock-out a gene in *Neurospora crassa*. Figure idea was derived from Colot, H. V. et.al. 2006 (COLOT *et al.* 2006)

After PCR amplification of these two flank regions they are co-transformed into yeast along with the *hph* cassette (green thick lines) and gapped yeast shuttle vector (yellow thick lines). Homologous recombination turns these four elements to the circular construct. Regions where homologous recombination should take place are marked with black cross marks. Finally linear deletion cassette is amplified using 5f and 3r primers. Thus knocking out a targeted gene was possible with this strategy and all the single knock-out strains used in this study were generated by this method. While setting a cross between two single knock-out strains it was made confirmed that two of the targeted genes are present in two different linkage group.

3.2.1.3 Ascospore germination & culturing fungi from single spore

When the ascospores are ejected out and collected on the lower surface of the petri dish's lid, the lid is removed carefully, flushed the ascospores and collected in a 1.5 ml tube. Then the spores are counted and diluted such a way that in a plate there would be 100 spores. Then the spores are mixed with top agar (temperature 60°C) in a glass tube and poured in petri dish. Alternatively after counting and diluting the spores, the tubes with spores are incubated in 60°C for 30 – 60 minutes. Then spores are inoculated on the SGF-agar plate and plated with such an amount that there would be 100 spores per plate. After 1-2 days of germination each ascospore will form a tiny fungal colony made of mycelium. Carefully those isolated colonies are cut out of the SGF-agar plate and transferred into VMM slant tubes and kept in 25°C for few days until they grow well and produce conidia spore.

3.2.1.4 Race tube experiment

Race tube experiment was performed to determine the differential growth rate among different single and double knock-out strains compare to the wild type strain (Fig. 8). First, long agar slant tubes were prepared containing VMM medium. A little fungal inoculum is placed at one end of the tube (opposite end of the tube from where agar is poured) and allowed to grow. The initial position is marked as the starting point and on regular time intervals the tubes are marked on the basis of the growth on fungi inside the tubes. The tubes are kept in the growth chamber at 25°C.

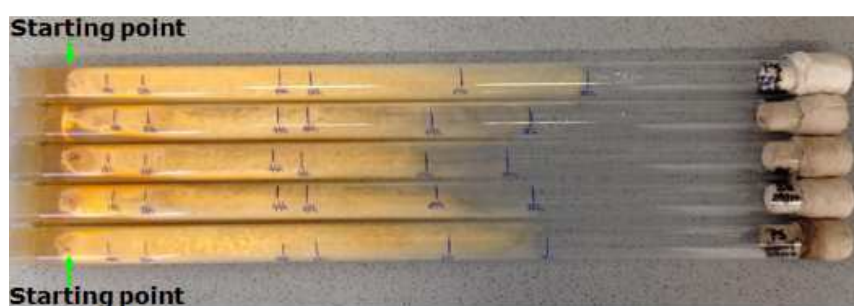


Fig. 8: One example of the race tube experiments. Fungi are growing inside the tube and on a regular time interval growth of the fungi was marked on the outside of the tubes.

Note: As shown in the picture, one end of the tube should be capped with cotton plug only. Otherwise improper aeration will affect the fungal growth.

3.2.2 General molecular biology methods

3.2.2.1 Genomic DNA isolation from *Neurospora crassa*

Fungi were first grown in liquid medium (50 ml VMM+S), @25 ° C @ 150 rpm for 3-4 days. Then mycelium were isolated from the medium by filtering through layers of cheesecloth and placed in liquid nitrogen followed by grinding in a mortar with a pestle. The powdery mycelium was collected in 13 ml tubes. Then 2.5 ml of lysis buffer (10 mM Tris- HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 2 % SDS) is added and mix well or vortex till the powder form of the mycelium is dissolved properly. 2.5 ml of phenol is added to that and centrifuge @ 5500 rpm for 10 minutes. Then carefully the supernatant was mixed with Phenol: Chloroform: Isoamyl Alcohol (25: 24: 1, v/v) and centrifuge @ 5500 rpm for 10 minutes. This step is performed twice. After that

the supernatant was mixed with chloroform - isoamyl alcohol and centrifuge @ 5500 rpm for 10 minutes only once. The supernatant was collected in a tube and 5 μ l of RNase A (20 mg / ml) is added to it, and incubated for 30 minutes to 1 h at 37 ° C. Then once again the supernatant was mixed with Phenol: Chloroform: Isoamyl Alcohol (25: 24: 1, v/v) and centrifuge at 5500 rpm for 5 minutes. The supernatant was taken out and mixed with Chloroform - Isoamyl alcohol and centrifuge at 5500 rpm for 5 minutes. Then 1/10 volume of 8 M ammonium acetate is added to the supernatant and mixed well and added 2 volume of absolute ethanol to it and kept for 30 minutes at -80 ° C (or 2 h incubation at -20°C) then centrifuged at 4800 rpm for 15 minutes. Once the centrifuge is over the tubes were taken out carefully and washed the pellets with 5 ml of 70 % EtOH and kept left for drying. Finally the pellets were dissolved in 300 μ l of 10 mM Tris- HCl pH 8.0. OD was measured and the DNA was stored in -20°C. Alternatively DNA isolation for screening purpose in search of positive knock-out strains was done using Quick-DNA™ Fungal/Bacterial Miniprep Kit (ZYMO RESEARCH, USA)

Note: All the reactions should be done under chemical hood only. After washing the pellets with 70 % EtOH the pellets should be allowed to dry but not too much dried.

3.2.2.2 Measurement of the DNA concentration with NanoDrop

To measure the DNA concentrations, NanoDrop 2000 Spectrophotometer was used. 2 μ l of the DNA sample volume was used to determine the concentration. Absorption was measured at the wave length of 260 nm. Absorption coefficient $A_{260} = 1.0$ corresponds to pure dsDNA content of 50 μ g/ml, with a layer thickness of $d = 1$ cm. The ratio of sample DNA absorbance at 260 and 280 nm was used to assess the purity of the sample. A ratio of about 1.8 is generally accepted as a good quality of DNA sample.

3.2.2.3 Primer designing for polymerase chain reaction (PCR)

Gene sequences of the individual genes were extracted from various database and imported in “Clone Manager” platform for further analysis. An optimal primer pair of length 20 nucleotide bases each was chosen for a particular PCR reaction. All the primer pairs were tested for primer

dimer, self-annealing, hairpin and optimal GC content prior to use. Details of all the primers are given in table 2.

3.2.2.4 Polymerase chain reaction (PCR)

The polymerase chain reaction is a routinely used molecular technique for researchers to specifically amplify a particular DNA fragment (BARTLETT and STIRLING 2003) with a set of oligonucleotide pair. The DNA template is first denatured and specific oligonucleotide/s bind to their complimentary sequences. Then using DNA polymerase the complimentary strands are synthesized. The chain reaction of denaturing template DNA, binding with complimentary oligonucleotide and extension of strands by DNA polymerase leads to exponential enriching of targeted DNA fragment. The composition of PCR reaction and PCR condition (temperature, duration etc.) is directly related to the length of the targeted sequence, the amount of initial DNA template and nucleotide base composition of the oligonucleotides. To amplify short fragment of genomic DNA (~550 – 1100 bp) Taq polymerase was used. Following reaction mixture was used for each 50 μ l of PCR reaction:

Reagents	Amount
10 \times PCR-Buffer + MgCl ₂	5.0 μ l
dNTP Mix (10mM)	1.0 μ l
Oligonucleotide 1 (5 pmol/ μ l)	1.0 μ l
Oligonucleotide 2 (5 pmol/ μ l)	1.0 μ l
PCR-Enhancer	0.5 μ l
Taq-Polymerase (5U/ μ l) in μ l	0.25 μ l
Genomic DNA (10 – 200 ng)	As per the DNA concentration
DD H ₂ O	Up to overall volume 50 μ l

After mixing all the reagents, the reaction was put in 50 μ l PCR tube and placed properly inside a thermal cycler. For DNA amplification always hot start method was used where the initial temperature of the reaction mixture is taken to higher in order to start the enzymatic activity of the Taq polymerase. Following thermal cycles were used for the PCR:

PCR condition that was used for amplifying targeted DNA fragments

Step	Temperature (°C)	Duration (Minute)	
Hot start	95	5	
Denaturation	95	1	45 Cycles
Annealing	Depends on the primer sets used	1	
Extension/elongation	72	2	
Final extension	72	10	

3.2.2.5 Confirmation for the knock-out strains

Confirmation for each knock-out strains regarding a positive knock-out was carried out by two way approach. First, PCR was done using the primer pair specific of middle part of the ORF. Secondly another primer pair was used which specifically amplify 5' UTR region of the targeted gene along with the *HygB* gene fragment (Fig. 7). For the second confirmation primer sets were derived from the information given in “FGSC Strain Database” regarding Primers used to make knock-out strains (http://www.fgsc.net/NeurosporaGenomeProject/primers_for_1kb_flanks.xls). After second PCR confirmation amplified PCR products were sent for “Sanger sequencing” to crosscheck the obtained results. For amplifying the 5'UTR respective to the ORF of the targeted genes the reverse primer (5'-ATCCACTTAACGTTACTGAAATCTCCAAC-3') which starts to amplify from the *HygB* gene is always the same and the forward primers are gene specific as targeted genes are different. Using primer pairs where one primer is always constant and hygromycin cassette specific and another is gene specific increase the authenticity of a knock-out strain in case found one.

Table 2. Description of primers used in PCR to check for the knockout strains

Sr. No.	Primer name	Sequence (5' ----> 3')	Description
1.	PS2596	CTCGAGTCAACCAGTCCGAAGGGC	Fwd. NCU00589 knock out, 5' flanking
2.	PS2597	GTCGACTGAGGCAGACGGGTTGAAAG	Rev. NCU00589 knock out, 5' flanking
3.	PS2598	GGCGCGCCGTATCGAACCACTTCAATC	Fwd. NCU00589 knock out, 3' flanking
4.	PS2599	ATTTAAATGGCCTCAGCCGGCAACACC	Rev. NCU00589 knock out, 3' flanking
5.	PS2660	TCGAATCCATCACCTCAAG	Fwd. NCU09116 ORF Posn. 392 (<i>tam1</i>)
6.	PS2661	AATACGGCTCGTCCTCAATC	Rev. NCU09116 ORF Posn. 1000 (<i>tam1</i>)
7.	PS2662	AAACCTCTACCGGTCTCTTC	Fwd. NCU01106 ORF Posn. 773 (<i>iaam</i>)
8.	PS2663	CGTCTTTAGAGGGCAAAGTC	Rev. NCU01106 ORF Posn. 1390 (<i>iaam</i>)
9.	PS2670	AACCCGGTTCACGGTACTCC	Fwd. NCU04092 ORF Posn. 796 (<i>ami1</i>)
10.	PS2671	AGGTCAGGTTGCCGGTTTCC	Rev. NCU04092 ORF Posn. 1375 (<i>ami1</i>)
11.	PS2738	TAGGAGGGCGTGGATATGTC	Fwd. HygB ORF posn. 122
12.	PS2739	CGTCTGCTGCTCCATACAAG	Rev. HygB ORF posn. 738
13.	PS2740	GCAGAACCAATGACCTTAGC	Fwd. to amplify NCU01106 (<i>iaam</i>) 5' UTR along with HygB gene
14.	PS2741	ATCCACTTAACGTTACTGAAATCTCCAAC	Rev. specifically on HygB gene
15.	PS2742	CCTGAACTGGTATCGAAAGG	Fwd, to amplify NCU09116 (<i>tam1</i>) 5' UTR along with HygB gene
16.	PS2743	ATGCTATCTGACCTGAGACG	Fwd. to amplify NCU04092 (<i>ami1</i>) 5' UTR along with HygB gene
17.	PS2755	CAAGTGCGCTGCCGAGTATC	Fwd. (NCU02193) ORF posn 615 (<i>ipd</i>)
18.	PS2756	CGACGAACAGGCTGGTCTTG	Rev. (NCU02193) ORF posn 1171 (<i>ipd</i>)

19.	PS2757	AAGTGTTAGGTCGCAGTAGG	Fwd. to amplify NCU02193 (<i>ipd</i>) 5' UTR along with HygB gene
20.	PS2760	CCTATGATGCCGTTGTCGTG	Fwd. (NCU03755) ORF posn 521 (<i>yuc</i>)
21.	PS2761	AAATCGCTGCCTGACTCTCC	Rev. (NCU03755) ORF posn 1078 (<i>yuc</i>)
22.	PS2809	GCTCATTACGGTCTGTTCC	Fwd. to amplify NCU03755 (<i>yuc</i>) 5' UTR along with HygB gene
23.	PS2811	CTATTGATCGTCAGCCAAGG	Fwd. to amplify NCU03415 (<i>iad1</i>) 5' UTR along with HygB gene
24.	PS2919	CTGGCGTTTCTCAACATCATC	Fwd. Primer for <i>iad1</i> (NCU03415) ORF Posn. 638
25.	PS2920	GATGAAGTAGCCCTTGTCAC	Rev. Primer for <i>iad1</i> (NCU03415) ORF Posn. 1137
26.	PS2928	AACTACCCGCTAGATATGGC	Fwd primer for mid part of <i>iad3</i> ORF
27.	PS2929	CCTCTTCCTTTCCGACATTG	Rev primer for mid part of <i>iad3</i> ORF
28.	PS2930	GTTGGACGACGAGGTGATTG	Fwd primer for mid part of <i>iad2</i> ORF (posn. 454)
29.	PS2931	CGCCGAACTTGAACGTCTTG	Rev primer for mid part of <i>iad2</i> ORF (posn. 1028)
30.	PS2932	CAAGTACCTGCGCGAGACTG	Fwd primer for mid part of <i>trpdc1</i> ORF (posn. 638)
31.	PS2933	CGTACACGGCCTGGTTGATG	Rev primer for mid part of <i>trpdc1</i> ORF (posn. 1218)
32.	PS2934	CGGTCAAGTCCCTAATCATC	Fwd primer for mid part of <i>trpdc2</i> ORF (posn. 242)
33.	PS2935	GAGCACTGGTGAGAAGAATC	Rev primer for mid part of <i>trpdc2</i> ORF (posn. 799)
34.	PS2936	AGGTACCAACAGTACCATGC	Fwd. To amplify NCU00378 (<i>iad3</i>) 5' UTR along with HygB gene
35.	PS2937	GTCAGCAGAATACTTGGACC	Fwd. To amplify NCU09648 (<i>iad2</i>) 5' UTR along with HygB gene
36.	PS2938	ATTTGTAGTGGCACAGCTCC	Fwd. To amplify NCU08275 (<i>trpdc1</i>) 5' UTR along with HygB gene
37.	PS2939	CCTAGAGGTACCTAATCACG	Fwd. To amplify NCU06112 (<i>trpdc2</i>) 5' UTR along with HygB gene

3.2.3 Microscopy

3.2.3.1 Plate preparation & inoculation

For microscopy very thin VMM-agar plates were prepared. Very little amount of molten agar medium was poured onto the plate and dispersed it by tilting the plate in a circular way. It was made sure that the medium was enough to cover the whole plate. Then the plates were left to cool down and stored in 4°C for later use. Inoculation of fungi on the plate was always done along the diameter of the plate so that fungi can grow on both sides and thus gives more area that can be imaged under microscope. Then the plates were kept in fungal growth chamber at 25°C for ~ 12-14 hours.

3.2.3.2 Sample preparation for microscopy

Both, light and fluorescent microscopy was done by Nikon and Zeiss Axiophot microscope. All the microscopy was live imaging. Depending on the need and situation two types of methods were used for sample preparation for microscopy. First, one small piece of agar was cut and placed on a microscope slide and second, the whole plate containing the fungi was placed under the microscope. For fluorescent microscopy, fluorescent dyes were added onto the fungi on agar slice.

3.2.4 Conidia isolation from *Neurospora crassa* & counting

Conidia or conidia spores of *Neurospora crassa* are means of asexual reproduction of the organism. First *Neurospora crassa* strains were inoculated in 150-200 ml Erlenmeyer flask with 50 ml VMM-Agar medium and kept for 10-21 days at 25°C in fungal growth chamber. Then in the flask 40 ml of 1 M sorbitol + 0.1 ml Tween® 20 solution is poured carefully and sealed with parafilm and shake well to take out all the spores with the solution. Then the mixture of spore, sorbitol and Tween® is poured on gauze and pass through funnel to filter and collected in 50 ml tubes and centrifuge at 4,000 rpm for 5 minutes. After the first round of centrifugation the spores were re-suspend and centrifuge with 50 ml of 1 M sorbitol only. This step is performed twice. Finally the conidia were collected in a 1.5 ml tube with 500 µl of 1 M sorbitol. Then the conidia

were diluted in a ratio of 1:100 and counted by hemocytometer. Formula for conidia counting is:
 n (number of spores in big square) / 4 = $K \times 10^8$ Spore/ml

Note: Mixture of sorbitol and Tween 80™ should not be poured harshly into the flask containing fungal culture. The solution should not touch the bottom of the flask directly. It will produce a “cloud” of spores and that will spread outside of the flask. The solution should be poured gently on the inner wall of the flask and let it flow through the wall to the bottom of the flask.

3.2.5 Culturing *Neurospora crassa* & extraction of indolic compounds for different chromatographic techniques

3.2.5.1 Extraction of indolic compounds from *N. crassa* growing in liquid medium

To maintain the uniformity in number of inoculated spores fungi were inoculated in such a way that there would be $\sim 10^6$ spores in each flask. First fungi were incubated in 100 ml flasks containing 50 ml of liquid VMM medium in the dark at 28°C for 72 – 96 hours @ 150 rpm. (Depending on the mode of study the liquid media was prepared with or without tryptophan). At various time points aliquots of 2 ml are taken from the main culture and stored in the dark at 4°C. Then the culture was filtrated with gauze and the flow through was collected in a 50 ml tube (Alternatively sample is centrifuged at 7000g for 15 minutes and supernatant is collected in another tube). Then pH of the flow through/supernatant is adjusted to 2.8 with 1M HCl. Then sample is mixed with ethyl acetate in a ratio of 1: 1 (Sample: Organic solvent, 1:1). The sample with the ethyl acetate is mixed in a tube and put in shaker at 200 rpm for 3 hours. After phase separation upper ethyl acetate phase was removed and collect in a light- protected (preferably) tube. Then the tubes were placed in a vacuum concentrator to let evaporate the organic phase. Finally the pallet was dissolved either in ethyl acetate (for TLC) or in 25% methanol, 1% acetic acid (for HPLC).

3.2.5.2 Thin layer chromatography (TLC)

Thin layer chromatography or TLC is a very popular and long practiced chromatographic technique among researchers. TLC is used to separate different compounds from a non-volatile mixture. Samples are loaded on a specific plate and the plate is placed in a running buffer. Then the mixture of the compounds also known as mobile phase is migrated up through the plate via capillary effect. Different compound migrates on the plate at different rate and thus the separation

of the compounds is achieved. In that sense TLC is rather a qualitative way to identify the presence of compounds in a given mixture. For separation of indolic compounds in this study, TLC silica gel 60 plate was used. As it is a qualitative method so detectable intensity/amount of compounds is directly proportional to the amount of the compound mixture loaded on the plate. Normally 8 – 10 μ l of sample was loaded on the plate in this study. Then TLC plate was placed on a buffer chloroform: methanol: water (84: 14: 1) in such a way that the bottom part of the plate is merged in the buffer while there is little space between sample loading point and the buffer. The sample is run for ~2 hours. Then the plate was taken out and spray with a mixture of van Urk and Salkowski reagents in the proportion of 1:3. Finally, the TLC plate is incubated at 90°C for ~10 minutes. Then immediately picture of the plate has been captured.

Note: The spray should be as aerosol. It should not create any droplet or smear on the plate. All the experimental steps should be done under chemical hood.

3.2.5.3 High-Performance liquid chromatography (HPLC)

3.2.5.3.1 THEORY

High-performance liquid chromatography or in short HPLC is a technique to identify and quantify different components from a chemical mixture. Formerly it was referred as high-pressure liquid chromatography as the technique uses high pressure to detect the analytes. The HPLC machine pumps a pressurized liquid solvent containing the compounds to be analyzed. Then the solvent is passed through a column that is packed with adsorbent materials and finally comes out of the column where a detector (usually UV, fluorescent, IR etc.) is present to detect the compound. Different compounds have different affinity towards the column matrix and interacts slightly different than the others. Due to this difference in interaction there is a difference in migration time of the compounds through the column which is also known as retention time. For same compound the retention time is always the same under same condition. In common practice first pure standard of the desired compounds in known concentration are run through the HPLC machine to find out the retention time and “peak area”. Then the mixture of unknown sample is run through the machine in order to identify as well as quantify a particular compound in that mixture of solution. For both qualitative and quantitative assessment of indolic compounds in this study a “Reversed Phase-HPLC” (RP-HPLC) technique was employed. Opposite of normal phase chromatography,

reversed-phase chromatography is based on hydrophobic interactions of the molecules. The separation technique depends on the hydrophobic binding of the molecule from the solvent in the mobile phase to the fixed hydrophobic ligands attached to the stationary phase (MCNAUGHT and WILKINSON 1997), known as the adsorbent or sorbent of the column matrix. In reversed-phase chromatography hydrophobic molecules in the polar mobile phase gets adsorb to the hydrophobic stationary phase on the other hand hydrophilic molecules in the mobile phase pass through the column freely and are eluted first.

3.2.5.3.2 HPLC COLUMN

In this study C18 column was used where octadecyl carbon chain (C18) is bonded with silica. Here silica is the stationary phase which has been surface-modified with RMe_2SiCl , where R is a straight chain alkyl group ($\text{C}_{18}\text{H}_{37}$) which serves as an inert non-polar substance. Column dimension is 4.6 mm ID (internal diameter) \times 250 mm length. The column is packed with silica particle with particle size of 5 μm and the particle pore size is 300 \AA .

3.2.5.3.3 HPLC SOLVENTS

In reverse phase HPLC the solvents are by convention is called HPLC solvent A and B. The A solvent by convention is the aqueous solvent and the B solvent by convention is the organic solvent (acetonitrile, methanol, propanol). For rest of the writings the convention A and B will be used to refer to the aqueous and organic solvents respectively. In this study solvent A and B was:

Solvent A: HPLC grade Water, 2.5% Acetic acid pH 3.8 set with 4 M KOH

Solvent B: HPLC gradient grade Acetonitrile, 20% HPLC grade water

3.2.5.3.4 FLOW RATE

Flow rate in HPLC mostly depends on the dimensions of the column used. The column with 250 mm length and 4.6 mm internal diameter flow rate was set to 1ml/minute.

3.2.5.3.5 HPLC GRADIENT

When running HPLC with an unknown sample with unknown concentration then usually a gradient of solvent B is run to know about the hydrophobic character of the unknown compounds. The % B in the gradient described below is used in this study:

Time (minute)	% B
0.00	20
25.00	50
29.00	100
34.00	100
37.00	20
45.00	STOP

It is important that the gradient should never be started at 0% B. As in 0% organic and in high aqueous environment the very hydrophobic, long C18 alkyl chains would repeal away from the high aqueous environment and mat down on the particle of the column. When these alkyl chains are settled down they are inefficient at capturing the analytes. Therefore the gradient should always be started with some % of solvent B in this case 20% (Fig. 9).

3.2.5.3.6 SAMPLE PREPARATION

The HPLC samples are usually reconstituted in solvent A to maximize the bonding between analytes and the column. For HPLC analysis extraction of indolic compounds from liquid media

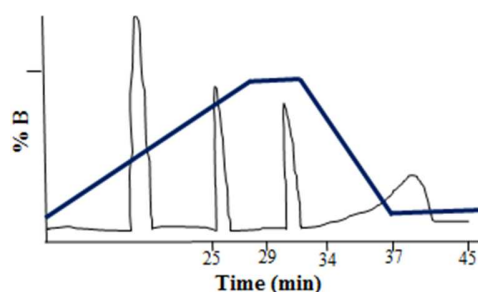


Fig. 9: Schematic representation of the HPLC gradient that has been used. The figure is representative only not proportionate to actual scale.

similar as described above. During sample preparation the pellet was dissolved in a mixture of 25% methanol and 1% acetic acid, pH 4.5. Same buffer was used for any subsequent dilution of the sample for HPLC analysis.

3.2.5.3.7 HPLC STANDARDS

For the purpose of plotting standard curves, identify individual peaks and internal control seven different standard indolic compounds were used. Tryptophan (Trp), Tryptamine (TAM), Indole-3-lactic acid (ILA), Indole-3-acetamide (IAM), Indole-3-acetic acid (IAA), Tryptophol (TOL) and Indole-3-acetonitrile (IAN). The standard stock solutions of IAA, IAM, IAN, ILA, TAM and TOL were prepared in methanol. The standard Trp stock solution was prepared in water. From the stock solution different other stocks and working solutions were made as per required. For dilution of the standard buffer with 25% methanol and 1% acetic acid, pH 4.5 were used in all the cases.

3.2.5.3.8 PLOTTING CALIBRATION CURVES

HPLC is a technique with both qualitative and quantitative advantages. Any analyte can be detected as well as can be quantified from a mixture of sample using HPLC. To quantify any particular compound first a calibration curve or standard curve is made using the same compound with known concentration and injection volume. In this study each standard compounds were measured in three different concentration with four different volumes of each concentration. Thus for each standard 12 (twelve) data points were obtained for calibration curves (table 3). Calibration curves were plotted with injected analyte mass vs peak area. Then the curves were fitted and linear regression equation as well as coefficient (r^2) was calculated.

Table 3. Injection volumes and concentrations of different standards used for preparing standard curves for each standard

Standards used	Concentration (μM)	Injection volume (μl)
1. IAA	5	2, 6, 10 & 15
	2	2, 6, 10 & 15
	1	2, 6, 10 & 15
2. ILA	5	2, 6, 10 & 15
	2	2, 6, 10 & 15
	1	2, 6, 10 & 15
3. TOL	5	2, 6, 10 & 15
	2	2, 6, 10 & 15
	1	2, 6, 10 & 15
4. TAM	5	2, 6, 10 & 15
	2	2, 6, 10 & 15
	1	2, 6, 10 & 15
5. TRP	5	2, 6, 10 & 15
	2	2, 6, 10 & 15
	1	2, 6, 10 & 15

4 RESULTS

4.1 Results of computational studies

4.1.1 Auxin biosynthesis mediating enzymes are present in wide range of fungi

A few reports about auxin production in filamentous fungi *Neurospora crassa* have been published earlier (GRUEN 1959; NAKAMURA *et al.* 1978; TOMITA 1984; NAKAMURA, T 1988; KOLLATH-LEISS *et al.* 2014). But none of the study mentioned functionality of the IAA producing gene/s before. This study aimed to comprehensively analyze the potential of *N. crassa* to produce auxin and identify the pathway(s) with gene functionality using both bioinformatics and experimental analysis of the genes involved in auxin biosynthesis pathway(s). *N. crassa* genes that were identified and used for bioinformatics as well as for experimental studies are *tam1* (NCU09116), *ipd* (NCU02193), *iad1* (NCU03415), *iad2* (NCU09648), *iad3* (NCU00378) and *yuc* (NCU03755). As mentioned before the genes were named as per the general enzyme nomenclature and used in this study for easy understanding. However, many of these genes had different names, based on previous work (see section 2.8 for details).

Site-specific binding of enzymes to their respective cofactors is essential to obtain their active states. The ligand binding sites for the group of homologous enzymes analyzed in this study were published previously (LIU *et al.* 1997; ESWARAMOORTHY *et al.* 2006; PHI *et al.* 2008; ZUTHER *et al.* 2008). Alignments of the amino acid sequences of four key enzymes i.e. tryptophan aminotransferase (TAM1), pyruvate decarboxylase (IPD), aldehyde dehydrogenase (IAD), and flavin-monooxygenase (YUC) from different fungal species were selected. These alignments demonstrate that all the key amino acid residues important for binding of the respective ligands are conserved in the fungal enzyme sequences. In many cases, specific groups of proteins are identified and categorized by their ligand binding motifs, which are known as signature motifs for that particular group of proteins. In this study, a motif searching approach was implemented to identify specific ligand binding motifs and categorize the studied enzymes accordingly. Three copies of IAD sequences (IAD1, IAD2 and IAD3) from *N. crassa* were identified and shown with six other species. Single copy of each TAM1, IPD and YUC from *N. crassa* was identified and shown along with eight other species. Gene *iaam* (NCU01106), *ami1* (NCU04092), *tdc1*

(NCU08275) and *tdc2* (NCU06112) were identified and used only for experimental analysis along with previous mentioned genes. Sequence-based functional conservation analysis suggests that these genes are conserved throughout fungal evolution (S1 Fig., S2 Fig., S3 Fig., and S4 Fig.). A phylogenetic analysis was also performed to check the closeness/relatedness of same enzyme among different fungal species. It was found that on a phylogenetic tree for a specific enzyme different fungal species appear group wise (S5 Fig., S6 Fig., S7 Fig., and S8 Fig.).

4.1.2 Key amino acid residues important for enzymatic function are conserved

Although the study was carried out using multiple species, the focal species was *N. crassa* because of the availability of thousands of knock-out mutants (COLOT *et al.* 2006) Aromatic L-amino acid decarboxylases are homologous to pyridoxal-5'-phosphate (PLP)-dependent enzymes that share a common ancient evolutionary origin that catalyze the decarboxylation of aromatic L-amino acids in different species lineages (FACCHINI *et al.* 2000). The same amino acid residues that are known to bind with the ligand of aminotransferase, PLP, are conserved in the putative tryptophan aminotransferase homolog in *N. crassa*. Data indicate that residues Y180, N302, D330, Y333, K381, and R388, present in the *N. crassa* homolog of tryptophan aminotransferase, are involved in ligand binding (Fig. 10).

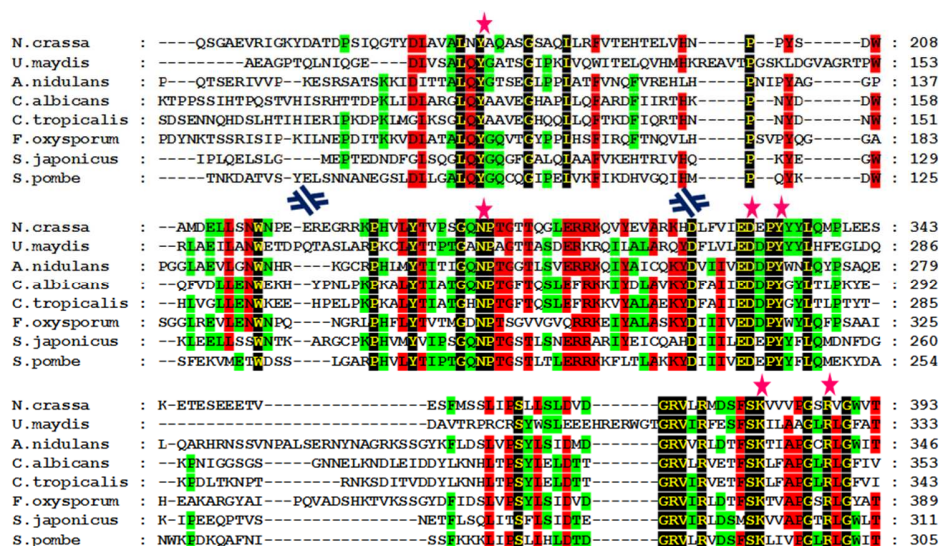


Fig. 10: Multiple sequence alignment of tryptophan aminotransferase homologs from different fungal species. Residues with 100% identity are shown in yellow font with black background and residues with 75% identity are shown in black font with red background and 50% identity is shown with green background and black font. PLP binding residues are marked with pink star on top. Sequences are shown in discontinuous fashion. Blue discontinuity symbol has been shown when there is a discontinuity in sequence.

The residues G36, E59, T81, V84, G396, W419, G420, I422, V449, G450, D451, and G452 in the pyruvate decarboxylase homolog from *N. crassa* were significantly conserved when aligned with known ligand binding residues (Fig. 11). NAD serves as a cofactor for aldehyde dehydrogenase enzymes. While compared with the known template it was found that IAAld dehydrogenase from *Neurospora crassa* has the conserved amino acid residues important in NAD binding inside the enzymatic catalytic site. Data suggest that the conserved residues from *Neurospora* aldehyde dehydrogenase (IAD1) involved in ligand binding are: N167, T242, G243, E266, L267, G268, C300, E397, F399, and F463. These residues are conserved in other species too (Fig. 12). Flavin monooxygenase or YUC family proteins are known to have a FAD binding signature motif. Data suggest that the YUC homolog from *Neurospora* has a nucleotide binding motif (GXGXXG) consisting of G17, A18, G19, P20, A21, and G22 (Fig. 13).

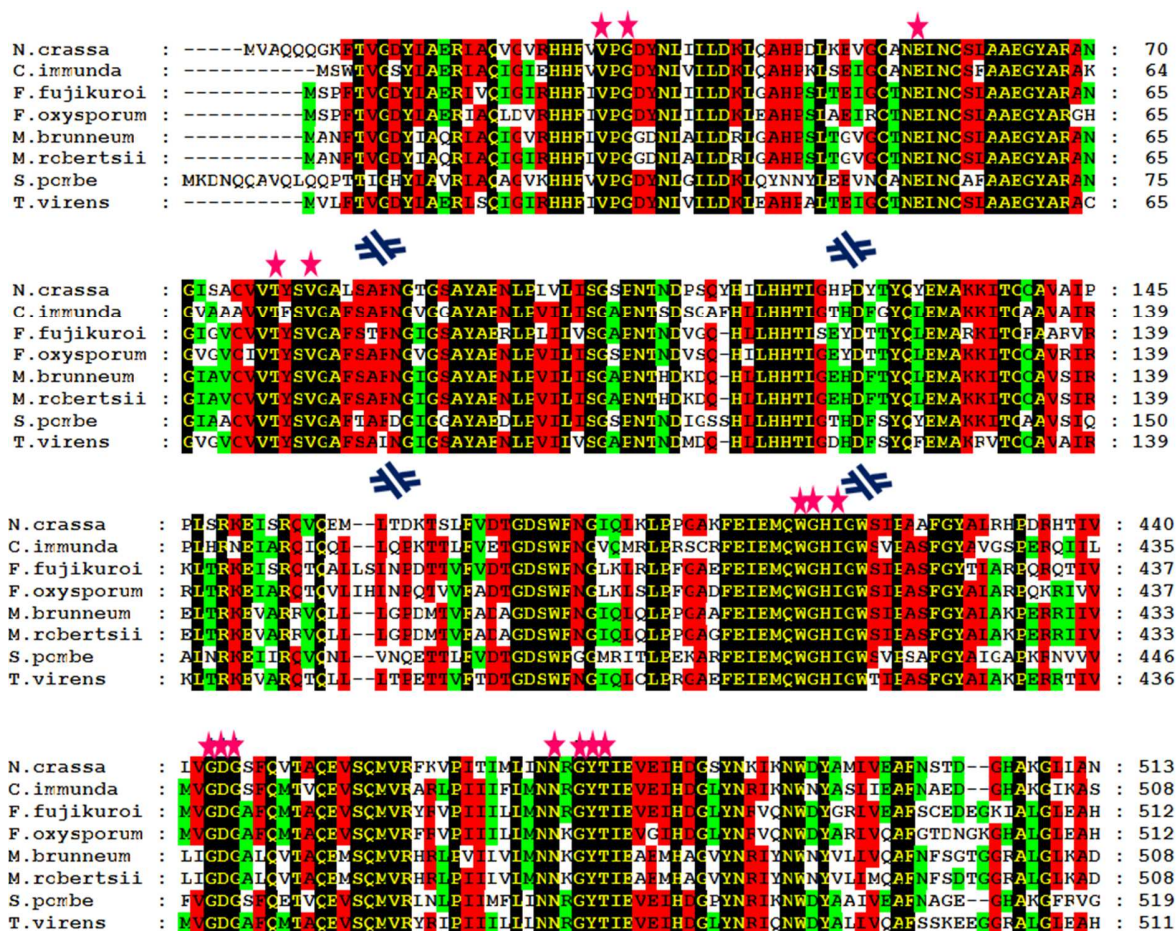


Fig. 11: Sequence alignment of homologous pyruvate decarboxylase from different fungal species. Black background with yellow font, red background with black fonts and green background with black fonts are representing 100%, 75% and 50% identity of the residues respectively. TPP binding residues are marked in pink stars. Blue discontinuous symbol has been shown when there is a discontinuity in sequence.

4.1.3 Structure-function correlation suggests the presence of an evolutionary conserved functional auxin biosynthesis pathway in fungi

The structure of any protein is principally determined by its amino acid sequence (EPSTEIN 1964). Changes in structures occur less often than changes in the associated amino acid sequence. In theory, distantly related homologous protein sequences can still be folded into similar structures. In this context, sequence and structural analysis data are supportive and complement each other. All key amino acid residues from each enzyme present in the auxin biosynthesis pathway are sequentially conserved in the focal species *N. crassa* as well as in other fungal species.

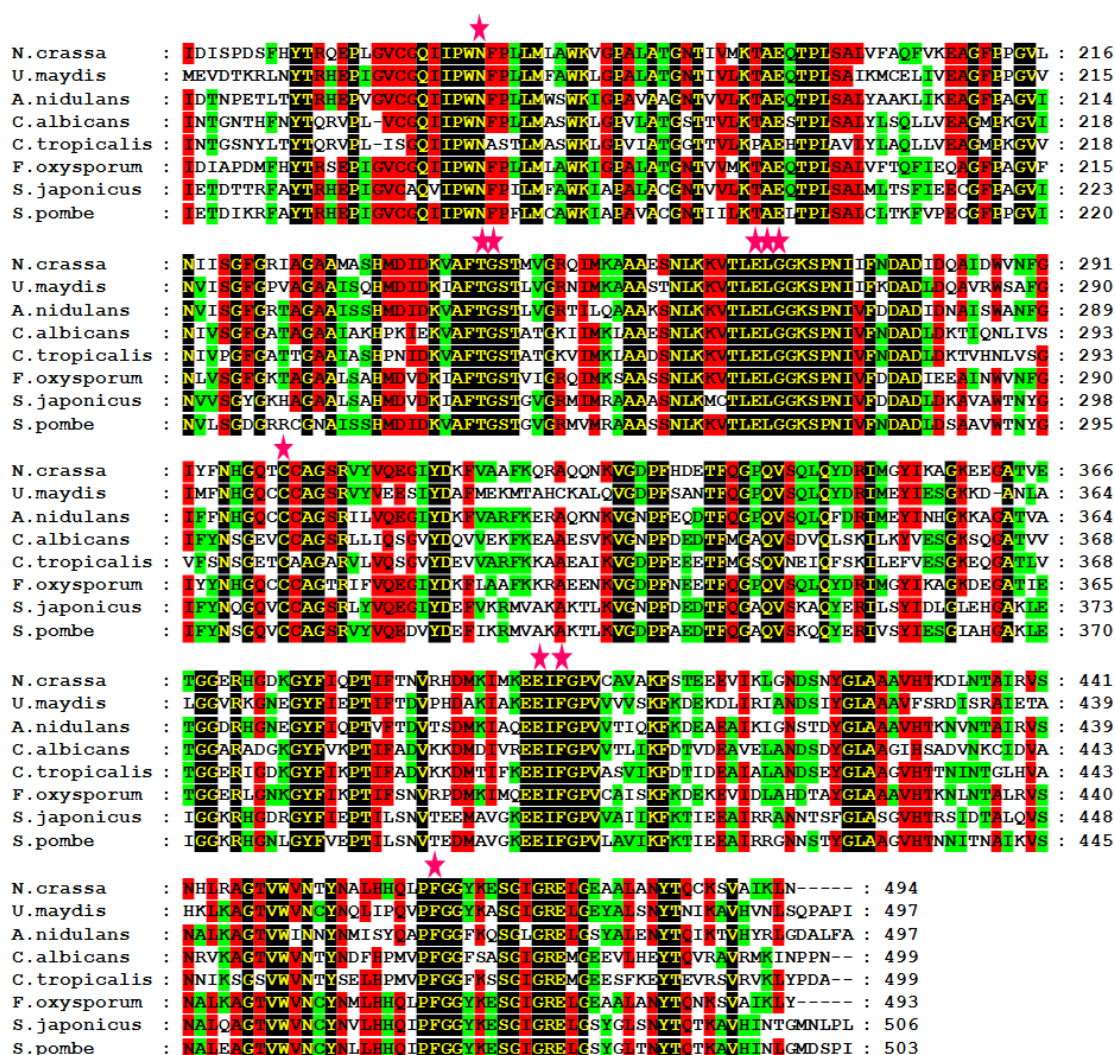


Fig. 12: Multiple sequence alignment of aldehyde dehydrogenase homologs from seven different fungal species. Amino acid residues with black background yellow font are representing 100% sequence identity, 75% identity is shown in red background in black font and 50% sequence identity has been shown in black fonts with green background. NAD serves as cofactor of the enzyme aldehyde dehydrogenase. NAD binding residues are shown with pink star on the top of the residues.

Evidence for structural conservation of the enzymatic catalytic sites of the analyzed proteins was also provided. All data were evaluated with a high level of authenticity using structural alignments of the predicted and known structures of enzymes. The results of structural analyses are presented in two ways. First, the overall structure of the predicted protein model is aligned with a known enzyme with a known function then, an in-depth analysis of each amino acid residue present in the enzymatic catalytic site and responsible for ligand binding was provided (Fig. 14 and Fig. 15, Fig. 16 and Fig. 17). It was also checked for the putative ligand binding site in the predicted structures using COFACTOR (ROY *et al.* 2012). COFACTOR in silico analysis makes predictions about the ligand binding sites and plausible ligands that can bind with the given protein structure. COFACTOR analysis of the putative *N. crassa* aromatic aminotransferase suggests that it has a strong binding match (BS-score 1.20) for PLP (a BS-score >1 signifies strong binding). PLP serves as a coenzyme in transamination, decarboxylation, and deamination reactions of amino acids. The enzyme aromatic aminotransferase Aro8 in a complex with PLP from *Saccharomyces cerevisiae* (ZUTHER *et al.* 2008) was used as a template (PDB ID: 4JE5) for structural alignment. A structural alignment between the predicted TAM1 from *N. crassa* and the template structure shows that in spite of having sequence dissimilarities, protein folding is very similar in both protein structures (Fig. 14a). Analysis of the PLP binding site indicated key amino acid residues are positionally conserved in the predicted structure and mimic a conformation similar to the template structure, which facilitates proper ligand binding (Fig. 14b).



Fig. 13: Multiple sequence alignment of homologous YUC from different fungal species. YUC or flavin monooxygenase is known to bind with FAD through its nucleotide binding motif (GXGXXG). GXGXXG motif has been shown with bar and conserved residues are highlighted with pink stars. Residues with black background and yellow font are 100% identical and residues with red background in black font are 75% identical.

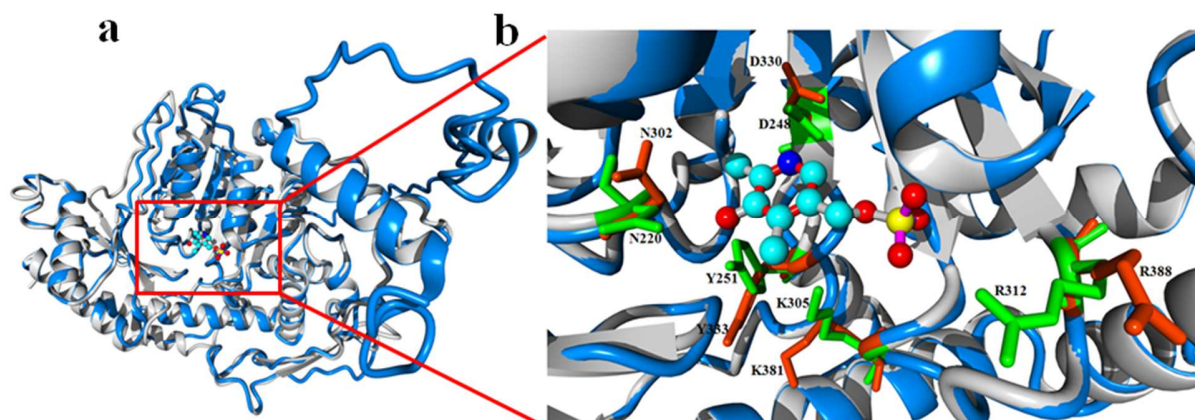


Fig. 14: Structural alignment between predicted enzymes and corresponding known structure of same enzyme homolog and characterization of ligand binding sites. Predicted structures and known templates are shown in blue and grey in color respectively. Residues present on predicted enzymes and templates are marked with orange and green respectively. All structures are rendered in ribbon. Key amino acid residues those involved in ligand binding has been rendered in stick model. Residues those are at close vicinity are only highlighted. Ligands has been shown in ball and stick model. (a) Overall structural alignment of predicted TAM1 structure with known enzyme structure (4JE5). PLP is bound inside enzymatic catalytic site. (b) Structural insight of ligand binding site of both predicted and 4JE5 as known structure.

Analysis of the ligand binding of the predicted structure of pyruvate decarboxylase from *N. crassa* indicated that thiamine pyrophosphate (TPP) has a higher potential to serve as a ligand (BS-score 1.46). Along with many other enzymatic reactions, TPP is known as a coenzyme for pyruvate decarboxylase. To explore the ligand binding site and compare the overall topology, pyruvate decarboxylases from *Enterobacter cloacae* (SCHUTZ *et al.* 2003) was used (Fig. 15) as a structural template (PDB ID: 1OVM). Structural alignment of pyruvate decarboxylase from *Neurospora* with the above mentioned template indicated that the TPP binding site is similar and all the key amino acid residues are positionally conserved in the enzymatic active site (Fig. 15b). Aldehyde dehydrogenase (IAD) comes under a wide class of enzyme namely dehydrogenase. We found a good amount of sequence conservation among three of the IAD enzymes from *N. crassa* (IAD1, IAD2 and IAD3) itself, therefore only the IAD1 structure has been shown here. The NAD binding pocket was also found in the predicted IAD1 homolog of *N. crassa* and was compared with the known NAD binding enzyme (PEREZ-MILLER and HURLEY 2003) (PDB ID: 1NZX) (Fig. 16). YUC, which is also known as FMO, is known to be functional when bound to the NADPH-FAD complex (ESWARAMOORTHY *et al.* 2006). The nucleotide binding sequence is represented as GXGXXG and the nucleotide binding sequence GAGPSG from *S. pombe* stabilizes binding of FAD (ESWARAMOORTHY *et al.* 2006).

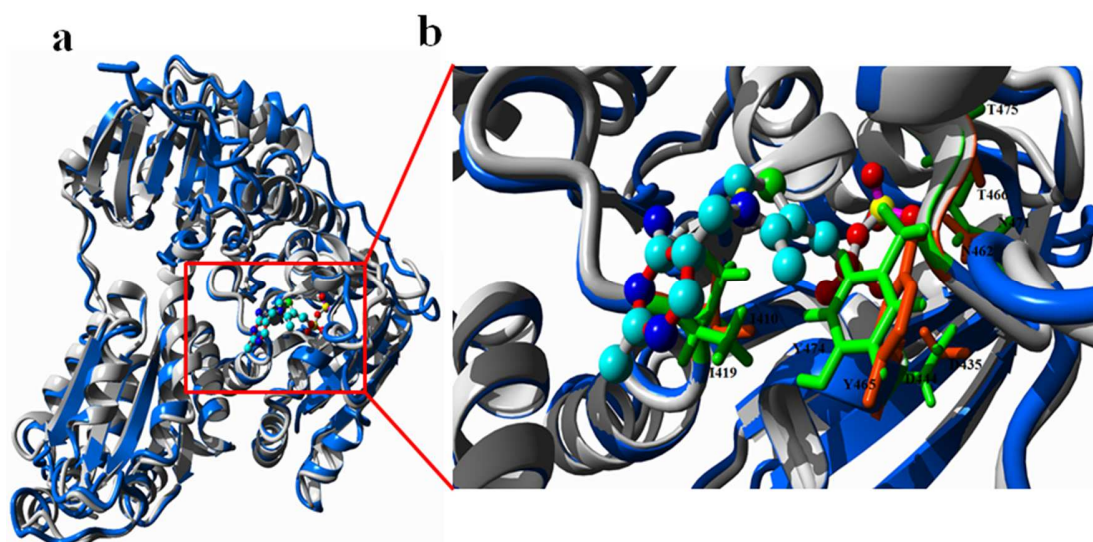


Fig. 15: Structural alignment between predicted enzymes and corresponding known structure of same enzyme homolog and characterization of ligand binding sites. Predicted structures and known templates are shown in blue and grey in color respectively. Residues present on predicted enzymes and templates are marked with orange and green respectively. All structures are rendered in ribbon. Key amino acid residues those involved in ligand binding has been rendered in stick model. Residues those are at close vicinity are only highlighted. Ligands has been shown in ball and stick model. (a) Structural alignment of predicted structure of pyruvate decarboxylase homolog from *N. crassa* with known pyruvate decarboxylase structure (1OVM). (b) Characterization of ligand binding site of predicted enzyme using 1OVM structure as template.

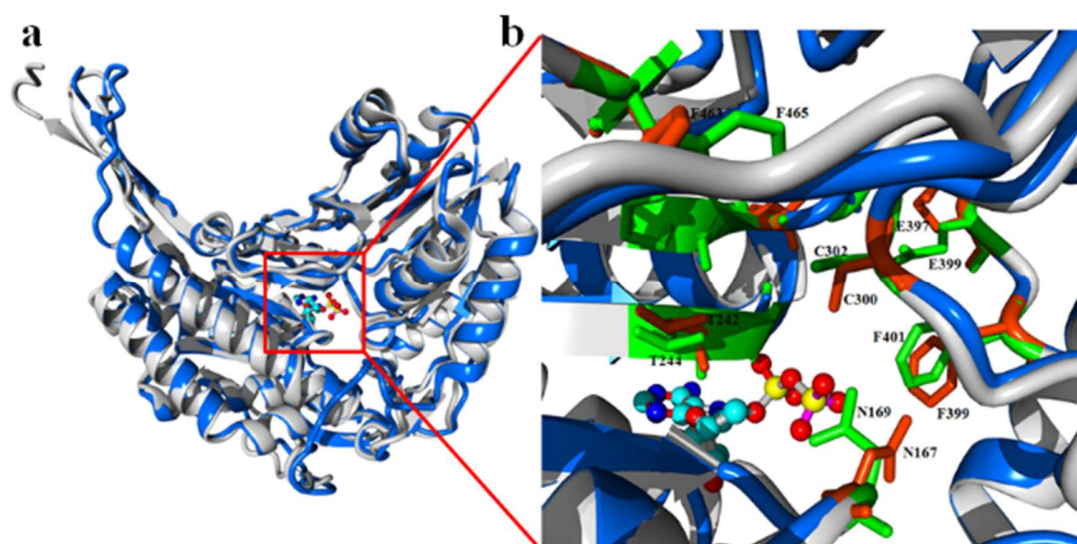


Fig. 16: Structural alignment between predicted enzymes and corresponding known structure of same enzyme homolog and characterization of ligand binding sites. Predicted structures and known templates are shown in blue and grey in color respectively. Residues present on predicted enzymes and templates are marked with orange and green respectively. All structures are rendered in ribbon. Key amino acid residues those involved in ligand binding has been rendered in stick model. Residues those are at close vicinity are only highlighted. Ligands has been shown in ball and stick model. (a) Overall structural alignment of predicted IAD1 structure with known enzyme structure (1NZX). NAD is bound inside enzymatic catalytic site. (b) Structural insight of ligand binding site of both predicted and 1NZX as known structure.

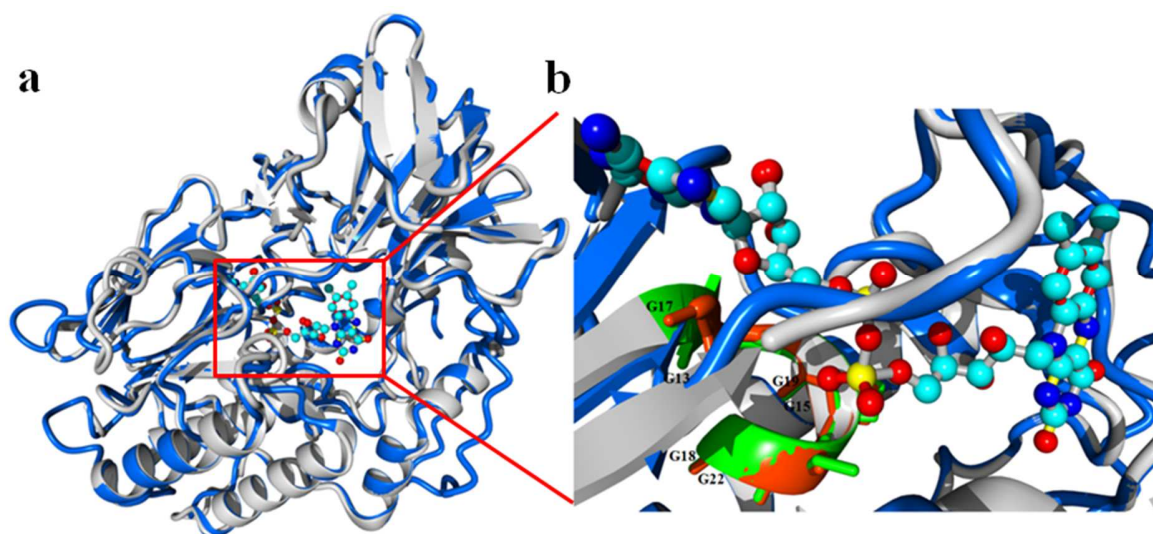


Fig. 17: Structural alignment between predicted enzymes and corresponding known structure of same enzyme homolog and characterization of ligand binding sites. Predicted structures and known templates are shown in blue and grey in color respectively. Residues present on predicted enzymes and templates are marked with orange and green respectively. All structures are rendered in ribbon. Key amino acid residues those involved in ligand binding has been rendered in stick model. Residues those are at close vicinity are only highlighted. Ligands has been shown in ball and stick model. (a) Structural alignment of predicted structure of flavin monooxygenase homolog from *N. crassa* with known flavin monooxygenase structure (1VQW). (b) Characterization of ligand binding site of predicted enzyme using 1VQW structure as template.

Results also show that the putative FMO from *N. crassa* is folded in such a fashion that the GXGXXG motifs of both the template (PDB ID: 1VQW) and predicted structure are aligned properly (Fig. 17). A detailed description of BS score, ligand and template of different structures is given in table 4.

4.1.4 Proposed auxin biosynthesis network based on all the IAA producing genes that has been identified in *N. crassa*

In bacteria and plants many IAA biosynthetic genes has been identified and based on that many pathways has been proposed too. In this study for the first time a comprehensive analysis in *N. crassa* has been done encompassing different IAA biosynthesis pathways that were proposed before in microorganism and plants. The IAA biosynthetic genes from different pathways in *N. crassa* has been identified and placed them in the IAA producing network for better understanding about the network (Fig. 18). The advantage of this proposed IAA producing pathway was taken for systemic analysis of IAA producing gene(s).

Table 4. Details of query, template, ligand and I-TASSER score for each of the predicted enzyme model

Serial No.	Query (Enzyme)	Query (NCU #)	Template (PDB ID)	I-TASSER Score	Ligand
1.	TAM1	NCU09116	4JE5	-1.75	pyridoxal-5'-phosphate (PLP)
2.	IPD	NCU02193	1OVM	1.16	thiamine pyrophosphate (TPP)
3.	IAD1	NCU03415	1NZX	1.19	nicotinamide adenine dinucleotide (NAD)
4.	YUC	NCU03755	1VQW	0.98	flavin adenine dinucleotide (FAD)

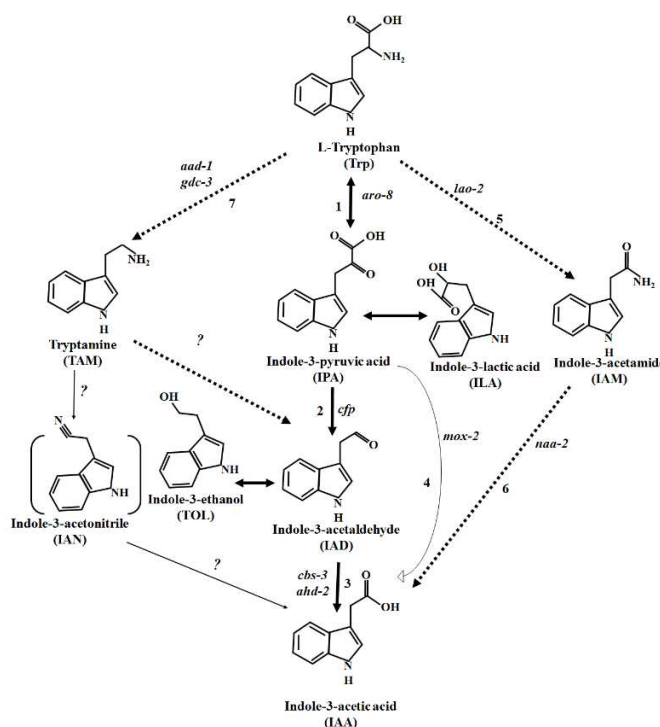


Fig. 18: Proposed tryptophan dependent IAA biosynthetic pathway in *N. crassa*.

The pathway with solid bold arrows has been functionally characterized by identification of novel genes and intermediate compounds were identified and estimated too. Pathways with dashed bold arrows are proposed on the basis of feeding test with IAM and TAM accordingly. Solid thin arrows are denoting pathways proposed in green plants but not known in fungi including *N. crassa*. Unidentified homologues of the genes in *N. crassa* for the respective biosynthetic steps are shown with question marks. IAN, with bracketed lines has not been identified from the *N. crassa* culture. All other indoles without any brackets (except IAM and TAM) are identified from the culture upon tryptophan feeding. Indole-3-pyruvic acid (IPA) and indole-3-acetaldehyde (IAAld) are very unstable compounds and spontaneously gets converted into indole-3-lactic acid (ILA) and indole-3-ethanol (TOL) respectively. IAAld has been readily identified from the culture as a proxy for IPA and IAAld respectively. 1. Tryptophan aminotransferase, 2. Pyruvate decarboxylase, 3. Aldehyde dehydrogenase, 4. Flavin monooxygenase, 5. L-amino acid Oxidase, 6. N-acylethanolamine amidohydrolase, 7. Aromatic -L-amino acid decarboxylase (*aad-1*) and Glutamate decarboxylase (*gdc-3*)

4.1.5 Predicted IAA transporter in *N. crassa* has an ortholog in *Arabidopsis thaliana* with similar annotation

After a bidirectional BLAST analysis it was found that gene NCU00589 which is annotated as “auxin efflux carrier superfamily” in *Neurospora crassa* has an ortholog in *Arabidopsis thaliana* with annotation “auxin efflux carrier family protein” (NP_565011). Moreover it was also found that homologous sequences of NCU00589 are also present in different fungi across the fungal kingdom (S9 Fig.). NP_565011 sequence was analyzed in UniProt (<http://www.uniprot.org/>) and found that it has a helical structure with ~40% transmembrane domains (Fig. 19). This finding was supported by the structure prediction approach.

After predicting structures of NCU00589 and NP_565011 from *Neurospora* and *Arabidopsis* it was observed that both of the structures consisted of mostly helices and very much similar to each other (Fig. 20). Structural alignment was done to check for the degree of similarity between these two predicted auxin transporters structures from *Arabidopsis* and *Neurospora* (Fig. 21). From the structural alignment it was found that most of the helices from each of the protein structure align perfectly with each other. Both of the protein structures resemble HEAT repeat domain (GROVES *et al.* 1999) with multiple helical structures.

Topology				
Feature key	Position(s)	Length	Description	Graphical view
Transmembrane ¹	16 – 37	22	Helical Sequence analysis	
Transmembrane ¹	49 – 67	19	Helical Sequence analysis	
Transmembrane ¹	79 – 103	25	Helical Sequence analysis	
Transmembrane ¹	115 – 133	19	Helical Sequence analysis	
Transmembrane ¹	153 – 172	20	Helical Sequence analysis	
Transmembrane ¹	356 – 379	24	Helical Sequence analysis	
Transmembrane ¹	391 – 414	24	Helical Sequence analysis	
Transmembrane ¹	426 – 447	22	Helical Sequence analysis	

Fig. 19: UniProt result of the sequence analysis of NP_565011. Topologically the protein sequence can be categorized as a transmembrane protein. UniProt prediction suggests that ~40% (total length 457aa) of the whole protein sequence is composed of helices.

HEAT repeat domain is a characteristic feature of the membrane proteins involved in intracellular transport. After topological analysis further physio-chemical assessment was done based on these predicted structures. For that purpose electrostatic properties of the protein surfaces were mapped and analyzed (Fig. 22).

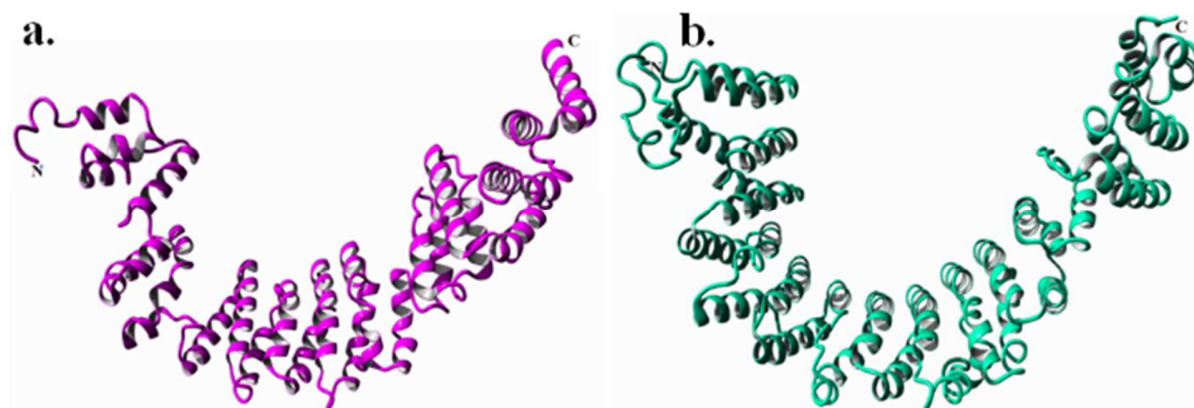


Fig. 20: Predicted structures of (a) auxin efflux carrier family protein and (b) auxin efflux carrier super family. N and C terminal of the proteins are marked accordingly.

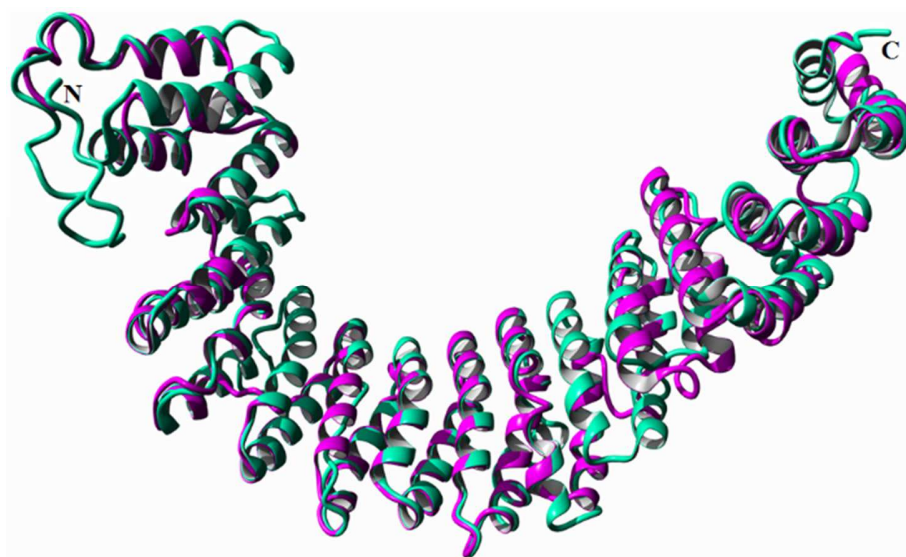


Fig. 21: Structural alignment between auxin efflux carrier family protein from *Arabidopsis thaliana* (magenta color) and auxin efflux carrier super family from *Neurospora crassa* (green color). The middle helices are aligned more precisely comparing the helices at both N and C terminus.

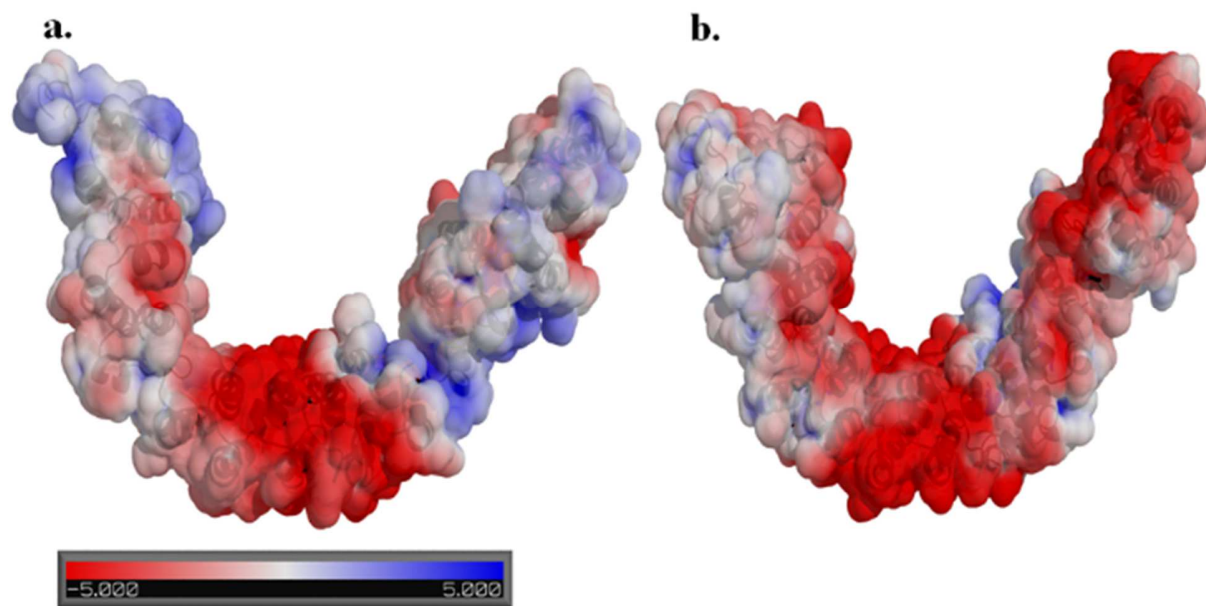


Fig. 22: Electrostatic potential of the surfaces of (a) auxin efflux carrier family protein from *Arabidopsis thaliana* and (b) auxin efflux carrier super family from *Neurospora crassa*. Irrespective of differences of charge distribution the mid part of both of the proteins are highly electro negative. In the scale, red to blue denotes highly electro negative (-5) to highly electro positive (+5).

4.2 Results from experimental studies

4.2.1 Double and triple knock-out strains were created by homologous recombination using *hph* cassette exchange

In this research project the IAA production in *N. crassa* has been studied through finding and examining the candidate genes present in IAA production pathway and thus IAA biosynthesis network has been established. It is well established in bacteria and plants, that there are several IAA pathways. It was found that none of the single knock-out strains has reduced level of IAA production therefore it is more likely that there are multiple and parallel IAA production pathways in *N. crassa*. In order to provide evidence for multiple and parallel pathways was decided to create double and triple knock-out strains encompassing different pathways. The knock-outs were created by crossing the appropriate strains. Double knock-out strains were created by setting crosses between two single knock-out strains as described in methods section. Luckily most of the important targeted genes were localized on different chromosomes and thus it facilitated higher probability of obtaining a double knock-out. After recombination chances of getting a double (and/or triple) knock-out individual would be very less if two targeted genes are present very close by or on same linkage group. If two single knock-out strains were set for a cross after homologous recombination theoretically 25% of the whole germinated spores would be double knock-out strains (assuming that none of the deletion mutations have effect on the survival of the germination of ascospores). Once successfully double knock-out strains were created then a cross between double and single knock-out *Neurospora* strains were set to make triple knock-out organisms. All single knock-out strains were created by homologous recombination using a *hph* cassette (see methods section for details). Hence, all double and triple knock-out individuals have a *hph* cassette gene in the position of corresponding deleted open reading frame of the respective gene(s). To confirm a double or triple knock-out in mycelia derived from crosses it is mandatory to show that the desired gene is deleted from the genome and the *hph* gene is being inserted in the exact location of the deleted gene(s) open reading frame.

The second confirmation is absolutely necessary because in case of a potential double knock-out it would always give a band on a DNA gel corresponding to hygromycin B resistance gene even if it was a single knock-out strains in actual. Additionally the location and confirmation of the hygromycin B resistance gene was tested by “Sanger sequencing” (SANGER and COULSON 1975) of the PCR amplified DNA band after agarose gel elution.

Two single knock-out strains with different matting types and deletion at different linkage groups were used for this purpose (table 5). During the screening procedure, first targeted genes were set for a PCR with primer sets specific for the respective ORFs and then further for subsequent testing to identify the presence of *hph* cassette at the right positions.

Table 5. Details of double knock-out strains that were created using the single knock-out strains

Details of single knock-out strains that were used for crossing and obtaining subsequent double knock-outs.

	<i>Atam1</i> (A)	<i>Aipd</i> (A/a)	<i>Adi1</i> (A/a)	<i>Adi2</i> (A)	<i>Adi3</i> (a)	<i>Ayuc</i> (A/a)	<i>Aiaam</i> (a)	<i>Aami1</i> (A)	<i>Atdc1</i> (A/a)	<i>Atdc2</i> (A/a)
<i>Atam1</i> (A)	■	<i>Atam1Aipd</i>					<i>Atam1Aiaam</i>			
<i>Aipd</i> (A/a)		■								
<i>Adi1</i> (A/a)			■	<i>Adi1Adi2</i>	<i>Adi1Adi3</i>			<i>Adi1Aami1</i>		
<i>Adi2</i> (A)				■						
<i>Adi3</i> (a)					■					
<i>Ayuc</i> (A/a)						■				
<i>Aiaam</i> (a)							■	<i>AiaamAami1</i>		
<i>Aami1</i> (A)								■		
<i>Atdc1</i> (A/a)									■	<i>Atdc1Atdc2</i>
<i>Atdc2</i> (A/a)										■

All the primers which were used in this study to amplify the ORF were designed in a way that they would produce a PCR amplicon with a size of ~600 - 650 kb, if the ORF is present in the genome. To check for the successful insertion and right location of the inserted *hph* cassette the primer set was designed in such a way that the forward primer lies on the 5` UTR of the targeted gene (ORF) and reverse primer is on the the hygromycin B resistance gene (Fig. 23).

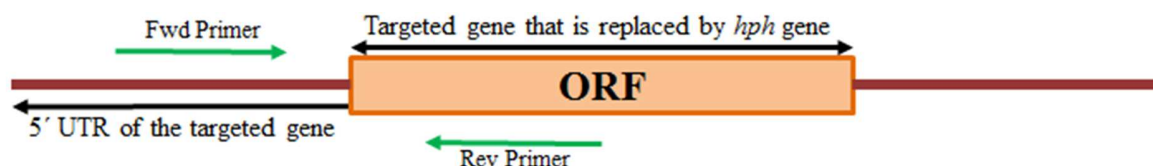


Fig. 23: Schematic diagram of the strategy explaining confirmation of the insertion of the *hph* cassette at right location at the targeted site. Forward primers were designed on the 5` UTR of the targeted ORF and reverse primers were designed to be on the *hph* ORF totaling 1000 – 1300 Kb amplicons (after PCR).

Table 6. Details of triple knock-out strains that were created using the double knock-out strains

Details of double knock-out strains that were used for crossing and obtaining subsequent triple knock-outs.

	<i>Atam1</i> (A)	<i>Aipd</i> (A/a)	<i>Aiad1</i> (A/a)	<i>Aiad2</i> (A)	<i>Aiad3</i> (a)	<i>Ayuc</i> (A/a)	<i>Aiaam</i> (a)	<i>Aami</i> (A)	<i>Atdc1</i> (A/a)	<i>Atdc2</i> (A/a)
<i>Atam1Aiaam</i> (A)										
<i>Aiad1Aami1</i> (a)						<i>Aiad1Aami1Ayuc</i>				
<i>Aiad1Aiad3</i> (a)						<i>Aiad1Aiad3Ayuc</i>				
<i>Atdc1Atdc2</i> (a)	<i>Atam1Atdc1Atdc2</i>									

Upon successful integration the *hph* cassette in the genome the primer sets corresponding to 5' UTR of different specific genes along with *hph* would produce ~1000 to 1300 kb PCR bands. The pair of highly specific oligonucleotides would produce an amplicon if the *hph* cassette is inserted exactly at the right location. An example is shown in Fig. 24 after agarose gel electrophoresis. Two samples namely S1 and S8 were confirmed through PCR and subsequent agarose gel electrophoresis. S1 is a double knock-out strain for genes *iaam* and *ami1* and S8 is a double knock-out strain for genes *iaam* and *tam1*. A PCR amplifying the 5' UTR of a targeted gene along with part of *hph* cassette has been shown as a confirmation of the successful insertion of the *hph* cassette in the targeted gene's location. The presence or absence of the targeted gene has been confirmed by the PCR amplification of part of the ORF of the gene. DNA from wildtype strain was used as a control for the PCR and subsequent gel electrophoresis. It is clearly visible in the agarose gel that both *AiaamAami1* and *AiaamAtam1* double knock-out strains have successful insertion of *hph* cassettes in the targeted region and all the targeted genes are deleted from the genome.

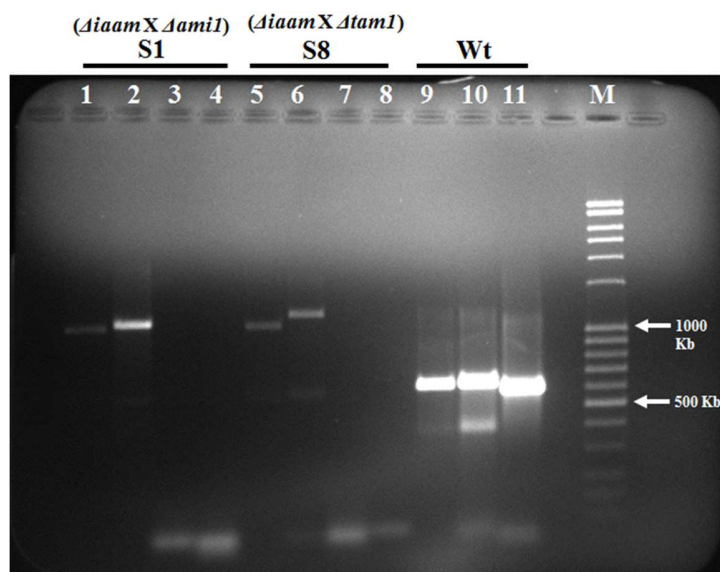


Fig. 24: An example of agarose gel picture after electrophoresis with the PCR products that confirms for two double knock-out strains. Two samples namely S1 and S8 were confirmed. S1 is a double knock-out strain for genes *iaam* and *ami1* and S8 is a double knock-out strain for genes *iaam* and *tam1*. Lane 1 and 2 were loaded with the PCR products of 5' amplifications of *iaam* and *ami1* genes ORF respectively along with the *hph*. Lane 3 and 4 were loaded with the PCR products of mid-part of *iaam* and *ami1* gene ORF respectively. Lane 5 and 6 were loaded with the PCR products of 5' amplifications of *iaam* and *tam1* genes ORF respectively along with the *hph*. Lane 7 and 8 were loaded with the PCR products of mid-part of *iaam* and *tam1* gene ORF respectively. Lane 9, 10 and 11 were loaded with the PCR products of mid-part of *iaam*, *ami1* and *tam1* gene ORF respectively from wild type strain (Wt). M is DNA marker.

4.2.2 Results from morphological and growth analysis of the strains

Morphological analysis of the single, double and triple knock-out (KO, DKO and TKO) strains were done by microscopic analysis to check for any altered phenotype that might have been introduced due to the knock-out gene(s). Plates containing the fungi which are to be imaged were kept for 10-15 minutes in the condition where imaging has been performed. This short time allow them to get adopted in the changed environment and normalize all the samples at the same time. The strains were specifically examined at the hyphal tip for any sorts of deformity and/or morphological alteration. During each microscopy session at least one wild type *N. crassa* strain was analyzed along with the batch of knock-out strains. During the analysis it was found that whenever any sort of morphological changes happened it was at the tip part of the growing hyphae. Very often most common type of deformity was found as splitting of the hyphal tip and further hyper-branching (Fig. 25).

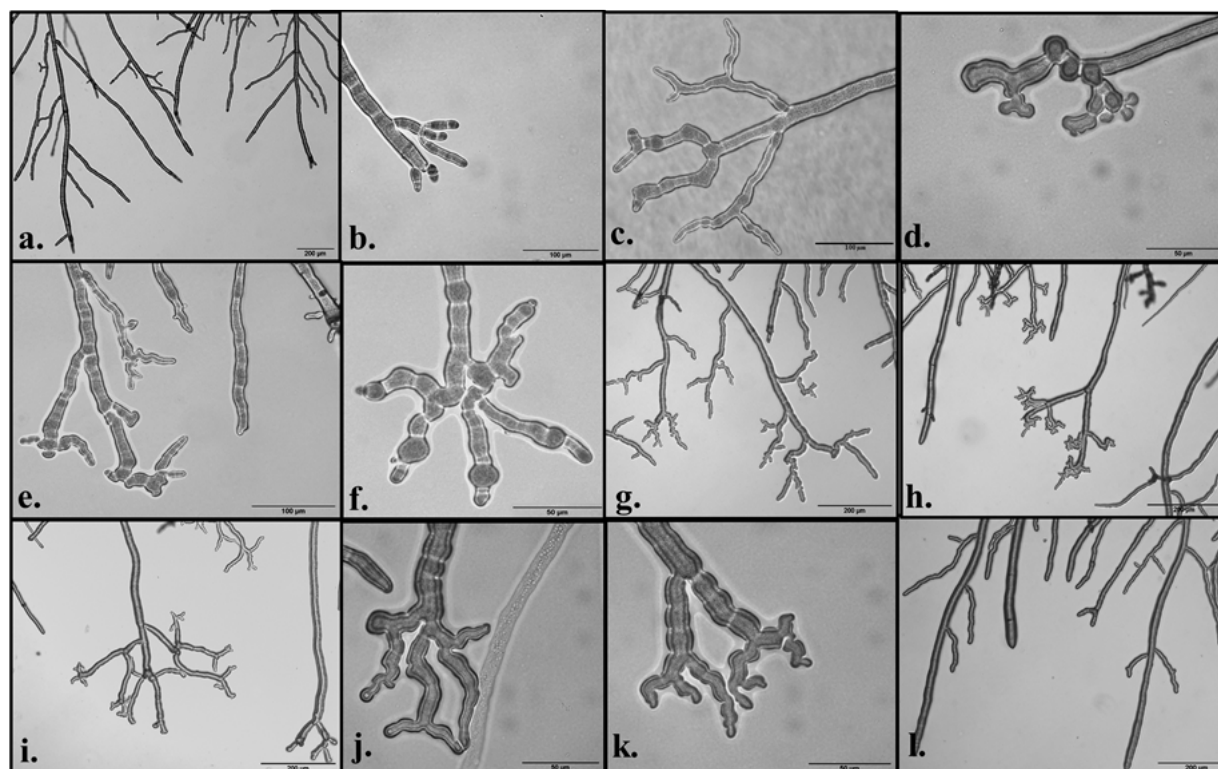


Fig. 25: Microscopic analysis of the growing hyphae of *N. crassa*. (a) and (l) Wild type strain. (b-c) Δyuc strain (d-e) $\Delta iaam \Delta tam1$ double mutant (f-g) $\Delta ami1 \Delta iad1$ mutant (h-i) $\Delta ami1 \Delta iad1 \Delta yuc$ triple knock-out strain and (j-k) $\Delta iaam \Delta ami1$ double knock-out strain.

Besides checking for the overall morphology of the knock-out strains, growth and conidia production was also checked for those too. It was found that $\Delta iad1\Delta iad3$ strains produce less conidia than the wild type strain (Fig. 26). In three days (Fig. 26a and Fig. 26b) and five days (Fig. 26c and Fig. 26d) interval overall growth were checked on the plates. The double knock-out strain for the genes $\Delta iad1$ and $\Delta iad3$ produces much less conidia than the wild type even after five days of inoculation. After culturing the strain on agar medium containing VMM and collecting spores after 10-15 days it was observed that $\Delta iad1\Delta iad3$ double knock-out strain produced least amount of conidia spores (Fig. 26e). Fungal growth was monitored and compared over time by race-tube assay.

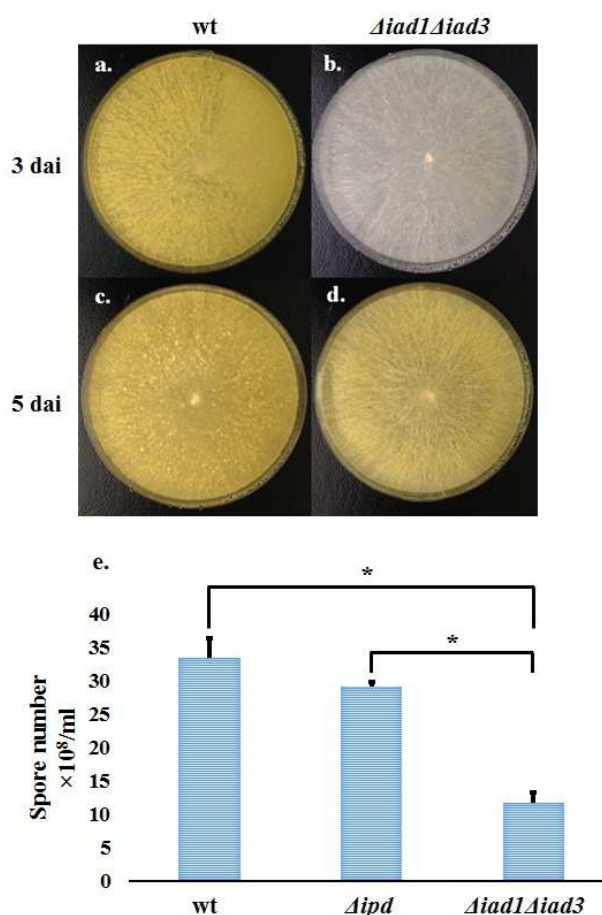


Fig. 26: Growth analysis of $\Delta iad1\Delta iad3$ double mutant strain compared to wild type. (a) and (c) Wild type strain (b) and (d) $\Delta iad1\Delta iad3$ double knock-out strain after inoculation of three and five days respectively. (e) Comparison of number of conidiospores among wild-type, Double knock-out strain produces less conidia that that of wild-type Δipd single knock-out and $\Delta iad1\Delta iad3$ double knock-out strains. dai: days after inoculation. * $p < 0.05$

It was found that *Δiad1Δiad3* double knock-out strain has a somewhat slower growth as compare to wild type as well as the other strains (Fig. 27). Morphologically the *Δiad1Δiad3* double knock-out strain was not different from the wild type strain (Fig. 28).

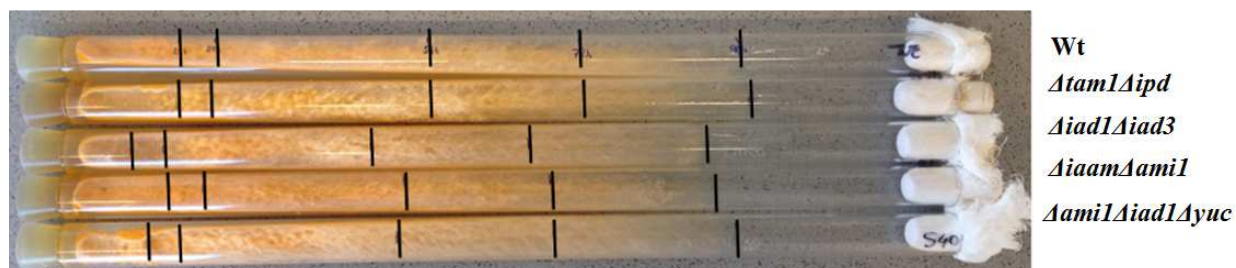


Fig. 27: Growth analysis of different knock-out along with wild-type strains. *Δiad1Δiad3* double mutant strain has been found to have a slower growth compared to wild-type as well as other double knock-out strains. Readings were taken in 24, 29, 54, 72 and 96 hours interval. All the readings are marked in black on the tubes respectively.

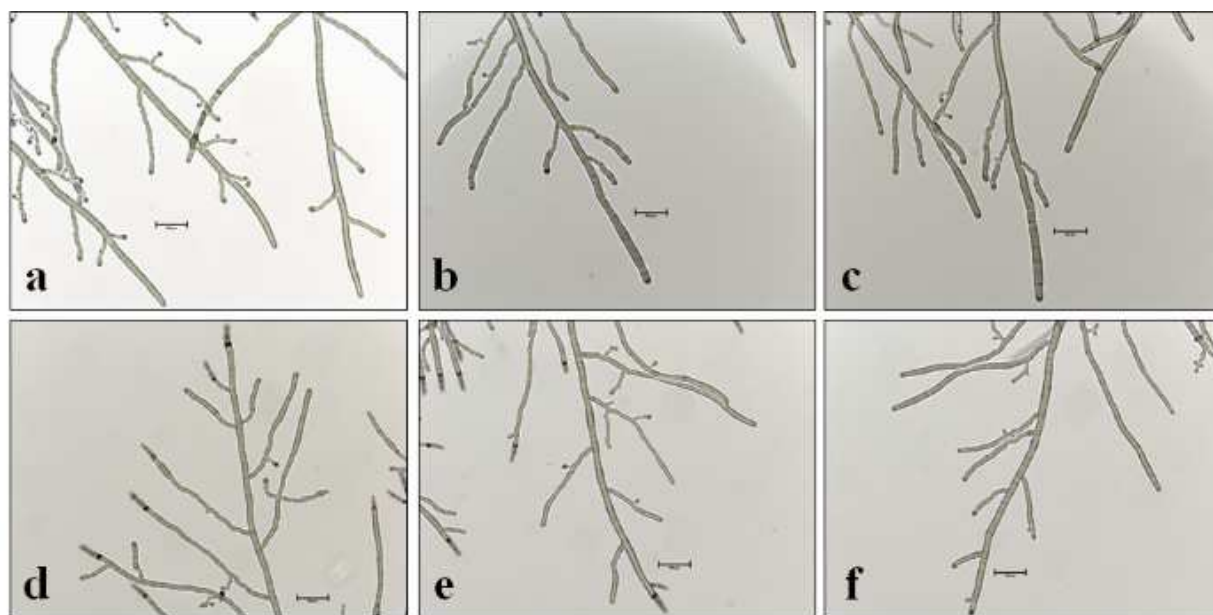


Fig. 28: Microscopic analysis of wild-type and *Δiad1Δiad3* double knock-out strains. (a-c) wild-type strain, (d-f) *Δiad1Δiad3* double knock-out strain.

4.2.3 Results from metabolite analysis

While it was previously known that external supplementation of tryptophan leads to IAA biosynthesis in *N. crassa* (TOMITA 1987; KOLLATH-LEISS *et al.* 2014), in this study for the first time the production of a wide range of indolic compounds including IAA, ILA and TOL by *N. crassa* has been analyzed employing different chromatographic techniques. *N. crassa* strains grown without supplementation of external tryptophan show no traces of IAA in the sample-extracts employing TLC and HPLC analysis (Fig. 29). While similar data have been reported much earlier (GRUEN 1959), those data were based on *Avena* curvature test and not by analytical techniques such as TLC or HPLC. Tryptophan feeding tests were performed with various concentration of tryptophan to identify the threshold concentration of tryptophan in IAA production by *N. crassa*. Cultures without any supplementation were used as control. Compounds were extracted from the culture and loaded on silica plates along with standard indolic compounds for TLC assay. I found that *N. crassa* can produce IAA with externally supplemented tryptophan even at a low concentration of 1 μ M (Fig. 30). Lower concentrations of 100, 50 and 25 nM tryptophan produced no IAA at all. Total indole including IAA production by the wild type *N. crassa* strain increases with increasing concentration of tryptophan supplementation (S10 Fig.).

For HPLC analyses a fixed tryptophan concentration level of 400 mg/L (1.96 mM) was used. 72 hours old wild type fungal cultures supplemented with and without tryptophan were analyzed by HPLC. Fungal cultures without tryptophan supplementation did not produce any of the indoles, while HPLC peaks for different indolic compounds appeared over time from the tryptophan supplemented culture. The different indolic compounds were identified by corresponding standards that were run along with the samples (Fig. 29a). Among all indolic compounds that were analyzed, IAA was the most abundant one. Apart from IAA, indole-3-lactic acid, and tryptophol were also identified under the same cultural and experimental conditions. All single knock-out strains, irrespective of their genetic background, produced IAA and related indolic compounds, but a few knock-out strains produced different indoles in higher or lower abundance (Fig. 29b). HPLC analysis from 72 hours old fungal cultures supplemented with 400mg/L (1.96 mM) tryptophan reveals that *Δipd* knock-out strain produces maximum amount of indole-3-lactic acid (Fig. 31a).

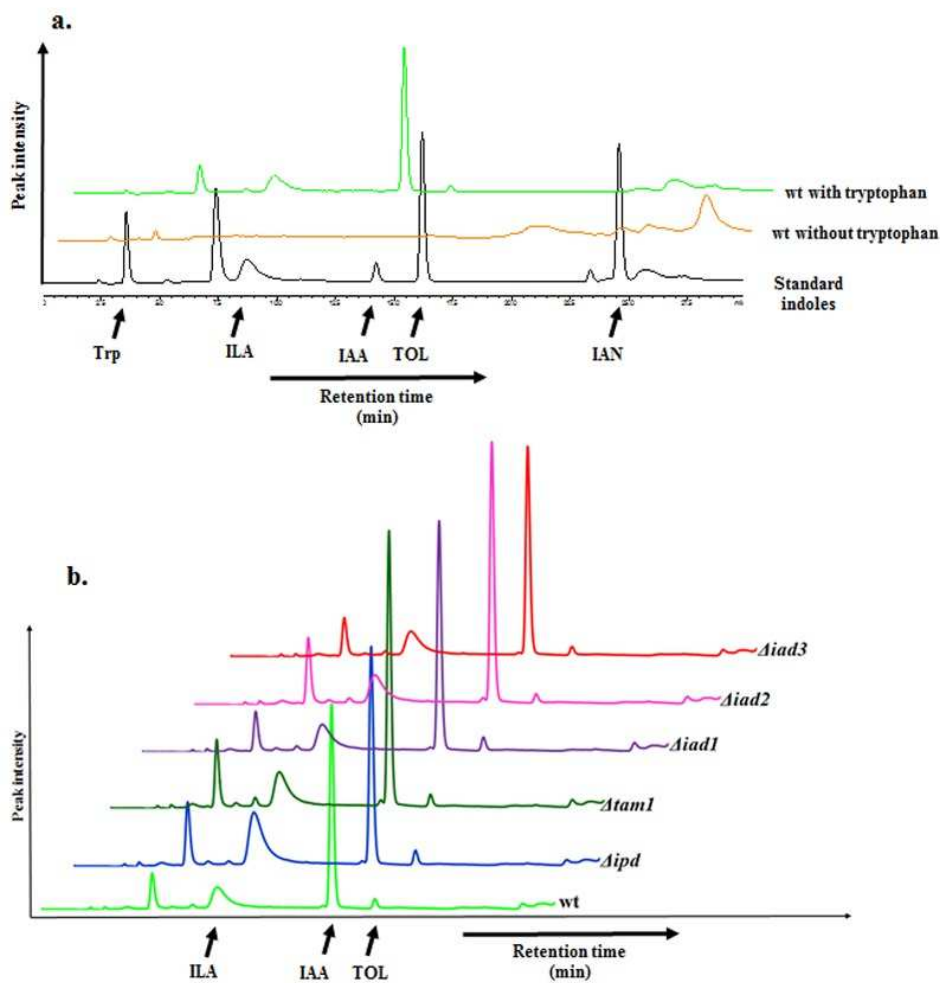


Fig. 29: IAA and related indolic compound production by *N. crassa* upon tryptophan supplementation.

Neurospora crassa produces IAA and other related indolic compounds while supplemented with tryptophan a. HPLC analysis of the metabolites produced by wild type *N. crassa* with and without tryptophan supplementation in 72 hours. b. Metabolite profiling of wild type as well as single knock-out *N. crassa* strains using HPLC. Single knock-outs for the genes specifically present in IPA pathway of IAA biosynthesis were used. Following standards were used Tryptophan (Trp), Indole-3-lactic acid (ILA), Indole-3-acetic acid (IAA), Tryptophol (TOL) and Indole-3-acetonitrile (IAN).

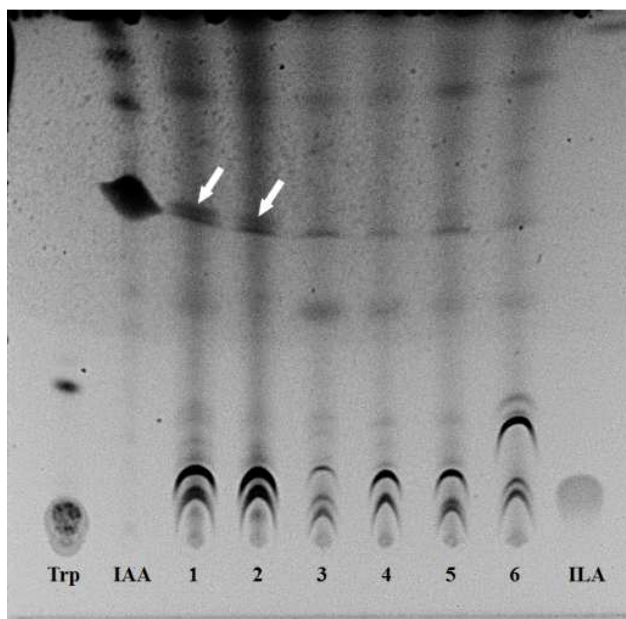


Fig. 30: *N. crassa* feeding assay with different concentration of tryptophan in 72 hours. White arrows in lane 1 and 2 are indicating traces of IAA. Following standards were used Trp. Tryptophan, IAA. Indole-3-acetic acid, ILA. Indole-3-lactic acid. Different tryptophan concentrations are: 1. 5 μ M, 2. 1 μ M, 3. 100 nM, 4. 50 nM, 5. 25 nM, 6. without tryptophan supplementation.

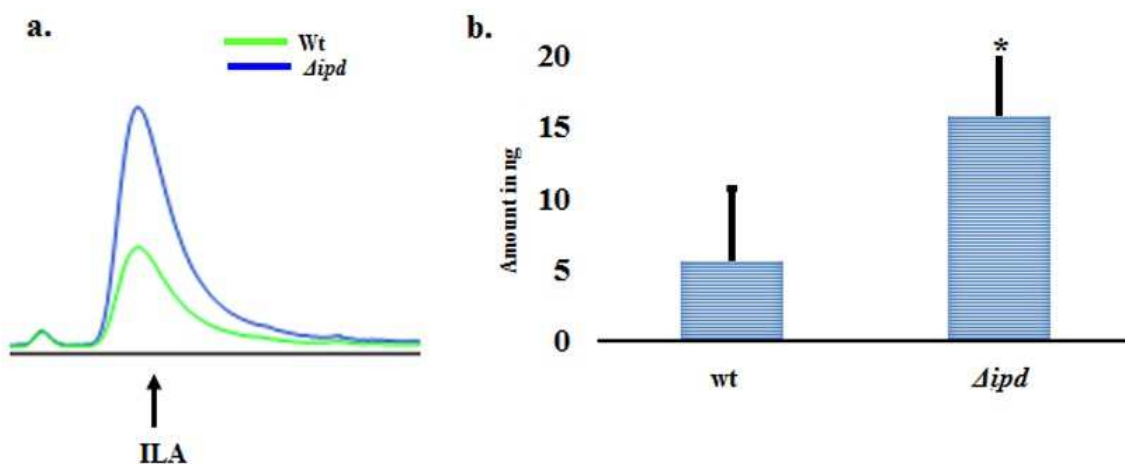


Fig. 31: Effect of Δipd strain on ILA production in *N. crassa*.

(a) HPLC peaks of indole-3-lactic acid (ILA) from wild-type and Δipd strain are shown with different colors. (b) Amount of produced ILA in Wild type and Δipd knock-out strains from 72 hours old cultures supplemented with tryptophan. * $p < 0.05$.

HPLC analysis from 72 hours old fungal cultures supplemented with 400mg/L (1.96 mM) tryptophan revealed that the *lipd* knock-out strain produces maximum amount of indole-3-lactic acid (Fig 31a). The estimated amount of ILA is 64.4% higher in *lipd* (15.78 ng) as compared to wild type (5.62 ng) (Fig 31b). While doing the pathway analysis with knock-out strains the most pronounced effect on the IAA production was found in the *liad1liad3* double knock-out strain (Fig 32). HPLC peaks for wild type, *liad1*, *liad3* and *liad1liad3* are shown in different colors as mentioned in the figure (Fig. 32a). Based on the HPLC peak areas and that of the standards we estimate that the amount of IAA in *liad1liad3* double knock-out strain (3.52 ng) is 44% lower than wild type strain (6.28 ng) (Fig. 32b) under the same cultural and extraction conditions.

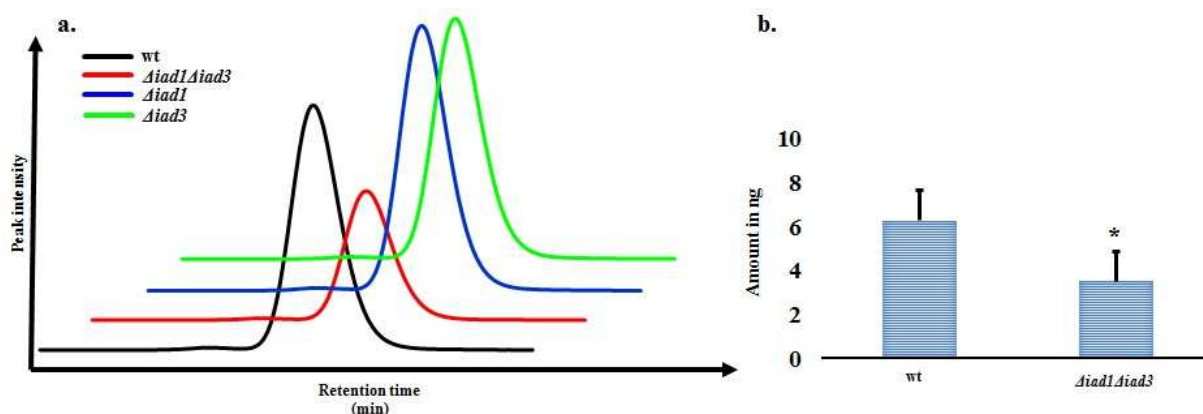


Figure. 32: Reduction in IAA production by synergistic effect of *liad1liad3* double knock-out in *N. crassa*
Individual knock-out strain for aldehyde dehydrogenase gene *liad1* and *liad3* does not have any significant effect in IAA production but synergistic effect of *liad1liad3* double knock-out results drastic reduction in IAA production. (a) HPLC peaks of IAA for wild-type along with other knock-out strains are shown in different colors as mentioned in the figure. IAA production has been decreased in *liad1liad3* double knock-out strain as compare to the wild-type strains. (b) Comparison of amount of IAA produced by wild-type and *liad1liad3* strains in 72 hours. * $p < 0.05$.

The other genes that were studied for IAA biosynthesis pathways are *yuc*, *iaam* (tryptophan-2-monooxygenase), *ami1* (indole-3-acetamide hydrolase) and *tdc* (tryptophan decarboxylase). Neither of the single knock-outs nor any of the double or triple knock-out strains (table 5 and 6) has any effect on the level of IAA production (Fig. 33).

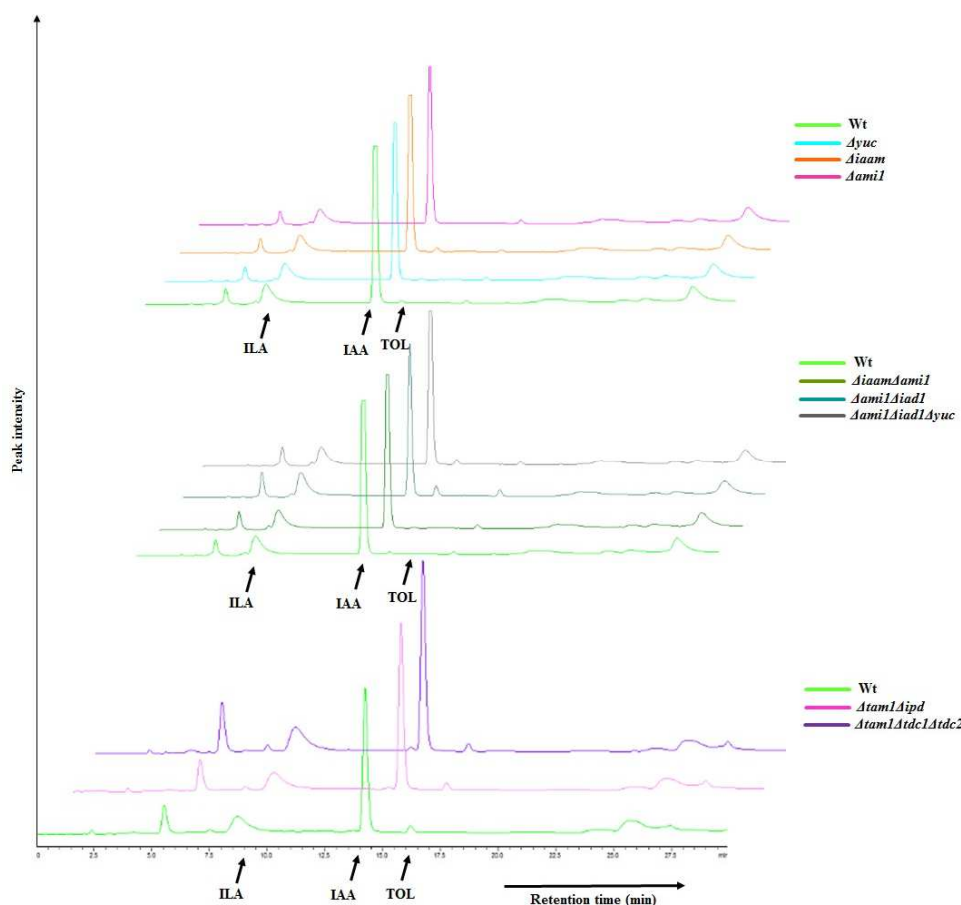


Fig. 33: HPLC analysis of the metabolites produced by different knock-out *N. crassa* strains supplemented with 400mg/L (1.96 mM) tryptophan in 72 hours.

Graphs with different colors are representing different knock-out strains. Specific graph for a particular knock-out strain is mentioned in the figure with color bars. Standard peaks are: ILA: Indole-3-lactic acid, IAA: Indole-3-acetic acid and TOL: Tryptophol.

4.2.3.1 Indole production in *Neurospora crassa* is time dependent and affected by the knock-out of the IAA producing genes

IAA production in fungi is time dependent and various indolic compounds start to accumulate into the culture media over time (TOMITA 1987; HILBERT *et al.* 2012). In this study, it was demonstrated how IAA and related indole production changes over time in different *N. crassa* strains with different mutation of the IAA producing genes (Fig. 34). Results suggest that production of IAA and related indolic compounds in wild-type culture media supplemented with tryptophan begins after ~12 hours of inoculation and reduces over time (Fig. 34a). *Aipd* strain shows similar indole production pattern as wild-type strain with notably higher ILA production

throughout the experimental time frame (96 hours) (Fig. 34b). A $\Delta iad1\Delta iad3$ double knock-out strain supplemented with tryptophan starts to produce IAA at a later hour as compared to wild-type strain (Fig. 34c). None of the 72 hours old fungal cultures shows any trace of tryptophan in HPLC and TLC analysis (Fig. 29b, Fig. 33 and Fig. 34a) but $\Delta iad1\Delta iad3$ double knock-out strain shows accumulation of tryptophan till 48 hours after inoculation compared to wildtype strain (Fig. 34c and 34d). These results suggest that $\Delta iad1\Delta iad3$ strain does not only have reduced level of IAA production but also lower efficiency in tryptophan utilization.

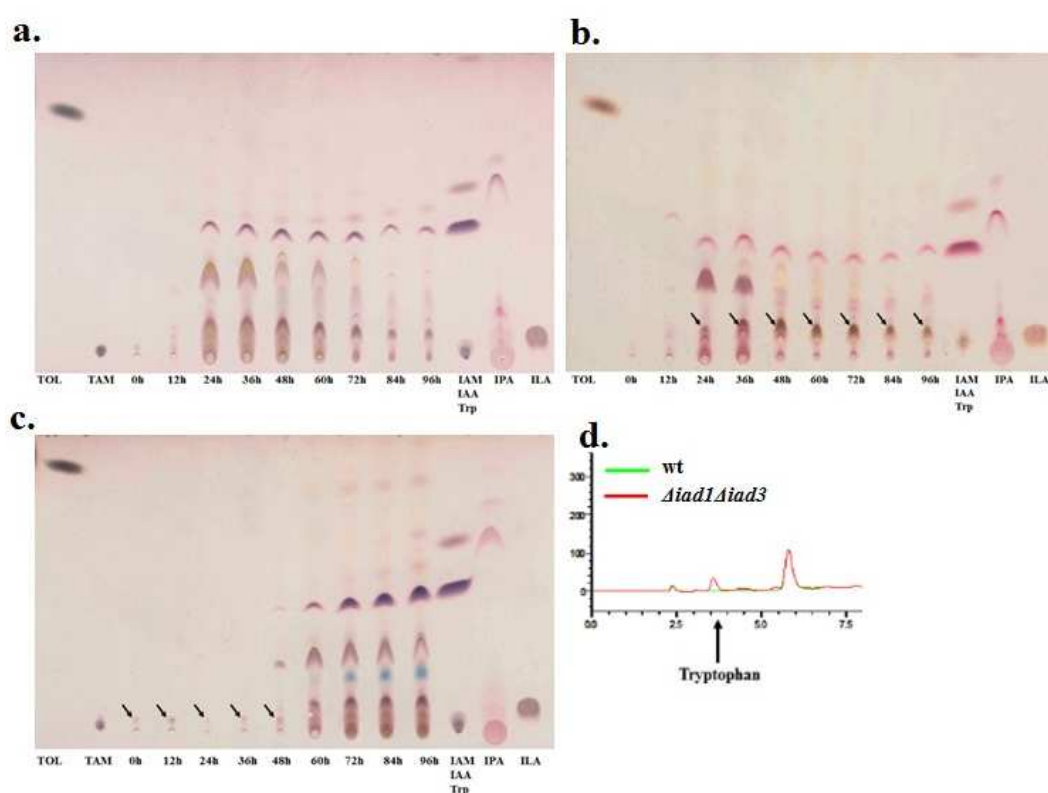


Fig. 34: Indolic compounds including IAA production by wild type and mutant *N. crassa* with tryptophan supplementation in 12 hours intervals for total of 96 hours.

Changes in IAA and indolic compounds metabolism over time in wild type and mutant *N. crassa* supplemented with 1.96 mM tryptophan. (a) Wild type *N. crassa* strain was grown with tryptophan supplementation, samples were collected in every 12 hours intervals for a total length of 96 hours. (b) Δipd knock-out strain was grown with tryptophan supplementation. In every 12 hours intervals samples were collected. Black arrow marks are indicating for ILA spots. (c) $\Delta iad1\Delta iad3$ double knock-out strain was grown with tryptophan supplementation. In every 12 hours intervals samples were collected and analyzed. Black arrow marks are indicating for tryptophan spots. (d) HPLC analysis of 48 hours old wild type and $\Delta iad1\Delta iad3$ double knock-out *N. crassa* culture supplemented with tryptophan. After 48 hours of inoculation $\Delta iad1\Delta iad3$ double knock-out strain shows a peak for tryptophan while corresponding peak from wild-type strain has not been observed. Following standards were used: tryptophol (TOL), tryptamine (TAM), indole-3-acetamide (IAM), indole-3-acetic acid (IAA), tryptophan (Trp), indole-3-pyruvic acid (IPA) and Indole-3-lactic acid (ILA).

4.2.3.2 Feeding test performed with other indolic compounds and the amino acid tyrosine

In earlier study it was mentioned that tyrosine may also act as a precursor for IAA production in fungi (GRUEN 1959), while some other studies utilized tryptamine. However, none of the fungi tested showed IAA production when supplemented with tryptamine (GRUEN 1959; HILBERT *et al.* 2012). In this study feeding experiments were performed with tyrosine along with indolic compounds such as tryptamine, indole-3-acetamide (IAM), indole-3-lactic acid, tryptophol (TOL) and indole-3-acetic acid (IAA) to check their ability to produce IAA. Results show that the wild type strain could not produce IAA even when supplemented with tyrosine at a relatively high concentration of 5 mM. (Fig. 35).

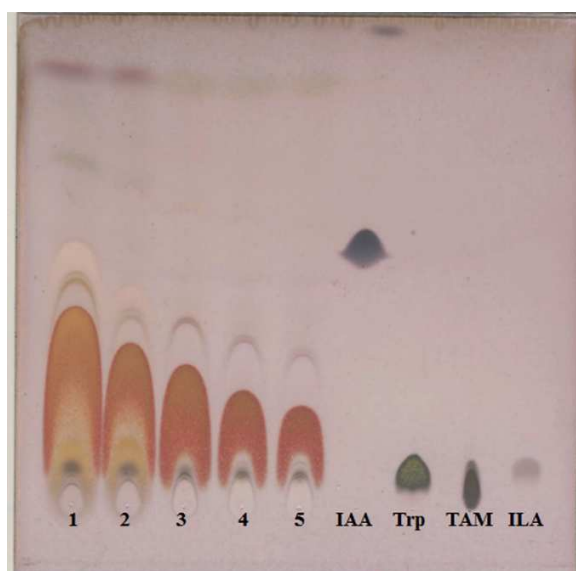


Fig. 35: Indole metabolite analysis on TLC plate extracted from 72 hours old *Neurospora crassa* cultures supplemented with various amount of tyrosine concentrations. Ethyl acetate extracted compounds were run on a silica plate along with standard indolic compounds. The tyrosine concentrations that were used in different cultures are: 1. 5 mM, 2. 2.5 mM, 3. 1.25 mM, 4. 625 μ M and 5. 312.5 μ M. IAA: Indole-3-acetic acid, Trp: Tryptophan, TAM: Tryptamine and ILA: Indole-3-lactic acid

The effect of tyrosine was also analyzed by feeding the wild type *N. crassa* strain with different tyrosine concentrations and performing subsequent metabolite analysis on TLC plates. But tyrosine mediated IAA biosynthesis was not found in the same experimental setup with any of the concentrations used. In contrast, both tryptamine and IAM supplementation led to IAA production, although in different concentrations: were as 1mM IAM is enough to induce IAA production, tryptamine had to be supplemented at 2.5 mM (Fig. 36). Tryptophan supplemented

culture produces maximum number of indolic compounds as compared to other supplements tested in this study (Fig. 36). We also observed that over time indolic compounds including IAA from tryptophan supplemented culture gradually depletes (Fig. 34a and Fig. 36b) but opposite happens to IAM supplemented culture (Fig. 36b and S11 Fig.) where IAA level tend to increase in time. These results clearly suggest that not only tryptophan but also tryptamine and indole-3-acetamide (IAM), the main precursor molecule for bacterial IAA biosynthesis (PATTEN and GLICK 1996; SPAEPEN *et al.* 2007) can be utilized as a precursor molecule for IAA biosynthesis in *N. crassa*.

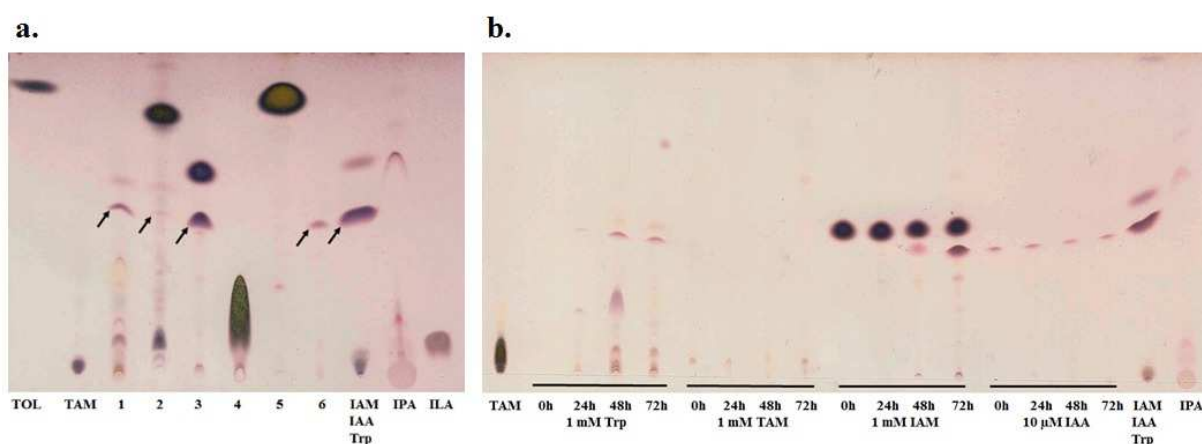


Fig. 36: Feeding effect of various indolic compounds on *N. crassa* in IAA production.

Different indolic compounds were supplemented to wild type *N. crassa* culture media for checking their role in IAA production. (a) TLC analysis to find the ability to produce IAA by various indolic compounds. Black arrows are indicating traces of IAA. Indolic compounds that were used as supplementation are: **1.** 1 mM tryptophan, **2.** 2.5 mM tryptamine, **3.** 1 mM indole-3-acetamide, **4.** 1 mM indole-3-lactic acid, **5.** 1 mM tryptophol, **6.** 10 μ M IAA. (b) The feeding experiment was extended by coupling with time course assay. Tryptophan (Trp), Tryptamine (TAM), Indole-3-acetamide (IAM) and Indole-3-acetic acid (IAA) were used for the assay. Samples were collected in 24 hours intervals. The numbers below the lanes represent the time in hours. Following standards were used: tryptophol (TOL), tryptamine (TAM), indole-3-acetamide (IAM), indole-3-acetic acid (IAA), tryptophan (Trp), and indole-3-pyruvic acid (IPA).

5 DISCUSSION

Indole-3-acetic acid (IAA) plays a crucial role as a phyto-hormone in green plants. It regulates many physiological roles such as root and shoot development, flowering, cell division, cell differentiation and more. Due to having these diversified role auxin has drawn attention of researches and lead them in synthesizing commercial auxin for various usages. In green plants physiological roles of IAA has been investigated in great details while IAA biosynthesis pathway is still elusive. Microorganisms including fungi and bacteria are also capable of producing indole-3-acetic acid (IAA) (GRUEN 1959; GUNASEKARAN and WEBER 1972; ROBINSON *et al.* 1998; TSAVKELOVA *et al.* 2006; REINEKE *et al.* 2008; HILBERT *et al.* 2012; MANO and NEMOTO 2012; TSAVKELOVA *et al.* 2012; KOLLATH-LEISS *et al.* 2014). Bacterial IAA biosynthesis pathway has been studied in great details and corresponding IAA biosynthesis genes were investigated too (COSTACURTA *et al.* 1994) but very little known about the function of IAA in those microbes. Researchers are trying to understand and exploit the potential of IAA-producing fungi for promoting plant growth, development and protection from infection and for a better sustainable agriculture (FU *et al.* 2015). While many fungi has been shown to produce IAA (GRUEN 1959) using various IAA biosynthesis routes (BASSE *et al.* 1996; ROBINSON *et al.* 1998; HILBERT *et al.* 2012; TSAVKELOVA *et al.* 2012) but limited number of IAA biosynthetic genes have been characterized in details (WOODWARD and BARTEL 2005; MANO and NEMOTO 2012). IAA synthesis via the IPA pathway has been described in a broad range of plant growth-promoting bacterial species (BASSE *et al.* 1996; PATTEN and GLICK 1996; SPAEPEN *et al.* 2007) and in few fungal species (BASSE *et al.* 1996; HILBERT *et al.* 2012). Only a few fungal genes involved in IAA production have been functionally characterized, and these include the tryptophan aminotransferase (*tam*) and aldehyde dehydrogenase (*adh*) genes from *U. maydis* and the aromatic aminotransferases (Aro8 and Aro9) from *Saccharomyces cerevisiae* (BASSE *et al.* 1996; ROBINSON *et al.* 1998; REINEKE *et al.* 2008; RAO *et al.* 2010).

There is strong evidence for multiple IAA biosynthetic pathways in fungi and bacteria, and the coexistence of these pathways suggests a differential spatial and temporal regulation of the production of IAA under various stimuli. For instance, in *Erwinia herbicola* *pv.* *gypsophylae*, the production of IAA via the IAM pathway correlates with disease severity, whereas the IPA pathway is involved in epiphytic fitness (CHALUPOWICZ *et al.* 2009) and endophytic fitness in *P. indica*. The IPA pathway has been well characterized in bacteria (KOGA *et al.* 1991). For green plant, IPA

has been identified as a native compound in *Arabidopsis* (TAM and NORMANLY 1998). *in vitro* assay has demonstrated that tryptophan aminotransferase of *Arabidopsis* (*TAAI*) catalyzes the reaction towards formation of IPA from L-Trp (STEPANOVA *et al.* 2008; TAO *et al.* 2008). Mutant *TAAI* strains accumulate less IAA in response to external stimuli like ethylene and shade (STEPANOVA *et al.* 2008; TAO *et al.* 2008; NORMANLY 2010). Sequence analysis and motif identification predicts five plausible pyruvate decarboxylases suitable candidates for an indole-3-pyruvate decarboxylase (IPDC) in *Arabidopsis* (NORMANLY 2010). All though all the five predicted genes were expressed in *E. coli* but an *in vitro* assay failed to demonstrate the IPDC activity for any of the targeted genes. Addition to that any IPDC activity was not detected in the protein extracts from wild-type *Arabidopsis* (NORMANLY 2010). These former studies suggest that it is not clear if the subsequent steps of IAA synthesis are similar in *Arabidopsis* to those found in the microbial IPA pathway.

However presence of IAA in *Neurospora crassa* has been documented in few studies (TOMITA 1987; KOLLATH-LEISS *et al.* 2014) but neither the biosynthetic genes nor the biosynthesis pathway has been explored. Here in this study for the first time it has been shown that genes from IPA pathway for IAA production are present in *Neurospora crassa* and functionally active. Although homologue of the gene that encodes for indole-3-pyruvate decarboxylase (*ipd*) is not found in green plants (MANO and NEMOTO 2012) but results from this thesis clearly demonstrated that *ipd* is present in wide range of fungi and fully functional in *Neurospora crassa*. TLC and HPLC analyses of *N. crassa* wild-type and knock-out strains for IPA pathway used in this study revealed that *N. crassa* produces IAA via the IPA route. Additionally it was also reported that alteration of the IAA producing genes in IPA pathway of *N. crassa* results in altered production in IAA as well as in related indolic compounds (Fig. 37). Tryptophan is needed as external supplementation for IAA production in *N. crassa* (TOMITA 1987; KOLLATH-LEISS *et al.* 2014). In this study the lower limit of tryptophan concentration was quantified as 1 μ M that *N. crassa* can utilize to produce IAA in the liquid medium (Fig. 30). The potentiality for tyrosine to produce IAA was also checked as tyrosine was mentioned somewhere else as a potential precursor candidate to produce IAA in fungi (GRUEN 1959). But result from this study confirms that *N. crassa* cannot use tyrosine as a precursor molecule to produce IAA (Fig. 35).

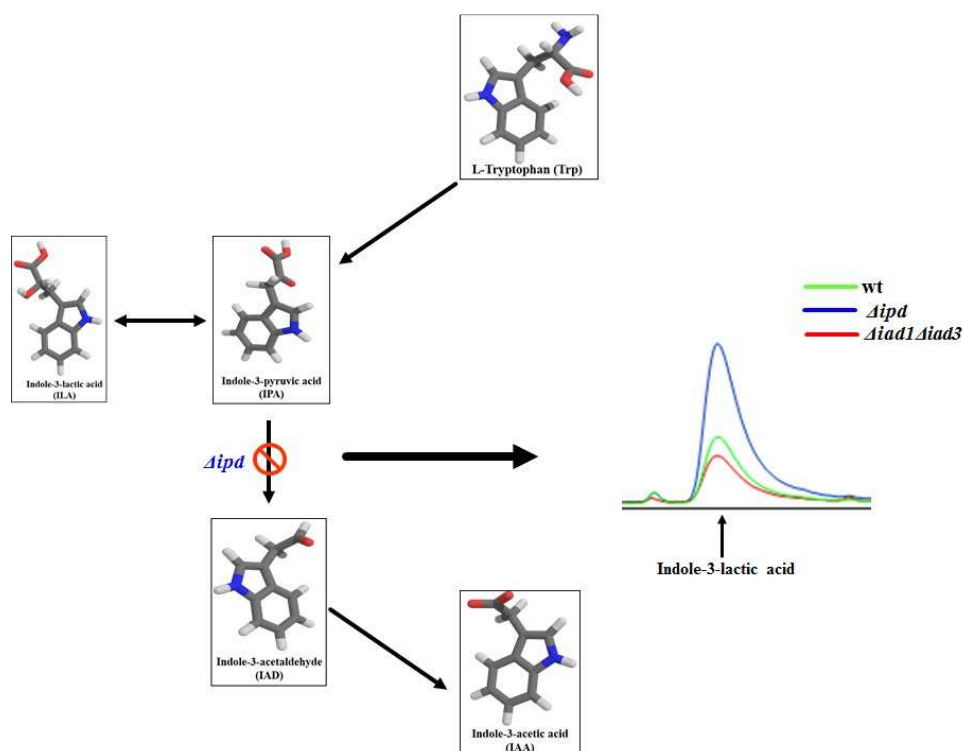


Fig. 37: *ipd* knock-out strain produces higher level of ILA due to absence of *ipd* gene that is responsible for conversion of IPA to IAD

A schematic diagram of result in ILA production by *ipd* knock-out *N. crassa* strain. *ipd* gene is responsible for conversion of IPA to IAD. IPA in nature is an unstable compound and there is a dynamic conversion between IPA and ILA so the status of IPA can be estimated by the level of ILA itself. As the *ipd* is missing from the knock-out strain therefore IPA cannot be converted to IAD and results in increase level of IPA hence higher level of ILA as compare to other strains where *ipd* is intact and functional. In the right side HPLC peaks are shown. Graphs with different colors are representing ILA peaks from different strains as mentioned in the figure.

5.1 Identification and functional characterization of indole-3-pyruvate decarboxylase in *N. crassa*

By employing homology searching a pyruvate decarboxylase gene (*ipd*) in *Neurospora crassa* (NCU02193) has been identified which is named as “cellular filamentous protein” (*cfp*) in FGSC as well as in “FungiDB”. In this thesis it has been shown that *ipd* from *N. crassa* contains binding sites for thiamine pyrophosphate (TPP or ThPP) which acts as a ligand for decarboxylase enzymes (Fig. 11 and Fig. 15). In the proposed IPA pathway from various studies (PHI *et al.* 2008; MANO and NEMOTO 2012) it has been shown that indole-3-pyruvic (IPA) acid is an unstable compound and there is a dynamic conversion between IPA and indole-3-lactic acid (ILA). Through HPLC analysis it has been shown that *ipd* *N. crassa* strain produces more amount of ILA as

compare to wild-type strain (Fig. 31). Hence this result perfectly matches with the fact that inability of conversion of IPA into indole-3-acetaldehyde (IAAld) by the fungi due to lack of *ipd* gene would lead to increased level of IPA and hence further accumulation of ILA (Fig. 37). These results unambiguously prove that *ipd* is present and functionally contributes its enzymatic role in IPA pathway of IAA biosynthesis in *N. crassa*.

5.2 Indole-3-acetaldehyde dehydrogenase genes (*iad*) are direct contributor for IAA production in *N. crassa*

Functional identification and synergistic effect of two *iad* genes in pathogenic fungus *U. maydis* has been studied earlier (REINEKE *et al.* 2008). In this thesis presence of more than one copy of the aldehyde dehydrogenase gene in *N. crassa* and their functional contribution towards IAA biosynthesis has been reported. Individual knock-outs of any of these aldehyde dehydrogenase genes does not have any significant effect on IAA production. $\Delta iad1$, $\Delta iad2$ and $\Delta iad3$ single knock-out strains as well as $\Delta iad1\Delta iad2$ double knock-out strain does not show any significant changes in IAA production (Fig. 32 and Fig. 38).

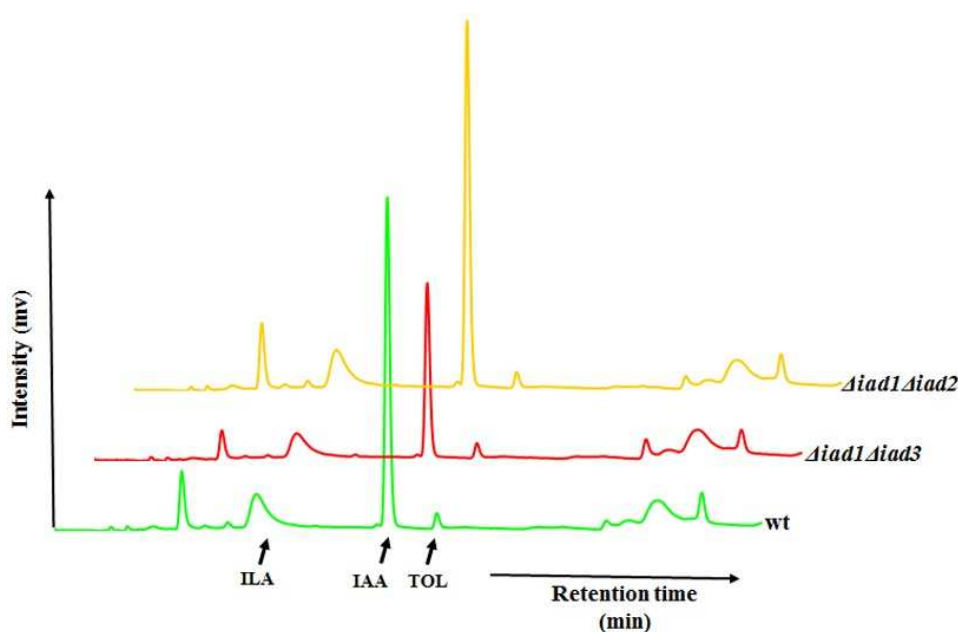


Fig. 38: The synergistic effect of $\Delta iad1\Delta iad3$ double knock-out strains

Only $\Delta iad1\Delta iad3$ strain shows rapid reduction in IAA production. On the other hand $\Delta iad1\Delta iad2$ strain has similar IAA production as wild-type strain. HPLC peaks of IAA and other indoles for Wild type along with two other knock-out strains are shown in different colors as mentioned in the figure.

Significant decrease in IAA formation has been observed only in *Δiad1Δiad3* double knock-out *N. crassa* strain. This study perfectly fit with the same scenario in a previous study done on *U. maydis* (BASSE *et al.* 1996). A single copy *iad* null mutant does not cause any reduction in IAA formation in *U. maydis*, but two copies of *iad* null mutant strain shows reduction in IAA formation by many folds (BASSE *et al.* 1996; REINEKE *et al.* 2008). Combining these results it can be concluded that there are more than one aldehyde dehydrogenase genes acting in the IAA biosynthesis pathway of *N. crassa* and they are very much interconnected and well regulated. The synergistic effect of only *Δiad1Δiad3* double knock-out implies that IAA production in *N. crassa* via IPA pathway proceeds through production of indole-3-acetaldehyde (IAld) and last step of this pathway is under tight regulation by multiple gene action. Here for the first time a novel aldehyde dehydrogenase gene (*iad3*) and its contribution towards IAA biosynthesis in *N. crassa* has been reported.

5.3 IAA biosynthesis in *N. crassa* involves multiple pathways but is activated and regulated spatiotemporally

In this study IAA production by *N. crassa* was examined with different supplements at different time points. Results have demonstrated that tryptophan is the only amino acid among typtophan, tyrosine and histidine that can be used as a precursor for IAA biosynthesis in *N. crassa* (histidine supplementation in S12 Fig.). Existence of multiple IAA biosynthesis pathways in microorganism has been reported earlier. For example in *Erwinia herbicola pv. gypsophilae* both IPA and IAM pathways exist (MANULIS *et al.* 1998) in which IPA pathway is involved in epiphytic fitness where IAM pathway is responsible for pathogenicity. In this thesis it has been shown that tryptophan supplemented culture medium produces many indolic compounds but IAM over time. On the other hand IAM supplementation resulted only in IAA production as major indolic compound along with very few other unidentified compounds. IAM pathway has been shown to be present in the filamentous fungi *Fusarium* (TSAVKELOVA *et al.* 2012) and *Colletotrichum gloeosporioides f. sp. Aeschynomene* (ROBINSON *et al.* 1998). In this thesis, no trace of *N. crassa* produced IAM was detected by TLC and HPLC analyses. However, it was found that IAM supplementation in the *N. crassa* culture medium also led to IAA production. These results clearly suggest that tryptophan is used as primary source for IAA production in *N. crassa* and primarily

proceeds through IPA pathway producing intermediate indolic compounds such as ILA, TOL which are side products of the IPA pathway of IAA production. The amounts of IAA, and other indolic compounds increased with increasing tryptophan concentrations (S10 Fig.). This result is supported by a previous study done on bacteria where the level of biosynthesis of indolic compounds like ILA, IAA and IAM was positively correlated with tryptophan concentration (SZKOP and BIELAWSKI 2013) providing further support for the fact that IAA biosynthesis in *N. crassa* is tryptophan dependent and tryptophan is used as the primary precursor. IAM pathway in *N. crassa* is activated only if IAM is supplemented to the media otherwise no trace of IAM from metabolite analysis was observed, which further suggests that IAM pathway in *N. crassa* is more like a reserved and very specific secondary pathway for IAA production.

In addition it was noticed that there is no change in IAA production in $\Delta iaam$, $\Delta ami1$, $\Delta iaam\Delta ami1$ and $\Delta ami1\Delta iad1\Delta yuc$ strains (Fig. 33). This might be explained by the fact that there are more than one genes for the same enzyme acting in the position of *ami1* (NCU04092) in *N. crassa*, as evidences has already been given for *iad* genes from *N. crassa* in support of this kind of effect.

Tryptamine (TAM) supplementation shows minute amount of IAA production when supplemented at a relatively higher concentration. (Fig. 36a). This suggests that although tryptamine supplementation leads to IAA production, TAM pathway itself is an inefficient and further secondary IAA production pathway in *N. crassa*. Genes from YUC and IAM pathway has an effect on morphology of growing hyphae (Fig. 25), but none of the knock-out strains encompassing these pathways shown any difference in IAA production (Fig. 33). IAA production has been studied using knocked-out strains for IAA-biosynthetic genes from IPA, YUC, IAM and TAM pathways. But in this study, only those genes present in the IPA pathway have been proven to be active and appear to produce IAA most efficiently in *N. crassa*.

5.4 IAA in general and in *N. crassa* from the point of evolution

Indole-3-acetic acid serves as main auxin in almost all the green plants. Therefore IAA is treated mainly as a 'phytohormone'. Auxins virtually influence every aspect of plant growth and development (WOODWARD and BARTEL 2005). Besides plant, green algae and many microorganisms including bacteria and fungi are also capable of producing IAA. But, unlike in plants function of IAA in fungi is least characterized. Like IAA production, IAA metabolism is also studied in details. A summarization of IAA metabolism states that seed plants produce the highest levels of total IAA metabolites (WICKETT *et al.* 2014). This highest level of IAA metabolite production in seed plants has been incorporated into plant kingdom by means of evolution of auxin metabolism (Fig. 39). IAA has been demonstrated as an ancient signaling molecule in previous studies (COOKE *et al.* 2002a; DE SMET *et al.* 2011). One such example of IAA to be a signaling molecule can be given by the presence of IAA responses in both the charophyte *Chara* and the brown alga *Fucus*. This suggests that IAA is present in these photosynthetic aquatic organisms as an ancient signaling molecule. Study has been conducted on *Fucus* rhizoids where IAA acts as the pheromone that regulates the mutual attraction of the rhizoids that are emerging from adjacent zygotes (JAFFE 1968). As IAA is a small organic molecule with a pK_a of 4.7, which gives it the potential for a rapid diffusion in aquatic environments as well as across the cell membranes. From these obvious facts it has been rightly hypothesized that IAA had originally evolved in photosynthetic aquatic organisms as a pheromone for regulating the growth of nearby members of the same species (JAFFE 1968; COOKE *et al.* 2002a). This hypothesis is supported by the rhizoids of *Chara* and *Fucus* itself. They exhibit similar IAA regulation and inhibitor properties. Such kind of similarities can be explained by either a common origin or convergent evolution. In the context of IAA transportation it is needed to analyze the origin and evolution of IAA-transporter. Due to the structural similarity between tryptophan and IAA, IAA can also be transported by general amino acid transporters. Due to this affinity towards general amino acid transporters it can reasonably be postulated that in the course of evolution generalized amino-acid transporters might have evolved into more specific IAA transporters by natural selection (COOKE *et al.* 2002b). This hypothesis is supported by the IAA influx carrier AUX1 in *Arabidopsis* which has indeed evolved from an amino acid permease (BENNETT *et al.* 1996).

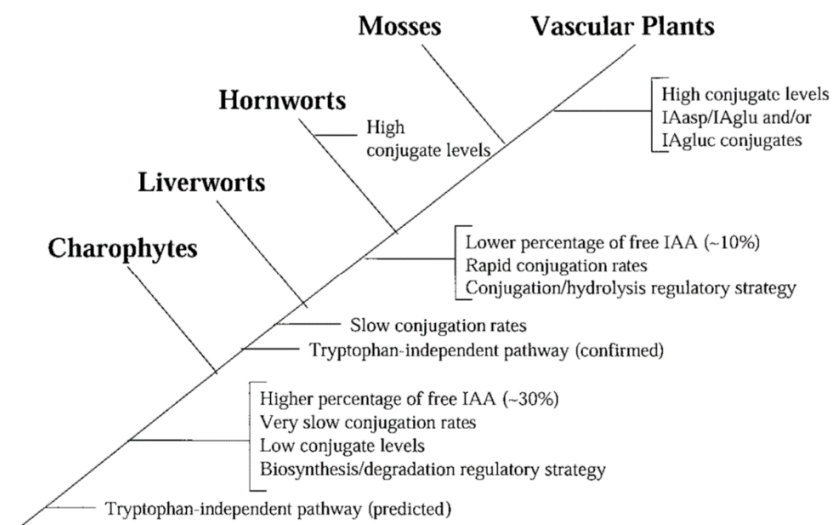


Fig. 39: The major evolutionary events in IAA metabolism of green plants has been shown in cladogram. (COOKE *et al.* 2002a).

There are multiple IAA production pathways existing in microorganisms. While phytopathogenic bacteria has been shown to produce IAA as a mode of infection to the plant tissues IAA producing fungi are rather classified in two groups. It has already been discussed that depending on type of fungi their IAA biosynthesis pathways also differs. For example plant-growth promoting beneficial fungi produces IAA through pyruvic acid pathway while acetamide pathway is common in phytopathogenic fungi. It has also been shown that even same fungal species has different effects on different plant species (FU *et al.* 2015). Irrespective of the plant-fungus association, the IAA remains same as the interaction molecule. The diversified roles of IAA produced by fungi in different plant-fungus interaction systems suggest that fungi may use IAA to interact with plants for pathogenesis or symbiotic strategies. This makes IAA a universal signaling molecule that leads to basal plant defense mechanism and/or plant growth promotion and modification. As an ancient signaling molecule it can be easily argued that IAA production in any species should also be through an evolutionary conserved biosynthetic pathway.

As mentioned previously biosynthetic pathway of IAA differs in different fungal species depending upon the type of fungal-plant interaction. In this study, *Neurospora crassa* which does not possess any plant interacting major property in particular has been used as focal species. IAA production in such a species has been shown to happen via a preexisting IAA biosynthesis pathway which involves many IAA biosynthetic genes. It has been successfully demonstrated that a non-plant-interacting fungi like *N. crassa* is capable of producing IAA via IPA, a well characterized

IAA biosynthesis pathway regulated by evolutionary conserved IAA biosynthetic genes. Therefore in general it can be stated that IAA biosynthesis pathway/s in *N. crassa* and possibly in other IAA-producing organisms is evolutionary conserved.

6 SUMMARY

The aim of this study was to reveal IAA biosynthetic pathway in *Neurospora crassa* and finding explanation from evolutionary background. In this study multiple IAA biosynthetic pathways of *N. crassa* were targeted using both computational and experimental approaches. For the same purpose different mutant strains for different IAA biosynthesis pathways has been used. Most of the double and triple knock-out strains did not show any effect on IAA production. Only prominent effects on IAA production were observed when knock-out strains from IPA pathway were targeted. With the data in hand it can be conclusively said that IAA production is mediated via IPA pathway and this pathway is the most prominent and primary pathway in *N. crassa*. *N. crassa* mutant was found with lesser amount of IAA production capability but no IAA auxotroph was found. This situation can occur if (1) there are multiple genes for a particular type of enzyme like aldehyde dehydrogenase as in here or (2) IAA production in *N. crassa* employs multiple pathways depending upon genetic background of the strain and the medium type where it is growing.

In this study for the first time it was shown that IAA production in *N. crassa* proceeds through IPA pathway. The pathway has been pinpointed with exact gene functionalities. It has also been shown that the enzymes involved in this pathway are functional with their designated enzymatic activity. Few genes were also identified in *N. crassa* with their novel functionality. A functional gene for pyruvate decarboxylase has not yet been found to fully support IPA pathway in plants but in this study a fully functional *ipd* gene has been explained in filamentous fungus *N. crassa*. It was also explained that *iad* gene, the other member of the same pathway (IPA) is present in *Neurospora crassa* in multiple copies and they have a synergistic effect on IAA production. This multimodal evidences from a single pathway gives enough support for identifying and establishing a functional IAA biosynthetic pathway in *N. crassa*. With this regard, it was also shown in this study that deletion of IAA producing gene(s) leads to altered morphology of the growing hyphae.

The function of IAA is well described in green plants. Phytopathogenic microbes including bacteria and fungi have been shown to produce IAA as a means of infection to plants. Some fungi has also been shown to benefit plants by promoting plant growths. In either way role of auxin has been shown in fungi as a means of plant-association. A very handful study has been conducted to

demonstrate the role of IAA in changing the virulence mode or internal physiology of the fungi. Few studies have demonstrated that IAA has an effect on hyphal length and conidia germination rate in *N. crassa* but no molecular data is available on that. IAA has been described as an evolutionary conserved signaling molecule for inter as well as intra species communication. Besides the numerous classical roles of IAA, it can also act as a means of infection and it also provides defense against infection depending on the physiobiological situation. For example, phytopathogenic bacteria use IAA for infecting plant tissue by blocking plant's defense mechanism on the other hand plants increase the IAA level in the infected tissues to eliminate the pathogen load. The biosynthetic pathway of IAA differs in species to species. In bacteria the IAM pathway is considered as the main IAA biosynthetic pathway. In fungi IAM pathway is common in phytopathogenic fungi but IPA pathway is more frequent in plant-beneficial fungi. *Arabidopsis* is more likely to involve a combination of both IPA and YUC pathway for IAA biosynthesis. In this scenario this study shows that a non-pathogenic, non-commensal fungi *N. crassa* produces IAA through IPA, one of the most studied pathway which argues towards a general role of IAA like a signaling molecule in *N. crassa* as well in other fungi. In fungi although IAA may not serve its 'defined function' as it does in green plants yet fungi have conserved IAA biosynthetic genes and defined pathway for it. This also implies that there is an evolutionary constraint on the IAA producing genes, its biosynthetic pathway and its universal function.

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Thesis Title	Auxin biosynthesis pathway in filamentous fungus <i>Neurospora crassa</i> and its evolutionary background

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Fields worked on and skills acquired	Bioinformatics, Bioinformatics of protein structures, Fungal biology, Metabolite analysis, Microscopic techniques, Molecular genetics and techniques, Molecular phylogeny
Name and type of the organization	Department of Molecular genetics in plants, Botany Institute, Christian-Albrechts-Universität, Kiel, University
Funded by	International Max-Planck Research School (IMPRS) for Evolutionary Biology, Plön, Germany
WORK EXPERIENCE	
Date	April 2016 – March 2017
Worked as	Teaching assistance
Responsible for	Bachelor courses: 1. Ligation und Klonierung von DNA in Escherichia coli. 2. Fungal diversity in marine and terrestrial ecosystem
Name and address of employer	Christian-Albrechts-Universität, Christian-Albrechts-Platz 4, 24118 Kiel, Germany

Publications

1. **Sardar P** and Kempken F. (2017) Characterization of indole-3-pyruvic acid pathway mediated biosynthesis of auxin in *Neurospora crassa*. **PLoS ONE** (Under Review)
2. Kumari S, Kumar A, **Sardar P**, Yadav M, Majhi R K; Kumar A, Goswami C (2015) Influence of membrane cholesterol in the molecular evolution and functional regulation of TRPV4. **BBRC**. Jan 456:312-9 (**Joint first author**)
3. Kollath-Leiß K, Bönniger C, **Sardar P**, Kempken F. (2014) BEM46 Shows Eisosomal Localization and Association with Tryptophan-Derived Auxin Pathway in *Neurospora crassa*. **Eukaryot Cell**. Aug 13:1051-63
4. Kumar A, Bhandari A, Sinha R, **Sardar P**, Sushma M, et al. (2012) Molecular Phylogeny of OVOL Genes Illustrates a Conserved C2H2 Zinc Finger Domain Coupled by Hypervariable Unstructured Regions. **PLoS ONE** 7: e39399.
5. **Sardar P**, Kumar A, Bhandari A, Goswami C (2012) Conservation of Tubulin-Binding Sequences in TRPV1 throughout Evolution. **PLoS ONE** 7: e31448.
6. Majhi R. **Sardar P**, Goswami L and Goswami C (2011) “Right time–right location–right move TRPs find motors for common functions”, **Channels** 5:1-7 (**Joint first author**)

10 DECLARATION

I, Puspendu Sardar hereby declare that this thesis is my own original work. I also declare that I have not used this work or part of works other than in the acknowledged resources and aids. This dissertation has not been submitted previously for the purpose of awarding any other degree or diploma in any other tertiary institution. Throughout this thesis, I followed the guidelines of “Good Scientific Practice” of the Deutsche “Forschungsgemeinschaft”.

Parts and information from this thesis had been used in the following articles for publication:

1. “BEM46 Shows Eisosomal Localization and Association with Tryptophan-Derived Auxin Pathway in *Neurospora crassa*”, Kollath-Leiss et. al. Eukaryot Cell. 2014 Aug 13:1051-63 and
2. “Identification and functional characterization of indole-3-pyruvic acid pathway mediated IAA biosynthesis in *Neurospora crassa*.” Puspendu Sardar and Frank Kempken (2017) (Under review).

Kiel,

Puspendu Sardar

11 Supplementary information

S1 Fig.

N. crassa	:	--MGEALINGSGAVSNVSALETGLPAAKLTADDPSEIPPASGPGR--ENAVTISD--ILERRA	:	57
U. maydis	:	-----MTPSAQ	:	6
A. macrogynus	:	-----MFAQAVASRGRVAVA	:	16
A. nidulans	:	-----	:	-
B. cinerea	:	-----MAPPTAIDIEGVVDTESIVLP-----DPLTVNGVSARRA	:	34
B. dendrobatidis	:	-----MTIASLLLRPVKHCKAILSSAAISSSTMN	:	29
C. albicans	:	-----	:	-
C. guilliermondii	:	-----	:	-
C. immitis	:	-----MSPHAAQDAAVQGAVDITKET---PEPLTVAN--VPARRA	:	35
C. neoformans	:	-----	:	-
C. tropicalis	:	-----	:	-
F. graminearum	:	MLSAVRTARLRSLIKHSRTIHSSSKHLSSTEAPAAPARPAHYDT---SOKLSAIDAVKERRL	:	58
F. oxysporum	:	-----	:	-
M. oryzae	:	-----	:	-
P. brasiliensis	:	-----MSPPGAVDPQIINNAD---P---PEPLTVAD--VPARRA	:	31
P. nodorum	:	-----MAPP SAEIVSAETDTSGITIPNPLTAPITSND--IFGRRK	:	38
P. triticina	:	-----	:	-
R. oryzae	:	-----	:	-
S. cryophilus	:	-----	:	-
S. japonicus	:	-----	:	-
S. pombe	:	-----	:	-
S. punctatus	:	-----	:	-
T. equinum	:	-----MHLEF	:	5
T. rubrum	:	-----MAPHAAAGAGTAGDAAQKAVV---EGTLTVAN--VAANRT	:	35
U. reesii	:	-----MAP	:	3
V. dahliae	:	--MSAIACQTAPVGLISRRSFHAKPKLNSAGEAAPVQQDAVLEN---KQIPISVFD S IRERRA	:	57

N. crassa	:	KAGRLIAPTASYADSDMFKGPO--VGKPKSKSMN--HHL SAESLSRHP CALKQA-----ARH	:	110
U. maydis	:	VANVDPNPYFGDKVGDLASTSETYPKASRVDP--RFLSQVSHDRPASAIRSL-----FPA	:	60
A. macrogynus	:	VPAPAARTLLGIRTMTTNNKTTTPIAKPIQDYT--KYL TAVSLARQPSAIRAL-----QPI	:	70
A. nidulans	:	-----MASLSPPLDLS--HFLSLTTKRAPSAVKDF-----YKY	:	32
B. cinerea	:	KAGKFBVAGVAAF-----TTSDFHFNPA--DRISLESRSRKP SNL KGA-----AKY	:	77
B. dendrobatidis	:	SFSSVASSEKSDSSSLVSMQLATAKPIIDYT--EYFSPRAAARPSAIRDL-----ATL	:	83
C. albicans	:	-----MSDPT--HLISKRAAGRTSVHFTNAPSDKPPANF	:	32
C. guilliermondii	:	-----MSEFVGHLLSKRALARKSSHFAAI--DKSPPKGF	:	33
C. immitis	:	KT--TMPTGVAASCHSDMFKSLACATKPKAKRWD--KYL SVE SKS R KASTL KQA-----AEY	:	88
C. neoformans	:	-----MPSVAAPVKLDYQ--SFLSQEAKGRIRSQLRDL-----RYF	:	35
C. tropicalis	:	-----MPTDPS--HLISKRAASRSSHFTNAPAGEVPIGF	:	33
F. graminearum	:	KAGKLVAGVAAASDSDMFKGPT--TGLPKSKRWD--NHL SHESK VREP CTLKQA-----ARH	:	111
F. oxysporum	:	-----MAPSATDATAQASPAATALQALPPP KDLS--HLFSKVTRNRKPSNIKAF-----YKF	:	50
M. oryzae	:	-----MQLPTRPLSRQDNGEDDSRPLPRDLS--HYYSQTTKNRKPSQVKEF-----YKF	:	48
P. brasiliensis	:	KT--KVPTGVAANSESDMFKSAACFTKPKAKRFE--HLL SAESKSRKGS SLKQA-----AKY	:	84
P. nodorum	:	KALQSQWGI AAASDTANFRQKSYENKPKAKRWD--HLLSQEALIRKGN SLKEA-----AKF	:	92
P. triticina	:	-----MVPVAVDYDGKFI SRAGKRWQPSGIRGL-----FPL	:	30
R. oryzae	:	-----MDYS--KHISEASKARNPSAIRAL-----MPY	:	25
S. cryophilus	:	-----MSIQPKDLS--HLLSVESASRRPSPLKAI-----AGS	:	30
S. japonicus	:	-----MVAYQPKDLS--HLLSLESARRPSPLKAA-----ALS	:	31
S. pombe	:	-----MDNLKKKYQ--HLLSLESASREYGPFOML-----GRI	:	30
S. punctatus	:	-----MTSEASNPDDYS--RFFT VRSAA RKP S AIRAL-----QPL	:	33
T. equinum	:	EKAHDVLHEDVRDAVNIRQAHFSAPKRLANRWD--HRLSDESLSQGASPLKNS-----VKT	:	59
T. rubrum	:	RC--ALPKGVAAFSDSSMFKSAASASQPKAKRWD--RYL TEESKLRKPS TLKQV-----ALY	:	88
U. reesii	:	MPTPEPLPGSHSDDLNRRRTAEPMLRPPHDFS--RYYS CATRRRVASSVKDF-----YKY	:	57
V. dahliae	:	KAGKLVAGVAAVSDSDMFKAPS--HDKPEAKRWD--HLLSHEAAV RKP CTLKQA-----ARF	:	110
N. crassa	:	LKNPNI-----ISLGGG L PCAEY P PIESITLKV-----TPPDFSEA	:	147
U. maydis	:	ELIPGM-----LSLLACKPNPDT P P ESLSLNL-----	:	88
A. macrogynus	:	MAMPGM-----ISLACG L PNPAT P P IANVELTL-----	:	98
A. nidulans	:	FMIPGI-----ANLACG L PNASY P P YDTLEASAA-----HPQRFPTI	:	69
B. cinerea	:	L--GPGM-----ISLGGG L PCDY P P IEEISIKVP-----TVPHFSEA	:	113
B. dendrobatidis	:	VNVPCM-----ISLCCG L PNPST P P ASIQYTL-----	:	111
C. albicans	:	KSHEKP-----LALS Y C M P NHGF P P IDSIDVNLVDYPFQKITTPSTTSSTAE EEPSSSLN	:	88
C. guilliermondii	:	KPADKN-----LILSWC L P NDGF P P IESIDLQLVDYPFE-----KSLSMSMTNASL	:	79

C. immitis : LKNPGL---ISLGGG---SPEYFPIDEISIKVP-----VAPNFSEE : 125
 C. neoformans : QAIPGM----ISFGGG---BHPSTWPNAMVLSVPFAKKS-----IFIPGYEST : 78
 C. tropicalis : KPHPNP---LLLSYGM---NHGFBPIDSIDVNVDPYFQ-----TTLDHAKPNGSSTS : 81
 F. graminearum : MKKPGL---ISLGGG---SSEVEFP AELGFKVP-----VAPKFSEK : 148
 F. oxysporum : MQIPGI---SNFAGGM---NPKYFPD TLEAQIS-----NADRWEPS : 87
 M. oryzae : FQIPGI---VNFAGG---PNLRFPPD TLEAQTA-----KPERWTPS : 85
 P. brasiliensis : LDKPGL---ISLGGG---SCEYFP EESISIKIP-----TPPHFSEE : 121
 P. nodorum : LATPGL---ISLGGG---SSEYFP EELSMRVP-----QVGHFSEA : 129
 P. triticina : ENRPGM---ISMLACKPNPATFP EESIAITL----- : 58
 R. oryzae : LNRKDM---ISLCAQDNPATFP FASLTLTL----- : 53
 S. cryophilus : VSKKGI---NVLISLACG---ENPQYFP ERKMGEIP-----SIGSWVRG : 69
 S. japonicus : KSKTGI---NIVSLACG---PHSDYFP IRTLSTAV-----YRPD : 65
 S. pombe : QSDSDI---KMLSFACG---EPNPKFP IHKLSVSFP-----EVNSWEKD : 69
 S. punctatus : LSIPCGMFGLVQISLGGG---ENPPTFP EKLSFTL----- : 66
 T. equinum : ATTFVP---IPLGVREASLFYFP WQSMTMTG----- : 87
 T. rubrum : M-GPNV---ISLGGG---SSEYFP EHEISVKAP-----SPROFADP : 124
 U. reesii : FAIPGI---HNLACG---BHVSYFP D SLEATVA-----LPNRIPKD : 94
 V. dahliae : LKTPGL---ISLGGG---SSEYFP ESEITMRVP-----TPPDFAEK : 147

N. crassa : AT---SQSGAE-----VRIGKYDAT-DPSIQGT--- : 171
 U. maydis : -----KPEAEA-----GPTQLNIQ-----G--- : 103
 A. macrogynus : -----KNGAKISMG-----D--- : 108
 A. nidulans : PGGKKAKGPQTS-----ERIVVPKESRSATS-KK : 97
 B. cinerea : QT---KESGTI-----LTAGKYDVS-EG--KGA : 135
 B. dendrobatidis : -----KSGEV-----IDIP-----T--- : 121
 C. albicans : GSENGHQTTPP-----SSIHTPQSTVHISRHTTDPKL--- : 121
 C. guilliermondii : ESLALSKTNGTV-----ANDSSGPKKITINRRSEDEKL--- : 112
 C. immitis : AT---HESGQV-----VTAGKYDIR-EG--RSE--- : 147
 C. neoformans : DPTSL-HPLAPY-----TPPTKLSIATDP---FA--- : 103
 C. tropicalis : DKINDLKSSDSE-----NNQHDSLHTIHERIPKDPKL--- : 114
 F. graminearum : DT---EESGQT-----VTIGKYDVR-DR--GGT--- : 170
 F. oxysporum : PNYPKVDPSTESLSGVSIGYSNSPDKNAPRNP DYNKTSRRISIPKILNEPDI--TKK--- : 143
 M. oryzae : ESTATNTDSRSN-----GDSRDRSESTHITVPLVAKETDPLRK : 123
 P. brasiliensis : ET---VKTGTV-----VTSKYDIR-NG--KSD--- : 143
 P. nodorum : ET---KESGVL-----IKAGKHDLA-DG--KSI--- : 151
 P. triticina : -----KPSVAT-----GEVPETLCLS-----G--- : 75
 R. oryzae : -----KTGKIEID-----D--- : 63
 S. cryophilus : A---ENEGKL-----SDFSVMDSSEDQDD--- : 90
 S. japonicus : GK---PENNNV-----SKIPLQELSLGMEPTEDND--- : 92
 S. pombe : TN---KDATVS-----YELS--NNANEGS--- : 88
 S. punctatus : -----KDEAGG-----GEIHVE-----S--- : 79
 T. equinum : -----TKESNT-----RNP MCVNIG-----EAA : 105
 T. rubrum : AP---ANPECV-----FSSTKHDVV-DG--TSE--- : 146
 U. reesii : GFVPPQPPTASP-----ASPPASQRVVVPKESVDDGLRR : 129
 V. dahliae : DNLSAQSPTAQT-----ISIGKYDVR-DG--KSE--- : 173

N. crassa : YDIAVALNYAQASGSAQLRFVTEHTELVHN---P-PY---SD---WRSCLTIGSTGA : 219
 U. maydis : EDIVSALQYGATSGIPKLVQWITELQVHMKREAVT---GSKLDGVAGRTP---RVVTTGNGSQDL : 164
 A. macrogynus : TMTRAALQYGPTPGHPDVAFFEKQARVHG---T-PYANKQLE---RKILVGNQSQDL : 160
 A. nidulans : IDITTAALQYGTSEGLPPLATEVNOFVREHLH---ENIPYA---GG---PGTLLTTGATDG : 148
 B. cinerea : YDLSIALNYGQSGSAQMLRWVTEHTEIVCN---P-PY---AD---WGCSLTVGSTSA : 183
 B. dendrobatidis : EQVKQALQYSATPGLKFEVWLKQVHEHGRD---P---LE---FDVCIGNSQDL : 169
 C. albicans : IDIARGLOYAAVEGHAPLQFARDFIIRTHK---P-NY---DD---NWFITTCASDG : 169
 C. guilliermondii : IDIAHGLQYSEPKGHPQILQFTREFISKIHP---P-KY---SD---WDTIVTTCASDG : 160
 C. immitis : YDLEIALNYGQSVCAAQLRFVTEHTEIVHN---P-PY---SD---WHCSLTAASTAS : 195
 C. neoformans : PDIAARDLQYSSNFGLPYVDWLQHVKRIHD---P-PY---PEDRWQVLATAGNTDA : 153
 C. tropicalis : MGLKSGLOYAAVECHQQLQFTKDFIQRTHN---P-NY---DN---WTSFTTNGASDG : 162
 F. graminearum : YDISIACNYGQATGSPQMMRYLTHETEIVYK---P-PY---AD---WKVCQTIIGSTGA : 218
 F. oxysporum : VDLATALQYQVTCYPPHSHFIRQFTNOVLH---P-SVPYQ---GG---ADVILNNGGTDG : 194
 M. oryzae : IDLATALQYGMADGYPLLFSFVRQFTREHLH---PDVPPYR---SG---PEVVLTIVGSTDG : 174
 P. brasiliensis : LDLEISLNYGQSNCAAPMRFVTEHTEIVHN---P-PY---SD---WQCSLTAGSTSS : 191

P. nodorum : FD IATAFN YGQAGCSAQ LRWMT EHTELVHN --- P PY --- ED --- WRCCMSIGS TSA : 199
P. triticina : RE IDAAL YGPTACQEEFLNWWYQLQQRCHC --- R GTP --- EEEGWSCAIGAGS OEL : 126
R. oryzae : QL FDRSL SLDLTS GQPL NQWLTELQRIEHA --- P P --- VD --- F DVSIGSGS ODL : 110
S. cryophilus : LP LSVAL YGKGS CCAQL T SFLKEHTQMHS --- P PY --- AN --- W DILLTTGNT QG : 138
S. japonicus : FGL SQGL YGQGF GALQAA FVKEHTRIVHQ --- P KY --- EG --- W DVMLTAGN TNA : 140
S. pombe : LD LLAGAL YGQCQGIPELVKFIKDHVGQIHM --- P QY --- KD --- W DIKITNGNTIG : 136
S. punctatus : GD LQKAL YSPTNGIPEFVAWLKELQTS EHS --- P LY --- DS --- F DVCVGN GSO DV : 127
T. equinum : FD LSSAL NYADPS GSTEL VACFRENTRLIHN --- P PY --- QD --- W DITLTCG S TSA : 153
T. rubrum : FD LEVAL NYGQSVGYAALVRWIT EHV EIVHN --- P PY --- GD --- W EITMAPG S TOG : 194
U. reesii : ID LATAL YGAVDCYPP LRSF IROFVREN LH --- P HVPYE --- GG --- P EVILTCG ATG : 180
V. dahliae : YD LSIAL NYGQSTCSAQMRFVTEHTELVYN --- P PY --- AD --- W KVCQTVG S TGA : 221

N. crassa : LEQTLRMLCDGPGRRD --- S LIT E EYSF STALETAGL --- DLKVVGVKM --- : 263
U. maydis : LNKTFDALL --- NPGD --- VII VE SPAYTGILPSLVMI --- KANIVPVTS --- : 205
A. macrogynus : LAKAINALV --- APGD --- TLIV E SPCYVGT LAILKPMVKAQ --- GVKLA E VPT --- : 205
A. nidulans : FSKAIEAF TETWNPDR --- DGIHLRQGITCEEFVYMNAIQTVKPR --- GLNIVPVAI --- : 199
B. cinerea : LEQTYRIFC --- ERGD --- FVLS E EYTFASA VETAAPL --- GVKFLGVKM --- : 224
B. dendrobatidis : LTKAIDAFV --- TDGG --- SVMMECPAYVGMIAVVRPT --- CANIVEIPS --- : 210
C. albicans : LNKAADVFL --- DDGD --- VII VE EFTFSPFLRFS DNA --- GAKAVPVKINFD --- : 213
C. guilliermondii : LNKVADALL --- DPGD --- VII IE EFTF TPELAHVANT --- GGIPVVRMNLDP T --- : 206
C. immitis : WDMVLR LFC --- NRGD --- YII IE EFTFSSA METAL E Q --- GIRLAPVKM --- : 236
C. neoformans : SDG VVRTLC --- SPGD --- SMIL E EFAFPGSLTHYKSL --- GINCQV GPM --- : 194
C. tropicalis : LNKACDALL --- DPGD --- VII VE EFTFSPFLRFS ENS --- GAIAISVRLNLN --- : 206
F. graminearum : LEEALRMFC DK --- DRGD --- SVIT E D F S STALETVGP L --- GIKAFG VSI --- : 261
F. oxysporum : FSKVLQL VDPWY EGL --- HPVVERP GMIC ETFVGNIL TQARPL --- GVNVPVEI --- : 245
M. oryzae : FAKTLELF VDPWSEGKDDVDRPGLCE RFAYGNVLTQARPK --- GVQVTV EA --- : 226
P. brasiliensis : WDMALRMFC --- ERGD --- YII LA E EYTFATALEAASAL --- GVRAAAVKM --- : 232
P. nodorum : LDMALRMFA --- RPGD --- VVLS E EYTFSAFVETARPM --- GVRVCGIPL --- : 240
P. triticina : MEKAFAAVC --- DPED --- TII ME TPVYSGT LGFLOS --- GRTLIEIES --- : 167
R. oryzae : LTKALEMMI --- NPGD --- AVMV E EPTYT GALAFLET M --- PCTLAHIAT --- : 151
S. cryophilus : LDMCLRM L M --- NRGD --- SII VE EKYTFPSALQAMRPM --- GLNAVSVDM --- : 179
S. japonicus : LDTCLRM L L --- NRGE --- SII VE EYTFPSALQOTIEPM --- GINCIPMPM --- : 181
S. pombe : LEYCLRLV --- NRGD --- CII IE EKYTYPAAITAMRPL --- GVKFIPIDM --- : 177
S. punctatus : LTKAFEM L L --- AEGD --- NII IE SPAYVGS LAF LKPL --- GCKFVEVPV --- : 168
T. equinum : IDIVLRMLC --- NRGD --- WII LA EAS TYTGTVMATKAH --- GLNIQSIEM --- : 194
T. rubrum : WDFVLRMLC --- KPGD --- MII TE EYTFCSA MEGAAAO --- GVTPEPVKV --- : 235
U. reesii : FSKTIEAF TNVWDENR --- DWIRERQGLICEEFAYMTAIQTARPR --- GLNIVPVAV --- : 231
V. dahliae : LEQALRMLCDR --- DRGD --- SVIT E EYSF STALETIGL --- GIKTFGVKM --- : 264

N. crassa : --DQG L I L E --- AMD E L I S N W N P E E R E G --- R R K E H V L Y T V E S G C N P T G T T O G L E R R K Q V : 316
U. maydis : --DQGMSS --- RLA E T I A N W E T D P Q T A S --- L A R E K C L Y T T P T G A N P A G T A S D E R K R Q I : 259
A. macrogynus : --DAH G I I D --- A L D K I I A D W P A T --- S P K E R V L Y T V E T G N P T C N M A P I E R R A I : 254
A. nidulans : --DDE G M L F Y G P G G L A E V L G N W N H R K --- G C R E H L M Y T I T I G C N P T G G T L S V E R R K Q I : 252
B. cinerea : --DAE G I L P E --- A L D E I L S N W D E Q A R - G --- A R E F V L Y T V E S G C N P T G A I Q G I E R R H A I : 276
B. dendrobatidis : --DAD G L N P D --- A M E K T I A N W P D V --- K T R E K I L Y T V E T A G N P T G V T S L A R K K R V : 259
C. albicans : --N D S D G I D L T --- Q F V D L I E N W E K H Y P - N --- L P K P K A L Y T I A T C N P T G F T Q S L E F R K K I : 266
C. guilliermondii : P D K S N G L D I D --- Y L T D L L E N W E T K K P - D --- L K P K A L Y S I P T G C N P T G L T Q S L E F R K K V : 260
C. immitis : --DEQ G I L P S --- S L D E V I T N W D A S A R - G --- A R E F V L Y T V E S G C N P T G A I Q D V E R R K E V : 288
C. neoformans : --D G E G I V P S --- A L E K I L A E W N E E A R - G --- G P K P K A I L I V F T C S N P S G I Y P T P R K N E I : 246
C. tropicalis : --K E S D G L D L D --- H L V G L I E N W K E E H P - E --- L P K P K A L Y T I A T G H N P T G F T Q S L E F R K K V : 259
F. graminearum : --DEQ G I L I E --- A M D E L I S N W D A K E R - G --- S R K P H L L Y T V E S G C N P T G A I Q G T E R R K A I : 313
F. oxysporum : --D E E G M I V E G S G G L R E V L E N W N P Q N --- G R I P H F L Y T V T M G D N P T S G V V G V Q R R K E I : 298
M. oryzae : --D S G C M L A D G P G G L E D V L A N W D D S K --- G R R E H L L Y T V T M G H N P T G I V L D V E R R K A L : 279
P. brasiliensis : --D D Q G M L P E --- S L D E V M T N W D A A A R - G --- A R K P H L L Y I V F S G C N P T G T H G P E R R K E I : 284
P. nodorum : --DAE G I L I E --- Q L D N I L S T W D V N V R - G --- A R R E H L L Y T V E T G C N P T G A I Q S A E R R R A L : 292
P. triticina : --D H H G I S A V --- E L E R V L A N W H S D P A T S S --- L K F E K V V Y S I P T G S N P T G C S A P L E R K R L I : 221
R. oryzae : --D E F G I V P E --- S L E N M L A H W P E S N P S G K K D Q P R E H V L Y T I P S G G N P T G I S A T Y E R K Q A V : 207
S. cryophilus : --D K N C M L P E --- S L E E I L N W K --- G --- G N K P R V L Y T I P S G C N P T G S T L S V E R K E K I : 227
S. japonicus : --D L N G I L P E --- K L E I L S S W N T K A R - G --- C P K P H V M Y V I P S G C N P T G S T L S N E R R A R I : 233
S. pombe : --D E N C M L P E --- S F E K V M E T W D S L --- G A R E H V L Y T I P T G C N P T G S L T L E R R K K F : 227
S. punctatus : --D K D G L D S D --- K L E E I I S G W K D V --- R T R E K V L Y T V E V G G N P T G V S T Y E R R K R I : 217
T. equinum : --D E E G L L V --- D I D N K I R H W D T L R --- S R K P E V L Y T I P S G H N P T G V T Q S T A R K A A I : 244
T. rubrum : --DAE G L L A T --- S L D E I T N W D P A A H G G --- A R K P I V L Y T V E S G C N P T G A I Q S A E R R R E V : 288
U. reesii : --D S Q G M K V E G E R G L A D V L D N W D F S R --- G O R E H L M Y T V T I G C N P T G A V L P L E R R Q I : 284

V. dahliae : --DAEGLLE---SMDLITWDEKAR-G---ARKPHVLYTVFSGCNPTGALQGPERRRAV : 316

* *

N. crassa : YEVARKHDLFVIEDEDPYVYLOMPL---EESKETESEEEET-----VESFMSS- : 359
 U. maydis : LALARQYDFLVLIEDDPYVYVH----FEGLDQDAVTRP----- : 292
 A. macrogynus : YALAQKHDLVMLEDDPYVYLOFALPPTDAATQQLTPPAD-----QTTDVALGDRYASA- : 307
 A. nidulans : YAICQKYDVIIVEDDPYVWNLQYPSAQELQARHRNSSVNPALSERNYNAGRKSSGYKFLDS- : 312
 B. cinerea : YKVAQKHDLVYIIEDEPYVFLQMOP---YTGPDADPVPPP-----ANNDEFLLKA- : 321
 B. dendrobatidis : YEVARKYNVVMIEDDPYVFLQF-----GEKRD--- : 286
 C. albicans : YDLAVKYDFATIEEDDPYGYITLTPK---YEKPNIGGSGSG---NNEKNDLEIDDYLNH : 319
 C. guilliermondii : YALAEKHDFIIEEDDPYGYITLTP---YKPEGVLKLDSE-----FLTVEEYLSNH : 307
 C. immitis : YKVAQKHDIIVIEDEPYVFLQMOP---YKGANAPQDPPP-----ANYNEFLKA- : 333
 C. neoformans : YAICRKNWLLIIEEDDPYCYLQVRP---NGADTP----- : 276
 C. tropicalis : YALAEKYDFATIEEDDPYGYITLPT---YTKPDLTKNPTR---NKSDITVDDYLNH : 309
 F. graminearum : YAVAQKHDIYIIEDEPYVFLQMOP---YTGRNQPDVPPP-----KTVDEFVSS- : 358
 F. oxysporum : YALASKYDIIIVEDDPYVWYLOFPSAAIHEAKARGYAIPO---VADSHKTVKSSGYDFIDS- : 355
 M. oryzae : YAICSKYDVLIVEDDPYVWYMOFPSAAREEAKSRNLPPPQ---VTSTYSRSTGYPFLLDS- : 334
 P. brasiliensis : YKVAQKHDFIIVIEDEPYVFLQMOP---YKGVDTPPDAPP-----ASYEAFIKS- : 329
 P. nodorum : YAVCQKHDIYIIEDEPYVFLQMOP---YTGPNAPVPPP-----SSHDEFLLNS- : 337
 P. triticina : LELVRKYDLLLIEDDAMVFLHFDR-----KN- : 247
 R. oryzae : YKICKSYDVIIEEDDAMVYLOY-----GEKRT--- : 234
 S. cryophilus : YALAQKHDFIIEDEPYVFLQMDSE---YQKPEAVDPKF-----TNEEFVNN- : 271
 S. japonicus : YEICQAHDIIEDEPYVFLQMDN---FDG-KIPEEQPT-----VSNETFLSQ- : 277
 S. pombe : LTLAKKYDIIIVIEDEPYVFLQMEK---YDANWKPKQAF-----NISSEKKK- : 271
 S. punctatus : YEIAKRYDIIIEEDDPYVYLOF-----GDKRA- : 244
 T. equinum : YQVAERHDLIIEEDDPYVFLRLNG---SFS-SLDINPPY-----SSFELKRS- : 288
 T. rubrum : YKVAQKHDIIVIEDEPYVFLQMEP---YTGLNTPTPPPP---ATTDEFLLKT- : 333
 U. reesii : YKLCQRYDIIIEEDDPYVWNLQYPSAQNALRQGSQSDSSP---HTPFSVHDEPSGFPLDS- : 341
 V. dahliae : YAVCQKHDIYIIEDEPYVFLQMOP---YAGKDAPVPPP-----ASVDQFLAD- : 361

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N. crassa : LIPSLLSLVD-----CRVLRMDSFSKVVVPGSRVGVVTA SENIIEQYLLHAEVCNCGA : 413
 U. maydis : RCRSIVSLEEEHRERWGTGRVIRFESFSKILAGLRLCFATGPNEILDVAVDANTAMSNLOP : 353
 A. macrogynus : LVPSYTSLSLTD-----ARVIRFESLSKVISACLRVGLCTGPAPLVDRIALDIQATSLOP : 361
 A. nidulans : LVPSYLSIDMD-----CRVVRLDTSKTIAPGCRLCGWITAPAFIERLARIASSTQAP : 366
 B. cinerea : LIPTYLSIDTD-----CRVMRMDSESKVIAPGCRVGCWITASEQIIVERFISNENSONP : 375
 B. dendrobatidis : --PSYFSFVDV-----CRVLRFDLSKVLSGCARTGWSGPKMLVERIILHSMGNSLHP : 338
 C. albicans : LTPSYLELDTT-----CRVLRVETFSKLFAPGLRLGFIVGHKEVIDAVKNYSDDVNRGA : 373
 C. guilliermondii : LVPSYITLDTT-----CRVVRLETFSKLFAPGLRVGFVVGHKVTEVIGQYAAVVTAP : 361
 C. immitis : LIPSFLSMVDV-----CRVVRLESFSKVLSPGSRVGLVASEQIIVERFLNAETCTONP : 387
 C. neoformans : LIPSFLSLVD-----CRVVRLDSESKFIAPGSRGCGWITGPKELVTAVMKAASSNGP : 330
 C. tropicalis : LTPSYLELDTT-----CRVIRVETFSKLFAPGLRLGFVIAHVKTIEAIKNYSEVINRGA : 363
 F. graminearum : LIPSLLSIDVD-----CRVMRMDSESKVLVPGSRVGLGWITASEQIIVERIIRHAEVASG : 412
 F. oxysporum : LVPSYLSIDVD-----CRVIRLTFSTKTVAPGSRVGLGWITAPAIERLTHIAETSTGQP : 409
 M. oryzae : LVPSFLSFVDV-----CRVVRLDTSKTVAPGCRVGLGWITAPAIERFVRIETSTQOP : 388
 P. brasiliensis : LIPSYLSIDVD-----CRVLRLESFSKVISPGSRMCGWIVGTAEIVERYTRHSEVSTONP : 383
 P. nodorum : LVPSYLSMDVD-----CRVMRLDSESKVVSPPGCRICGWITACAEIVEKYTKHADVSTONP : 391
 P. triticina : RAPSIFYALEKSEGGQ---TRVVRFDLSKIISSGIRLGFVTGPARTLEIINLHTSNTNLOP : 306
 R. oryzae : --PSYLSLVD-----CRVLRCDMSKILSSGLRIGWVSGPQLIERMNMHTMVTNLOP : 286
 S. cryophilus : LITSFLHIDVD-----CRVIRMDSESKVVAPGSRVGVITAPLFIERSLYTETASONA : 325
 S. japonicus : LITSFLSIDTE-----CRVIRLDSESKVLVAPGTRVGLWLTGHPLFMERALRQNEVNIQSA : 331
 S. pombe : LIPSLHLHLDLTD-----CRVLRVDSFSKLVIPGCRVGLGWITGNSLFDIRITHYAEVCTESE : 325
 S. punctatus : --ATYFSMDAD-----KRVLRFDLSKIFSAEMRIGWVTGPKPLVDRIVLHGQATMLHP : 296
 T. equinum : LPSSYLSLKS-----CRVIRVDSFSKILAPGCRVGLWLTASKQIIVEIFENFAEVGPAPP : 342
 T. rubrum : LIPSYSLSMDTD-----CRVIRLDSESKVISPGSRVSVFLVSSAQFTERMIRQATSTQAP : 387
 U. reesii : LVKSYLSIDTA-----CRVVRLDTSKIIAPGCRVGLWLTAPDIIVERILRISETSTQOP : 395
 V. dahliae : LVPSLLSMDTD-----CRVLRMDSFSKVLVPGSRVGLGWITASAQMVERYIRHAEVANOGP : 415

N. crassa : NCISQVILYKLL-----DEQW-GHE-GYFK-WLMNLRQEYTKR : 448
 U. maydis : SGLAGVVAYTLL-----NYW-GIP-GFLR-HVDNVARYYAKR : 387
 A. macrogynus : TGLSQAVALALA-----QHSWHGVPAGFLA-HAARVAAFYRSR : 398
 A. nidulans : SGFVQAMVAELILGQQVDDGSAD-----SAKSKDTERAW-KMD-GWVR-WLEGLRAGYEQR : 419

B. cinerea : SGISOMVLYKLL-----DESW-GHG-GYLO-WLINLRLEYTKR : 410
 B. dendrobatidis : SGISOIMMYSLL-----EKN-GIE-GFLE-HNRKVAIFYKEK : 372
 C. albicans : SGLTOTIVNNVI-----QENFKGVD-GWLE-WILKMRLNYSYR : 409
 C. guilliermondii : SGLSOMVLNVI-----EQKYKGVY-GWLQ-WILKMRLTYTHR : 397
 C. immitis : SGMSOILFKLL-----DEAW-GHE-KYLO-WLVHIRMAYTNR : 422
 C. neoformans : SGFAVASISAVI-----KAWGCHE-GLERDYLPHISDTYSKR : 366
 C. tropicalis : SGLTOTIVNNVI-----QDNFKGVE-GWLE-WILKMRSNYAYR : 399
 F. graminearum : SGFSOVILYKLL-----DETW-GHE-GYLR-WLMNLRLEYTKK : 447
 F. oxysporum : SGFVQALIAQAILGPHSS-TLETFSLS-PESEKPSFTGW-QLD-GWVR-WLEGLRGEYER : 465
 M. oryzae : SGFVCSMIGELVMSSQPTETKTTFLGLRTHKDRFTAFTGW-QMD-GWVR-WLEGLRGVYEDR : 446
 P. brasiliensis : SGMSOILFKLL-----DESW-GHA-GYFD-WLINLRMEYTR : 418
 P. nodorum : SGMSOLILFKLL-----EDHW-GHA-GYLD-WLIHIRMQYTKR : 426
 P. triticina : SVTCAITGKLL-----CHW-GFD-RFEA-HCDQVSKFYSDK : 340
 R. oryzae : SGVSOVMAYQLL-----ANW-GHQ-GFFE-HVKRVADFVKEK : 320
 S. cryophilus : SGISQAMHLAF-----KTW-GHD-GYLA-WLKHIRYSYTER : 359
 S. japonicus : SGLSOVLLHGIL-----HTW-GQE-GYLE-WLKQIRFOYTR : 365
 S. pombe : SGVSOVLYAIL-----NRW-GQN-GFLE-WLQDLQNSYTR : 359
 S. punctatus : SGLSOMLAYQLL-----KKW-GRD-GFLK-HTRSVAGFYEQK : 330
 T. equinum : SGPSOVMYKLF-----VESW-GQE-GFAN-WVNHLSGEYRSR : 377
 T. rubrum : SGMAOILYKLL-----DESW-GHE-GYLR-WLIHIRIEYTKR : 422
 U. reesii : SGFVCSMVAKLIMGDQADMVKK-----PC-TEGSPGW-RVD-GWVR-WLEGLRGGYEKR : 446
 V. dahliae : SGFSOVMWKLIL-----DETW-GHE-GYLR-WLIDLRMNYTR : 450

N. crassa : RDTMVTAAE--KFLP-----K-EVVSWTVPVAGMFWLWKIDHSQ : 484
 U. maydis : RDNFEAKAN--KVLG-----AAGVAQWVTPVAGMFLNLRNLPP : 424
 A. macrogynus : RDALLKAMSAARLVE-----D-GLVEMSVPPQAGMFLWLKL---- : 432
 A. nidulans : MQQMCSGLEEGKYIALHTRSDEFSLSGNEEDSWEVLEKVMYDFAWPTGGMFWVVKVNYES : 480
 B. cinerea : RNVILAAACE--KYLE-----K-DIVSWNPPSAGMFLNLSVDWHK : 446
 B. dendrobatidis : CDAFMSSAK--RHLD-----GIAEFVAPKAGMFWMKL---- : 403
 C. albicans : KDLLYSIFESQAYK-----K-GYVDVIDPKAGMFTFKINLPK : 447
 C. guilliermondii : RDVLVNAIEESDAYK-----K-NYVSIIDPRAGMFLSLVVNFP : 435
 C. immitis : RNVMLEACE--KYLE-----T-SVASWHPAAAGMFWIKVDWKK : 458
 C. neoformans : SNLMVSLIR--KYVP-----VEAAECPDGGMFLNVLRLVES : 402
 C. tropicalis : KDLLYSIFESEAYK-----K-GYVDVIDPKAGMFTFLINLPP : 437
 F. graminearum : RDALLAAACE--DHLP-----S-DLASWTPPVAGMFMINIDYTK : 483
 F. oxysporum : MTRMCSILEENAFSL-RQSTSONP-----DLWDVSIKIRLDFDWPVGGMFWVVRVHFEK : 520
 M. oryzae : MNRVCRILDDGKYQL-KQGTPVVPGSHPLDADWGVITKPKLFEFDWPRGGMFWLRFVFEF : 506
 P. brasiliensis : RNTMLDACE--KYLE-----K-SIASWNPAAAGMFWIKVDWKR : 454
 P. nodorum : RDVILQACT--KYLE-----T-EVMSWKPMPAGMFWMQIDFKK : 462
 P. triticina : LEVVNAAK----- : 349
 R. oryzae : RDDFVECLD--KHMT-----GRAVWTVPNAGMFWLRL---- : 351
 S. cryophilus : RNALLYAMD--KHLP-----K-SICSYIAPEAGMFWFEVDKSA : 395
 S. japonicus : RNALLAMN--KYLE-----E-GLCSYIAPEAGMFWFEVDRSR : 401
 S. pombe : RNALLAAD--KHLP-----K-SVCKYHSPKAGMFLWVLDKNR : 395
 S. punctatus : RDLFLQAS--KRLQ-----GVAEWTVPDAGMFWLKL---- : 361
 T. equinum : RDIMIAACS--QHLP-----K-GLCTWTPPTHMFLWITIDLLR : 413
 T. rubrum : RNCIMDACE--EFLP-----K-AVASCNPPTAGMFWNMRVDWKK : 458
 U. reesii : MQTMCITILEEGRFSIVQ---GDALSNDNLDEWQVVDNVQMFDFDWPVAGMFWVVKFCLET : 504
 V. dahliae : RNMLLAACE--DHLP-----K-DIVSWTPPAAAGMFLNWKVDHTK : 486

N. crassa : HFGIQA-----GKGILEVEEELFNSCIARGVIVAR--SWFR----AE-Q-DTEP : 526
 U. maydis : TGQ-----GESEGDSFALISDKARAAGVLAVPCVAFI----PD----GS : 460
 A. macrogynus : -----KTVDASALVKGYATDRKVLVLLPGIEFL----PN--AQPGE : 467
 A. nidulans : HFLROI-----YGPERSKALWVHFTQKPOLCLLPGTIFS----PT-DEVQAR : 524
 B. cinerea : HFAAKT-----KPIADIEQEIFLSGIDQGVILICPSWFT----AE-KDGFVP : 488
 B. dendrobatidis : -----IGIDSDTLIKRKALEKKVLLVPGIEFL----PN----AR : 435
 C. albicans : -----DVDVLQMKLLLWKLISYGIILVVP--GYNMT----VD-LEFSKD : 484
 C. guilliermondii : -----GTPIIIEKIRLLNWKLLAFGVHAVPCINMA----TD-SEFSKE : 472
 C. immitis : HFLALA-----GVSYSIEEELFKAAVGEGLVLSR--SWFF----AD-K-STEP : 500
 C. neoformans : HFLQST-----LSPEISDKVFHTLIGEKVMVAP--SSYFKTPGGPVWSKDEES : 449
 C. tropicalis : -----DVDVLAKMKLLLWKLISYCVSVVPG--GYNMT----VD-QEYSKA : 474
 F. graminearum : HFDAGK-----RSIVDIEEELFNLCIENGVLIR--SWFL----TE-K-DKAP : 524
 F. oxysporum : HFLYQT---RGGDIAPVIDGTALANAF--LHSTHAPHLVLSG--PSMFS----ATPEIREKR : 573
 M. oryzae : HFLWKAPRPGKDGEPAVMDGGVLSGALLAMLTKKPWLVLVAP--GFMFS----ANEIQRKD : 563
 P. brasiliensis : HFGVRE-----GKDHAATENAFKATIEEGALVSA--SWFV----AD-S-SIPQ : 496
 P. nodorum : HFOYPE-----KSIEEIEEELFYRNIHGTMCMR--SWFY----AD-A-DEEH : 503

P. triticina : -----NT----- : 351
R. oryzae : -----LGGITDSYDLVMTKVLKENVLAIPGLAFM---PR---GN : 384
S. cryophilus : YKFADQ-----GKSIAEIESDIHEEAIQDGANVACGNWFV---VD---SSIN : 436
S. japonicus : YLHSDK-----FKTVPEIETDIHQAVDAGVNLACGHWFI---VD---SSKN : 442
S. pombe : LICSNM-----DKSISEIEMEIFVELVNNGVKPVCCQLFM---GE---PNSA : 436
S. punctatus : -----LGITDSSDLIKQKAMEKKVLLVPGFEFF---PN---PT : 393
T. equinum : HFDYLE-----KRTHLSLDLEDKIYERAASHGVAVAKGSWFN---VE---TCL : 455
T. rubrum : HPAVQQ-----GKDHKAIEMIFLAAVDAGVIVSRGSWFL---AD-Q-SATE : 500
U. reesii : HPLWSQ-----VPAAKLANALWKYLLINAPYRVIVGPGWLFA---PT-EDVKKY : 548
V. dahliae : HEDYPQ-----RSLLDIEEDIFNKAIGNCVLCARGSWFR---TE-P-GTPA : 527

N. crassa : NELFFRATFAAAT-AENMTEAIRFGEAIRESEKL----- : 560
U. maydis : KSCYVRTSIIIA-EDDVEEAFNRLRNVVLDADWDAGKMPPELA----- : 503
A. macrogynus : PCAFVRAIYSTAT-DEQMGEQMKRLRECIDLAMRDQKQKQAPKK----- : 512
A. nidulans : GHQYFRLCFAAMP-ADDVVPITQVVVDGFRSFWQRKNLDGLEDDGIAQTMEQLQMEPSAN : 584
B. cinerea : VDMFFRATFAAAS-EEKMTEAIEFVAVRNSFGIE----- : 523
B. dendrobatidis : ATPYVRASYSLAT-KEDIDLALQLRELVLLEARDKK----- : 470
C. albicans : RSNFFRLCYALANDEEILESCKRLTDAVYEFFSNGLEF----- : 523
C. guilliermondii : TANFFRLTVAANTDEELIAAGTQLAKAVTEFFENGLEF----- : 511
C. immitis : TDMFFRATFAAAP-ADKIQAIAKIFGDTLRAQFQLQ----- : 535
C. neoformans : KRIFVRLSFSFST-ADEMEEGVKRFARGLRKEWGIQNLDEVEH----- : 491
C. tropicalis : RSNFFRLCVAIANNEEILESCKRLTDGVLEFFNNGLEY----- : 513
F. graminearum : PGLFFRATYASAT-PENMNKAIERFGKAVRDSFGRK----- : 559
F. oxysporum : GWQYIRLCFAAES-DENIDGGASRFARSVGEFFKINDTAQIEKLEELPS----- : 622
M. oryzae : GWAYFRLCFAAEA-EEAVDPCTORFVDGIQRFWKIKTVREIEDLLKGFPISSGEASSMNVA : 623
P. brasiliensis : TDMFFRATYAAP-GDKIEEAIIRIGVALRSEFKLD----- : 531
P. nodorum : DTLFFRATYASP-EDKMEEGIRLIGESVREEGLGKK----- : 540
P. triticina : ----- : -
R. oryzae : KNEYIRVSGSNVN-KPQDDEALRRLAMVIDKEAEIHNVKI----- : 423
S. cryophilus : DKIFFRVTFAAYE-LPEFETAIERFANVLKFFKS----- : 470
S. japonicus : DRVFFRVTFAAAN-FEDFNVAIERFAAVLNKNFK----- : 475
S. pombe : DKIFFRFAVSLAD-LSTFEAGLERFTSTIQKYFQL----- : 470
S. punctatus : TTPYVRASYIAE-AEDMDEALRRLRELVEEARS----- : 426
T. equinum : DKVFFRITFVSTTCLDDLENGVKRLGAAVRDEFRLAQA----- : 493
T. rubrum : EDMFFRSTFAAAP-ADKIREAIRFGEVLKAQFNI----- : 534
U. reesii : AWRYMRLCFAPVD-EGAVATSSHNFIEGCRSFWQVKDIHDIDDLEDDNEE----- : 597
V. dahliae : KDMFFRATFASAS-EEAMSTAIQRLGAAIQTSYRID----- : 562

N. crassa : ----- : -
U. maydis : ----- : -
A. macrogynus : ----- : -
A. nidulans : FLGMGC----- : 590
B. cinerea : ----- : -
B. dendrobatidis : ----- : -
C. albicans : ----- : -
C. guilliermondii : ----- : -
C. immitis : ----- : -
C. neoformans : ----- : -
C. tropicalis : ----- : -
F. graminearum : ----- : -
F. oxysporum : ----- : -
M. oryzae : DQDVGNMGMYMGC : 636
P. brasiliensis : ----- : -
P. nodorum : ----- : -
P. triticina : ----- : -
R. oryzae : ----- : -
S. cryophilus : ----- : -
S. japonicus : ----- : -
S. pombe : ----- : -
S. punctatus : ----- : -
T. equinum : ----- : -
T. rubrum : ----- : -
U. reesii : ----- : -
V. dahliae : ----- : -

S1 Fig. Multiple sequence alignment of Tryptophan aminotransferase homologs from 26 different fungal species. Residues with 100% identity are shown in yellow font with black background and residues with 75% identity are shown in black font with red background and 50% identity is shown with green background and black font. PLP binding residues are marked with pink star on top.

S2 Fig.

N. crassa	:	-----MS	:	2
U. maydis	:	-----	:	-
A. gossypii	:	-----	:	-
A. nidulans	:	-----MA	:	2
A. niger	:	-----MAT	:	3
A. oryzae	:	-----MAT	:	3
B. cinerea	:	-----MV	:	2
C. albicans	:	-----	:	-
C. glabrata	:	-----	:	-
C. globosum	:	-----MT	:	2
C. tropicalis	:	-----	:	-
F. oxysporum	:	-----	:	-
K. africana	:	-----	:	-
K. lactis	:	-----	:	-
M. acridum	:	-----MA	:	2
M. gypseum	:	-----MAS	:	3
M. thermophila	:	-----MT	:	2
N. dairenensis	:	-----	:	-
N. tetrasperma	:	-----MS	:	2
P. anserina	:	-----MAI	:	3
S. cerevisiae	:	MVMAHFCINLAVLVEHRKKXIYKQGSFTLLAIRFGFLPFIIFLVIFFSQLLFSTHNLTON	:	61
S. cryophilus	:	-----	:	-
S. japonicus	:	-----	:	-
S. macrospora	:	-----MS	:	2
S. pombe	:	-----	:	-
T. blattae	:	-----	:	-
T. delbrueckii	:	-----	:	-
T. terrestris	:	-----MGL	:	3
U. hordei	:	-----	:	-
V. polyspora	:	-----	:	-
Z. rouxii	:	-----	:	-
*				
N. crassa	:	DIRTQSLQKPVVAEYLERRLHGI G I R S V H G L P G D E N V A L D Y V P K A --- G L K W V G S V N E L	:	60
U. maydis	:	---MSNESNOIKIGAYLLERLVOLGSQSVCVPGDENMGFLDLIEEH--PKLKIENSNEL	:	56
A. gossypii	:	-----MSEITLGRYLFERLQKQVEVRTIFGVPGDENSLLDKIVYEV--DCMRWAGNANEL	:	52
A. nidulans	:	DIATREL R Q P I D I A E Y L E R R L H E V G I R S V H G V P G D Y N L A A L D Y L P K C --- G L H W V G N C N E L	:	60
A. niger	:	DIATRD L R K P I D V A E Y L E R R L H E V G V R S V H G V P G D Y N L A A L D Y L P K C --- G L H W V G N C N E L	:	61
A. oryzae	:	DIATRD L R K P I D V A E Y L E R R L H E V G V R A V H G V P G D Y N V A L D Y L P K C --- D L H W V G N C N E L	:	61
B. cinerea	:	DLRTE S L K S P V D L A E Y L F T R L K O I G I D S V H G L P G D Y N V A L D Y I P K L --- G L K W V G N C N E L	:	60
C. albicans	:	-----MSEITLGRFFFERLHQLKVDVTFGLPGDENLALLDKIYEV--ECMRWAGNANEL	:	52
C. glabrata	:	-----MSEITLGRYLFERLQVVDVKTIFGLPGDENSLLDKIYEV--ECMRWAGNANEL	:	52
C. globosum	:	DIREQGLKPPIDATEYLFERLHEIGIRSVHGLPGDENVALDYIPKA---GLRWVGSVNEL	:	60
C. tropicalis	:	-----MSEITLGRFFFERLHQLQVDTVTFGLPGDENLALLDKIYEV--DCMRWAGNANEL	:	52
F. oxysporum	:	---MSNSNLQKPIDVAEYLFERLQYEVGVRSVHGVPGDYNVALDYLPQC---NLKRWGSVNEL	:	57
K. africana	:	-----MAEITL GK Y L F E R L A Q V D V K T I F G L P G D E N S L L D K I Y E V --- P G M R W A G N A N E L	:	52
K. lactis	:	-----MSEITLGRYLFERLQKQVEVQITIFGLPGDENSLLDNIYEV--PCMRWAGNANEL	:	52
M. acridum	:	DIRVQSLRKPIDVSEYLFERLQYEVGVRSVHGVPGDYNVALDYLPKC---GLKRWGSVNEL	:	60
M. gypseum	:	DIVTRELEEPDVAEYLFERLHGMGKSVHGVPGDYNLAALDYLPKC---GLHWVGNANEL	:	61
M. thermophila	:	DIREQGLKPPVVAEYLFERLHEIGIRSVHGLPGDENVALDYIPKA---GLKRWGSVNEL	:	60
N. dairenensis	:	-----MSEITLGRYLFERLQVVDVKTIFGLPGDENSLLDKIYET--PCMRWAGNANEL	:	52
N. tetrasperma	:	DIRTQSLQKPVVAEYLERRLHGI G I R S V H G L P G D E N V A L D Y V P K A --- G L K W V G S V N E L	:	60
P. anserina	:	DIRTQGLKPPVVAQYLFERLHEIGIRSVHGLPGDENVALDYIPKA---KLKRWGSVNEL	:	61
S. cerevisiae	:	NTVKSIMSEITL GK Y L F E R L K Q V N V T I F G L P G D E N S L L D K I Y E V --- E C M R W A G N A N E L	:	120
S. cryophilus	:	-----MAGEILVGEYLFERIRQLGVKSIQGVPGDENLALLDLIDNVGDESFRWVNTNEL	:	55
S. japonicus	:	---MSGKEILVGEYLFERLLELGVKSIICVPGDENLALLDLIEKIGDSSFRWVNTNEL	:	57
S. macrospora	:	DIRTQSLQKPVVAEYLERRLHGI G I R S V H G L P G D E N V A L D Y V P K A --- G L K W V G S V N E L	:	60
S. pombe	:	-----MSGDILVGEYLFERLEQLGVKSIICVPGDENLALLDLIEKVGDEKFRWVNTNEL	:	55
T. blattae	:	-----MAEITL GK Y L F E R L N Q V K V Q T I F G L P G D E N S L L D K I Y E V --- P G M R W A G N A N E L	:	52
T. delbrueckii	:	-----MSEITLGRYLFERLQKQVDTNTIFGLPGDENSLLDKIYEV--PCMRWAGNANEL	:	52
T. terrestris	:	DIRERGLKPPVVAEYLFERLHEIGIRSVHGLPGDENVALDYIPKA---GLRWVGSVNEL	:	61
U. hordei	:	---MSSNEIKIGAYLLERLVOLGTSIQGVPGDENMGFLDLIEDH--PSLEWIGNSNEL	:	54
V. polyspora	:	-----MSEITLGRYLFERLQKQVQITIFGLPGDENSLLDKIYEV--PCMRWAGNANEL	:	52
Z. rouxii	:	-----MSEITLGRYLFERLQKQVDTNTIFGLPGDENSLLDKIVYEV--QGLRWAGNANEL	:	52
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N. crassa	:	NAAYAADGYARA--KGISALVTFFGVGELSAINGVAGVSEHVPVIVHIVGCPSTIEQRNCGM	:	119
U. maydis	:	NAAYAADGYARVK-RTIAAVVTFFGVGELSALNGTAGSFSERLPVIVVGVVPSGAGQSHS	:	116
A. gossypii	:	NASYAADGYARV--KKISCLVTFFGVGELSALNGTAGSVAEHVGVLVHVVGVPSISACAKOL	:	111
A. nidulans	:	NAGYAADGYARV--NGIAALVTFFGVGELSAINATAGVSEFVPIIHHVGVOPHSRQKDCGL	:	119

A. niger : NAGYAADGYARV--NCIGALITTFGVGELSALNATAGSYSEFVPIVHIVGPNTRKQKDCM : 120
 A. oryzae : NAGYAADGYARI--NGMSALVTFGVGELSALNATAGAYSEFVPIVHIVGQPHTRKQKDCM : 120
 B. cinerea : NAGYAADGYARV--KGISALMTTFGVGELSALNATAGAYSERVPIVHIVGTPTSTISQKDCM : 119
 C. albicans : NAGYAADGYARVNPNGLSALVSTFGVGELSALNATAGSYSEHVGVLINLVGVPSSSACAKQL : 113
 C. glabrata : NAGYAADGYARI--KGMSCIIITFGVGELSALNGTAGSYAEHVGVLHVVGVPSISSQAKQL : 111
 C. globosum : NAGYAADGYARV--KGISALITFGVGELSALNGTAGAFSEHVPVVEHIVGCPSTISORNGM : 119
 C. tropicalis : NAGYAADGYARVNPNGLAALVSTFGVGELSALNATAGSYSEHVGIIINLVGVPSSSACAKQL : 113
 F. oxysporum : NAGYAADGYARV--TKMAALITFGVGELSALNGVAGSYSEHIPVVEHIVGCPSTISORDQM : 116
 K. africana : NAGYAADGYARI--KGMSCIIITFGVGELSALNGTAGSYAEHVGVLHVVGVPSISSQAKQL : 111
 K. lactis : NAGYAADGYARL--KGMSCIIITFGVGELSALNGTAGSYAEHVGVLHVVGVPSISSQAKQL : 111
 M. acridum : NAGYAADGYARI--KOIGALITTFGVGELSALNGVAGAFSEHIPVVEHIVGYPSITLORDQM : 119
 M. gypseum : NAGYAADGYARI--NGMSALVTFGVGELSALNATAGAYSEFVPIVHIVGQPHTRKQKDCM : 120
 M. thermophila : NAGYAADGYARV--KGISALITFGVGELSALNGTAGAFSEHVPVVEHIVGCPSTISORNGM : 119
 N. dairenensis : NAGYAADGYARI--KGMSCIIITFGVGELSALNGTAGSYAEHVGVLHVVGVPSVSSQAKQL : 111
 N. tetrasperma : NAGYAADGYARA--KGISALVITFGVGELSALNGVAGAYSEHVPVVEHIVGCPSTISORNGM : 119
 P. anserina : NAGYAADGYARA--LGISALVITFGVGELSALNGVAGAYSEHVPVVEHIVGCPSTISORNGM : 120
 S. cerevisiae : NAGYAADGYARI--KGMSCIIITFGVGELSALNGTAGSYAEHVGVLHVVGVPSISSACAKQL : 179
 S. cryophilus : NAGYAADGYARV--KGLSALVITFGVGELSALNGLAGAYAEHVPVVEHIVGMPSITKACSSGA : 114
 S. japonicus : NAGYAADGYARV--KGISALVITFGVGELSALNGLAGAYAEHVPVVEHIVGMPSITKACSSGA : 116
 S. macrospora : NAGYAADGYARA--KGISALVITFGVGELSALNGVAGAYSEHVPVVEHIVGCPSTISORNGM : 119
 S. pombe : NAGYAADGYARV--NGLSALVITFGVGELSALNGVAGSYAEHVPVVEHIVGMPSITKACSSGA : 114
 T. blattae : NAGYAADGYARI--KGMACIITFGVGELSALNGTAGSYAEHVGVLHVVGVPSLSSQAKQL : 111
 T. delbrueckii : NAGYAADGYARV--KGMALVITFGVGELSALNGTAGSYAEHVGVLHVVGVPSISSQAKQL : 111
 T. terrestris : NAGYAADGYARI--KGISALVITFGVGELSALNGVAGAFSEHVPVVEHIVGCPSTISORNGM : 120
 U. hordei : NAGYAADGYARIK--RTISAVVITFGVGELSALNGTAGSFSERLPVVEHIVGVPSITAAOGAHS : 114
 V. polyspora : NAGYAADGYARI--KGMACIITFGVGELSALNGTAGSYAEHVGVLHVVGVPSLSSQAKQL : 111
 Z. rouxii : NAGYAADGYARV--KGLALVITFGVGELSALNGTAGSYAEHVGVLHVVGVPSVSSQAKQL : 111

N. crassa : LLHHTLGNLDFHVEANMSENISCDVAKLVKPSIAYQIDHAIRECWIRSRPVYIWFPTDMV : 180
 U. maydis : LLHHTLGDGRFSABENMSKETSADSAI LKSKQAGAGESIDRILITAMKSARPVYLAEPDILV : 177
 A. gossypii : LLHHTLGNLDFHVEHRMSANISDITAVITDISSAPAEIDRCIRACYVHQRPVYLGEPANMV : 172
 A. nidulans : LLHHTLGNLDFHVESSMNKGISVTTANINITYDAATLIDNAIRECWIHSRPVYLAEPDIMI : 180
 A. niger : LLHHTLGNLDFHVEAKMSAGISCTLGRNETLEAATLIDNAIRECWIRSRPVYIWFPTDMI : 181
 A. oryzae : LLHHTLGNLDFHVESTRMSADISCTLGCNSTHEVATLIDNAIRECWIRSRPVYIWFPTDMV : 181
 B. cinerea : LLHHTLGNLDFHVEADMSKETSACAMAKINPHEAALIDHTLQOCVWHSQRPVYITPTDMV : 180
 C. albicans : LLHHTLGNLDFHVEHRMFKNISQTSAFIADINSAPAEIDRCIRDAYVYQRPVYIGLPSNLV : 174
 C. glabrata : LLHHTLGNLDFHVEHRMSANISSETTAMVITDIATAPAEIDRCIRTTYITQRPVYLGEPANLV : 172
 C. globosum : LLHHTLGNLDFHVEANMSQISCDMARLNKPAEIPDOIDHALRECWIRSRPVYIMPTDMA : 180
 C. tropicalis : LLHHTLGNLDFHVEHRMFKNISQTSAFISIPNTAASEIDRCIRDAYVYQRPVYIGLPSNLV : 174
 F. oxysporum : LLHHTLGNLDFHVEANMSAQISCNVAKLNKPSIEAEOIDTALRTCWLRSRPVYIMVPTDMV : 177
 K. africana : LLHHTLGNLDFHVEHRMSANISSETTAMVITDIATAPAEIDRCIRTTYITQRPVYLGEPANLV : 172
 K. lactis : LLHHTLGNLDFHVEHRMSNISSETTAMITDIATAPAEIDRCIRTTYVSRQRPVYLGEPANLV : 172
 M. acridum : LLHHTLGNLDFHVEANMSQISQVAKLNKPSIEAEOIDHALRECVWHSQRPVYITPTDMA : 180
 M. gypseum : LLHHTLGNLDFHVEANMSAGISCSVAKLNPRDAAYIDSTLRECVWHSQRPVYITPTDIV : 181
 M. thermophila : LLHHTLGNLDFHVEANMSQISCDVARLNKRAEIDOCIDHALRECWIRSRPVYIMPTDMV : 180
 N. dairenensis : LLHHTLGNLDFHVEHRMSANISSETTAMVITDIATAPAEIDRCIRTTYITQRPVYLGEPANLV : 172
 N. tetrasperma : LLHHTLGNLDFHVEANMSENISCDVAKLVKPSIAYQIDHAIRECWIRSRPVYIWFPTDMV : 180
 P. anserina : LLHHTLGNLDFHVEANMSQISACNTARLNPAEIAEOIDFALRECWIRSRPVYIWFPTDMV : 181
 S. cerevisiae : LLHHTLGNLDFHVEHRMSANISSETTAMITDIATAPAEIDRCIRTTYITQRPVYLGEPANLV : 240
 S. cryophilus : LLHHTLGNLDFHVEVMNRPVSAITTVITNGEDAALKIDEALTIYRKARPVYIGLPSADTA : 175
 S. japonicus : LLHHTLGNLDFHVEEMSEKVSAYTVMITGETAADKIDKALSISYRKARPVYIGLPSDVG : 177
 S. macrospora : LLHHTLGNLDFHVEANMSEHISCDVAKLVKPSIEAEOIDHALRECWIRSRPVYIWFPTDMV : 180
 S. pombe : LLHHTLGDGDFRTEMDMFKKVSAYSIMIDNGNDAEKIDEALSIYKARPVYIGLPSDAG : 175
 T. blattae : LLHHTLGNLDFHVEHRMSAETSETTAMITDIATAPAEIDRCIRTTYVQRTVYLGEPANLV : 172
 T. delbrueckii : LLHHTLGNLDFHVEHRMSANISSETTAMITDIINSAPAEIDRCIRTTYVSRQRPVYLGEPANLV : 172
 T. terrestris : LLHHTLGNLDFHVEANMSQISACNTARLNKPAEIDOCIDHALRECWIRSRPVYIMPTDMV : 181
 U. hordei : LLHHTLGDGRFSABVNMKSETSADSAV LKQKGVGEAIDRILV SAMKKARPVYLAEPDILV : 175
 V. polyspora : LLHHTLGNLDFHVEHRMSANISSETTAMITDIANAPAEIDRCIRTTYITQRTVYLGEPANMV : 172
 Z. rouxii : LLHHTLGNLDFHVEHRMSANISSETTAMITDIATAPAEIDRCIRVAYVNRQRPVYLGEPANLV : 172
 N. crassa : EKIIEGARLDTEIDLSEPNDEPREDYVVDVVLRYLHGAKNPVILVDCAIRHRVTEEVKR : 241
 U. maydis : HATIPAEALKTLDYSIDNDKAEYVLOVAQKHIADAKSAVILVDCAAHRHGCIQETHE : 238
 A. gossypii : DLTVPASLLNTEIDLSLKPNDEAEVEVSTVLELVANAKHPVILVDCASRHDVKAETKQ : 233
 A. nidulans : TKIIEGERLKTIDLSLHPNDEPKEDYVVDVVLKYLHAAKNPVILVDCAIRHRVLEEVHD : 241
 A. niger : VKQIEGRDLKFLDLSLHPNDEPKEDYVVDVVLKYLHAAKNPVILVDCAIRHRVLEEVHD : 242
 A. oryzae : TKIIEGERLDTFLDLSLPPNDEPKEDYVVDVVLKYLHAAKNPVILVDCAIRHRVLEEVHD : 242
 B. cinerea : QKKIEGERLKTIDLSLHPNDEPKEDYVVDVVLKYLHAAKNPVILVDCAIRHRVLEEVHD : 241
 C. albicans : DMKVPSKLLDKKIDLSLHPNDEPKEDYVVDVVLKYLHAAKNPVILVDCAIRHRVLEEVHD : 235
 C. glabrata : DLKVPKLLKTPIDLSLHPNDEPKEDYVVDVVLKYLHAAKNPVILVDCASRHDVKAETKQ : 233
 C. globosum : EKKVIEGARLDTEIDLSEPNDEPREDYVVDVVLKYLHAAKNPVILVDCAIRHRVLEEVHD : 241
 C. tropicalis : DVKVPKLLDKKIDLSLHPNDEPKEDYVVDVVLKYLHAAKNPVILVDCAIRHRVLEEVHD : 235

F. oxysporum : QEKVEGARLDTPIDLSEPNDEGNEDEFVVDIEILKAMYARQRPVILVDSCAIRHRVVKVEVHQ : 238
K. africana : DLKVPAKLLETPIDLSIKPNDAEAEKEVVDITILSLIKDAKNPVILSDACASRHDVKAETTK : 233
K. lactis : DLTVPASLLDTPIDLISLKNDEPAEEVEIENVLQLIKEAKNPVILADACCSRHDVKAETTK : 233
M. acridum : DAKVEGARLDTPIDLTEPONDPEKEDYVVDVIMKYLNAAKNPVILVDAICAVRHRVLEEVHD : 241
M. gypseum : KQKIEGKRLKTPIDLQLPANDEEKEDYVVDVVLKYLQAARKPAIIVDAICAIRHNVLDEVHD : 242
M. thermophila : ERKVEGARLDTPIDLTEPANQSEREDYVVDVVLRYLHAAKQPVILVDAICAIRHRVLEEVHD : 241
N. dairenensis : DLKVPASLLETPIDLSLKNDEPAEETEVDNTILELIKDAKNPVILADACASRHDVKAETTK : 233
N. tetrasperma : EKKIEGARLDTEIDLSEPNDEPREDYVVDVVLRYLHGAKNPVILVDAICAIRHRVLEEVKR : 241
P. anserina : EKOIEGARLDTPIDLSDPPNEPEREDYVVDVVLRYLHAAKNPIILVDAICAIRHRVLEEVNRN : 242
S. cerevisiae : DLNVPAKLLQTPIDMSLKNDAEAEKEVVDITILALVKDAKNPVILADACCSRHDVKAETTK : 301
S. cryophilus : YFKTSSANLRKLETEEPENEVSEQEVITVAEMIEKSKKPVILVDAICAIRHRVLEEVKQ : 236
S. japonicus : YFKTSSAGLKKLQLEEPANDEPKIEEVEHTIVQMINASKKPVILADACVTRHRVLEEVKHE : 238
S. macrospora : EKKIEGARLDTEIDLSEPNDEPREDYVVDVVLKYLHGAKNPVILVDAICAIRHRVLEEVNK : 241
S. pombe : YFKASSNLGKRLKLEEDTNDPAVEQEVNHISEMVMNAKPVILVDAICAVRHRVLEEVHE : 236
T. blattae : DLTVPASLLETPIDLSLKNDAEAEENEVETVLELVREAKNPILVDAICCSRHDVKSSETEE : 233
T. delbrueckii : DLKVPASLLDTPIDLISLKNDAEAEQEVVDVVLALIKAKNPVILADACCSRHDVVKDETRK : 233
T. terrestris : EKKIEGARLDTPIDLTEPENEAEEREDYVVDVVLKYLHAAKCEIILVDAICAVRHRVLEEVHD : 242
U. hordei : HATIPADALKLKYDAEPNEEAAEKYVLNVAKKHIEQAKSAVILVDAICADRHGCIARETRE : 236
V. polyspora : DLMVPADLLKTPIDLISLKNDEPAEETEVDITILAMVKEAKNPILVDAICASRHDVKAETTK : 233
Z. rouxii : DQKVPASLLNTPIDLISLKNDEPAEETEVDITVLELIKEAKNPVILADACCSRHDVKAETTK : 233

N. crassa : FIEKTKLPVFEVPMGKCAFDETSEHYGGVYACTGSLF-EVAKRVEGSDLVLSIGALKSDFN : 301
U. maydis : LIEKSGLPVFAVPMGKTIVDEDDHAQYGGIVGSLTSE-KVKNVVEQADVLITVGSLSKSDFN : 298
A. gossypii : LIDVTFQFPAFVPMGKGSIDQHPFRFGVYVGTLSAF-DVKEAVESADLILSVGALLSDFN : 293
A. nidulans : LIEVSGLPTEFVPMGKCAVNDTHRCYGGVYACTGSNF-GVREQVESDILSILGALKSDFN : 301
A. niger : LMEASGLPTEFVPMGKCAVDETRNYGGVYACTGSNA-GVREQVESDILSILGALKSDFN : 302
A. oryzae : FVEKSGLPTEFVPMGKCAVDETHKNYGGVYACTGSNF-GVREQVESDILSILGALKSDFN : 302
B. cinerea : LIEKTKLPVFEVPMGKCAVDETSENYGGVYAGSGSH-DVKHRVESDILITIGALKSDFN : 301
C. albicans : LIEETQFPVFTPMGKSSVDESNPRFGVYVGTLSKPF-EVKESVESADLILSILGALKSDFN : 295
C. glabrata : LIDATQFPSEFVPMGKGSIDQHPFRFGVYVGTLSRF-EVKEAVESADLILSVGALLSDFN : 293
C. globosum : LVEKTKLPVFEVPMGKCAINENHPSYGGVYACTGSSQF-AVAERVESADLVLSIGALKSDFN : 301
C. tropicalis : LIAETQFPVFTPMGKSSVDESNPRFGVYVGTLSSP-DVKEAVESADLVLSVGMALSDFN : 295
F. oxysporum : LIDKLDLPVFEVPMGKCAVNDHPNYGGVYACTGSDGSHPARAQSDIVEGSDLLITIGALKSDFN : 299
K. africana : LIDATQFPFAVPMGKGSIDQHPFRFGVYVGTLSRF-EVKEAVESADLILSVGALLSDFN : 293
K. lactis : LIDLTFQFPAFVPMGKGSIDQHPFRFGVYVGTLSRF-AVKEAVESADLVLSVGAALLSDFN : 293
M. acridum : LLAKTNLPVFEVPMGKSAVNDQHDNFGVYVGTLSGSHPEVKNIVESDILVSVGALKSDFN : 302
M. gypseum : LVSKGLPTEFVPMGKCAVDETLNFGVYVGTLSGSHPEVQKHIEASDILSILGALKSDFN : 302
M. thermophila : LVEKTKLPVFEVPMGKCAINENHPSYGGVYACTGSSQF-AVAERVETADLVLSIGALKSDFN : 301
N. dairenensis : LIDLTFQFPSEFVPMGKGSIDQHPFRFGVYVGTLSRF-AVKEAVESADLILSVGALLSDFN : 293
N. tetrasperma : FIEKTKLPVFEVPMGKCAFDETSEHYGGVYACTGSLF-EVAKRVEGSDLVLSIGALKSDFN : 301
P. anserina : LVDKALPTEFVPMGKCAVNDSSPTYGGVYACTGSSQF-AVQELVESADLVLSVGAALLSDFN : 302
S. cerevisiae : LIDLTFQFPAFVPMGKGSIDQHPFRFGVYVGTLSKPF-EVKEAVESADLILSVGALLSDFN : 361
S. cryophilus : LIELTHFPSTVPMGKSAINENHPSYGGVYACTGSSQF-AVQELVESADLVLSVGAALLSDFN : 296
S. japonicus : LINLTHFPSTVPMGKSSVDESEWFDGVMYVGTLSIDF-AVKDRIESDILSILGALKSDFN : 298
S. macrospora : FIEKTKIPVFEVPMGKCAFDETCEHYGGVYACTGSLF-EVAKRVEGSDLVLSIGALKSDFN : 301
S. pombe : LILKTHFPSTVPMGKSAIDETSQFFDGMVYVGTLSIDF-EVKDRIESDILSILGALKSDFN : 296
T. blattae : LINATQFPSTVPMGKGSIDQHPFRFGVYVGTLSRF-EVKAAVESADLVLSVGAALLSDFN : 293
T. delbrueckii : LIDLTFQFPSEFVPMGKGSIDQHPFRFGVYVGTLSRF-EVKEAVESADLILSVGALLSDFN : 293
T. terrestris : LVEKTKLPVFEVPMGKCAINENHPSYGGVYACTGSHPE-DVAERVESADLVLSIGALKSDFN : 302
U. hordei : LIEKSGLPVFAVPMGKAIVDEDDHQYGGVYVGTLSSE-KVKEVEGADVLITVGSLSKSDFN : 296
V. polyspora : LIDTTFQFPSEFVPMGKGSIDQHPFRFGVYVGTLSRF-EVKAAVESADLILSVGALLSDFN : 293
Z. rouxii : LIDLTFQFPSEFVPMGKGSIDQHPFRFGVYVGTLSRF-EVKEAVESADLVLSVGAALLSDFN : 293

N. crassa : TAGFSYHTSOLNTIDLHSDHCTVRYSEYPGVAMRGVIRKVTTERIDMSKL-SITSEFPVEN- : 360
U. maydis : SENFSYRTPKSSITLHSDYTTIGYSHPGIGMKKLPKLSALLETNGDARREETKIVPK : 359
A. gossypii : TGSFSYSYKTKNIVEFHSDYTKIRSATFPGVQMKFALOKILLNAVPEAIK-GYKVEVPARV : 353
A. nidulans : TAGFSYRIGOLNTIDFHSYTVRVRYSEYEDTNMKGVLQKIVQRMG--FI-KADVPHISNA : 359
A. niger : TGSFSYHIGOLNTIDFHSYTVRVRYSEYEDTNMKGVLQKIVQRMG--AV-NAAPVHLSNT : 360
A. oryzae : TTGSFSYRIGOLNTIDFHSYTVRVRYSEYEDTNMKGVLQKIVQRMG--NL-NVGFVSPSNL : 360
B. cinerea : TAGFSYKTSOLNTIDFHSYTKIAVRYSEYGVHMRGVLOKIIKKVDLRKL-SAVEGFKMEN- : 360
C. albicans : TGSFSYGYKTRNIVEFHSDYTKIRQATFPGVQMKFALOKILLTTVKKSINPNYTPVVPETK : 356
C. glabrata : TGSFSYKTKNIVEFHSDYTKIRNATFPGVQMKFALOKILLNAVPEAIK-GYKVEVPARV : 353
C. globosum : TAGFSYRTSOLNTIDFHSYCTVRYSEYPGVAMRGVIRKVNERNVDLTKL-CRPSSEVDAN- : 360
C. tropicalis : TGSFSYNYKTRNIVEFHSDYTKIRQATFPGVQMKFALOKILLTKVKSVPKPYVFAVPAT- : 355
F. oxysporum : TTGSFSYRTSOLNSVDFHSTCKVRYSTYPGVAMRGVIRKVIDRVPKSM-PAPSIPEVRN- : 358
K. africana : TGSFSYKTKNIVEFHSDYTKIRNATFPGVQMKFALOKILLASVADAAG-GYKVEVPARV : 353
K. lactis : TGSFSYSYKTKNIVEFHSDYTKIRSATFPGVQMKFALOKILLTKVADAAG-GYKVEVPSEP : 353
M. acridum : TAGFSYRTSOLNSIDLHSDHCVRYSTYPGVAMRGVIRKIVKRVDPKQL-SIRASFTVRN- : 361
M. gypseum : TTGSFSYRVSRLNTIDFHSNEMVRYSEYPGVSMKGVIRKVVNRMG--KL-NVS-APKPHEN : 359

M. thermophila : TAGFSYRTSOLNTIDFHSDECTVRYSEYVGVAMRGVLRKVVVERVDLSKL-SRPPSEEVVN- : 360
 N. dairenensis : TGSFSYKTKNIVEFHSDIKIRNATFPVQMKFALOKILGSAEAAK-GYKPVAVPAKP : 353
 N. tetrasperma : TAGFSYHTSOLNTIDLHSDHCTVRYSEYVGVAMRGVLRKVTERIDMSKL-SITPSEPVEN- : 360
 P. anserina : TTGFSYRTSOLNTIDFHSDECKVRYSEYVGVAMKGVTRKLIERVDTKLSSEKLAEKVVN- : 362
 S. cerevisiae : TGSFSYKTKNIVEFHSDEKIRNATFPVQMKFVLOKLLTTIADAAG-GYKPVAVPART : 421
 S. cryophilus : TGSFSYHLSOKNSVEFHSDEKIRYALPEVSMKYVTRKLMNVLNPEKC-RAKAAETLGYD : 356
 S. japonicus : TGSFSYHISOKNTIEFHSDEKIRYALPEVSMKYLRVVISSLDAKAM-RAKAAKAIGFH : 358
 S. macrospora : TAGFSYHTSOLNTIDLHSDHCTVRYSEYVGVAMRGVLRKVTERIDTSKL-SITPSEOVEN- : 360
 S. pombe : TGSFSYHLSOKNAVEFHSDEKIRYALPEVSMKYILRKLKVLDAASC-HSKAAFTIGYN : 356
 T. blattae : TGSFSYAYKTKNIVEFHSDIKIRNATFPVQMKFVLOKLVSKIGAAIK-GYKPVAVPAGI : 353
 T. delbrueckii : TGSFSYKTKNIVEFHSDIKIRNATFPVQMKFALOKILAKVGDVAK-DYKPVAVPART : 353
 T. terrestris : TTGFSYRTSOLNTIDFHSYTCQVRYSEYVGVAMRGVLRKVVVERVDLSKL-SQPSSEQVNN- : 361
 U. hordei : SGNFSYRTPKESTIEFHSDEYTTVGYSHYPIGKMKGLEPKLSAILQPDQARRLEETKTVVVK : 357
 V. polyspora : TGSFSYSYQTKNVVEFHSDEKIRKASFPVQMKFVLOKLVQAQIGAAVK-DYKPVAAAPALP : 353
 Z. rouxii : TGSFSYKTKNVVEFHSDIKIRNATFPVQMKFVLOKLLQAVPEAVK-NYKPGVAVPAPP : 353

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N. crassa : --AVAENRDDSQAITTOAFLWRVGEYFQEGDVVVETETGTANFGIWESEFPKDVMMVTOVLW : 419
 U. maydis : F-ENALPHDSSSTITTOEFLWRMGOFFQEQDQVIVETGTSFSGMLEAKLPAKTRWVSQVLW : 419
 A. gossypii : --APNEDADPKTLPKOEFLNNOVSSFLKEGDIVITETGTAAFGINQTRFPSSITIGISQVLW : 412
 A. nidulans : L-PEHEKNSSEQRITTHAWFMVMVQWLKENDIVITETGTANFGIWDTRFPPSGVTALSOVLW : 419
 A. niger : L-PESEKSSSQEITTHDWLWVNVQWLKENDIVITETGTANFGIWEIRFPANVTALSOVLW : 420
 A. oryzae : L-PDNEKASTEQAITHAWFLWVYVQWLKENDIVITETGTANFGIWDTRFPAGVTALSOVLW : 420
 B. cinerea : --KV TANEDSSETITTOAFLWRVGEYFQEGDIIIVTETGTANFGIWEIRFPKGVTAISOVLW : 419
 C. albicans : --LINTPAAPSTPLTTOEFLWRVGEYFQEGDIIIVTETGTSAFGIVQSRFPKNSIGISQVLW : 415
 C. glabrata : --PENKCDPATPLKOEFLNNOVSKFLOEGDVIIVTETGTSAFGINQTRFPNNAYGISQVLW : 412
 C. globosum : --EVTKNRDSSETITTOAFLWRVGEYFQEGDIIIVTETGTSNFGIWEIRFPKGVTAISOVLW : 419
 C. tropicalis : --KAITTPGNNDPVSQEYLWRKVSDFWQEGDVIIVTETGTSAFGIVQSKFPKNAIGISQVLW : 414
 F. oxysporum : --EVEKNTDDEIITTOAFLWRVGEYFQEGDIIIVTETGTANFGIWDTRFPNVTALSOVLW : 417
 K. africana : --PENKACDPTPLAOWEMNQCAKFFQEGDVIIVTETGTSAFGINQSLFPNNSVGTISOVLW : 412
 K. lactis : --EHNEAVADSTPLKOEFLNNOVSKFLOEGDVIIVTETGTSAFGINQTRFPNNTYGISQVLW : 412
 M. acridum : --EAKEHHDSEATITTHAWFLWRVGEYFQEGDIIIVTETGTSNFGIWDTRFPKGVTAISOVLW : 420
 M. gypseum : L-PEENPQFPAPTISHSLWVAVGNWLOKENDIVITETGTSNFGIWEIRFPKGVTAISOVLW : 419
 M. thermophila : --EVTKNRDSSETITTOAFLWRVGEYFQEGDIIIVTETGTSNFGIWEIRFPKGVTAISOVLW : 419
 N. dairenensis : --AANAAVDAATPLKOEFLNNOVSKFLOEGDVIIVTETGTSAFGINQTRFPNNTYGISQVLW : 412
 N. tetrasperma : --AVAENRDDSQAITTOAFLWRVGEYFQEGDVVVETETGTANFGIWESEFPKDVMMVTOVLW : 419
 P. anserina : --EVSENDRDSTETITTOAFLWRVGEYFQEGDIIIVTETGTSNFGIWESEKFPDVTGTOILW : 421
 S. cerevisiae : --PANAAPASTPLKOEFLNNOVSKFLOEGDVIIVTETGTSNFGIWEIRFPKGVTAISOVLW : 480
 S. cryophilus : IKPKHAEGYSQQITTOAFLWRVGEYFQEGDIIIVTETGTANFGIWDTRFPKGVTAISOVLW : 417
 S. japonicus : VKPVHAEGFPKDSITTHQWFLWRVGEYFQEGDIIIVTETGTANFGIWDTRFPKGVTAISOVLW : 419
 S. macrospora : --AVAENKDDSETITTOAFLWRVGEYFQEGDIIIVTETGTANFGIWESEKFPKGVTAISOVLW : 419
 S. pombe : IKPKHAEGYSNEITTHCWFWRVGEYFQEGDIIIVTETGTANFGIWDTRFPKGVTAISOVLW : 417
 T. blattae : --PANKEVPANTPLKOEFLNNOVSKFLOEGDIIIVTETGTSNFGIWEIRFPKGVTAISOVLW : 412
 T. delbrueckii : --PENAATADSTPLKOEFLNNOVSKFLOEGDIIIVTETGTSNFGIWEIRFPKGVTAISOVLW : 412
 T. terrestris : --KVAENWDDSETITTOAFLWRVGEYFQEGDIIIVTETGTANFGIWEIRFPKGVTAISOVLW : 420
 U. hordei : F-QNALPDSDDVITTOAFLWRVGEYFQEGDIIIVTETGTSNFGIWEIRFPKGVTAISOVLW : 417
 V. polyspora : --ASNAECPASTPLKOEFLNNOVSKFLOEGDIIIVTETGTSNFGIWEIRFPKGVTAISOVLW : 412
 Z. rouxii : --SPNAEVADSTPLKOEFLNNOVSKFLOEGDIIIVTETGTSNFGIWEIRFPKGVTAISOVLW : 412

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N. crassa : GSGICWSVGAAGCAALAVKD-LEQDRRTILEVGDGSLTTCOEVSTMLKHN-LRVTMLH--- : 475
 U. maydis : GSGICWSVGAAGCAALAAARE--NALGRTHLEVGDGSLTLTVOEISTMIRHW-LKPYLFLVNN : 477
 A. gossypii : GSGICFTTGACLGAAFAAEE-IDPNKRIVILEIGDGSLOLTVOEISTMIRHW-LKPYLFLVNN : 471
 A. nidulans : GSGICYSVGACQCAALAAAKE-QG-NRRIVLWVGDGSLTLTVOEISTMIRNN-LNPITFVICN : 477
 A. niger : GSGICYSVGACQCAALAAAKE-IG-NRRIVLWVGDGSLTLTVOEISTMIRNN-LNPITFVICN : 478
 A. oryzae : GSGICYSVGACQCAALAAAKE-QG--RRTVLEVGDGSLTLTVOEISTMIRNN-LNPITFVICN : 477
 B. cinerea : GSGICYSVGACQCAALAAARD-AGSDSRRTILEVGDGSLTLTVOEISTMIRHW-LKPYLFLVNN : 478
 C. albicans : GSGICYTVGATCGAAMAAQE-IDPKKRIVILEVGDGSLTLTVOEISTMCKWECNNTYFLVNN : 475
 C. glabrata : GSGICFTTGACLGAAFAAEE-IDPKKRIVILEIGDGSLOLTVOEISTMIRHW-LKPYLFLVNN : 471
 C. globosum : GSGICWSVGAAGCAALAAKD-MGTDRTTILEVGDGSLTLTVOEISTMIRHW-LKPYLFLVNN : 478
 C. tropicalis : GSGICYATGATCGAAMAAQE-IDPKKRIVILEIGDGSLOLTVOEISTMCKWDCYNTYLYVNN : 474
 F. oxysporum : GSGICWSVGAAGCAALAAKD-AGKEGRTILEVGDGSLTLTVOEISTMIRHH-LKPYLFLVNN : 476
 K. africana : GSGICFTTGAVLGAFAAEE-IDPKKRIVILEIGDGSLOLTVOEISTMIRWN-LKPYLFLVNN : 471
 K. lactis : GSGICFTTGACLGAAFAAEE-IDPKKRIVILEIGDGSLOLTVOEISTMIRHW-LKPYLFLVNN : 471
 M. acridum : GSGICWSVGAAGCAALAAARE-MGEKRRRTILEVGDGSLTLTVOEISTMIRHE-LSPITFVICN : 479
 M. gypseum : GSGICYSVGACQCAALATKE--GASRRTILEVGDGSLTLTVOEISTMIRNG-LTPTFVICN : 477
 M. thermophila : GSGICWSVGAAGCAALAAKD-MGVDRTTILEVGDGSLTLTVOEISTMIRHW-LRITFLIFN : 478
 N. dairenensis : GSGICFTTGACLGAAFAAEE-IDPKKRIVILEIGDGSLOLTVOEISTMIRWN-LKPYLFLVNN : 471
 N. tetrasperma : GSGICWSVGAAGCAALAVKD-LEQDRRTILEVGDGSLTTCOEVSTMLKHN-LRVTMLH--- : 475
 P. anserina : GSGICWSVGAAGCAALAVKD-MGEDRTTILEVGDGSLTLTVOEISTMIRHW-LRITFLIFN : 480
 S. cerevisiae : GSGICFTTGACLGAAFAAEE-IDPKKRIVILEIGDGSLOLTVOEISTMIRHW-LKPYLFLVNN : 539

S. cryophilus : **GSICYSVGAFFGAALAVKDSSEPDRTILVVDGSLHLLTATEISTCVRFQN-LKPIIFVINN** : 477
S. japonicus : **GSICYSVGAFFGAALGVYDSAEPPDRRTILVVDGSLCLTLTTEISTFIRQG-LKPIIFILNN** : 479
S. macrospora : **GSICWSVGATCGAALAVKDLQDRRTILEVVDGGSFLLTCEVSTMMKHN-LRVTFILIYN** : 478
S. pombe : **GSICYSVGAFFGAVLAVHDSKEPDRRTILVVDGSLCLTITEISTCIRHN-LKPIIFIIINN** : 477
T. blattae : **GSICFTTGCATLCAAFAAEE-IDPNKRTILEVVDGSLCLTVOEISTLIRWN-LKPYLFLVNN** : 471
T. delbrueckii : **GSICFTTGCATLCAAFAAEE-IDPKKRVILEVVDGSLCLTVOEISTVVRWG-LKPYLFLVNN** : 471
T. terrestris : **GSICWSVGAACGAALAARD-MGVDRRTILEVVDGGSFLLTAEVSTMRHN-LKVTFLIYN** : 479
U. hordei : **GSICWSVGATLGVLAARE--NKLGRCLLEVVDGSLCLTVOEIGTMIRHG-LTPLYLFLVNN** : 475
V. polyspora : **GSICFTTGCATLCAAFAAEE-IDPKKRTVILEVVDGSLCLTVOEISTMIRWN-LKPYLFLVNN** : 471
Z. rouxii : **GSICYTTGATLCAAFAAEE-IDPKKRVILEVVDGSLCLTVOEISTMIRWG-LKPYLFLVNN** : 471

N. crassa : -----YRTLH-PWHGRGL-----QRC----- : 490
U. maydis : **DGYTIERQIHGQRSYNDIP-PYDHSLLDFFGN--EKF-HSDAKSHENDPHQSKQYYRV** : 534
A. gossypii : **DGYTIERLIHGETAQYNCIC-PWKHLDLPTFGA--KDY-----ETHRV** : 512
A. nidulans : **EGYTIERFIHGWDESYNDC--TWDIKGLPVAFGGK-GRY-----KGYKV** : 519
A. niger : **NGYTIERYIHGWDESYNDC--PWDieGLPRVFGAK-DKY-----KGYKV** : 520
A. oryzae : **EGYTIERYIHGWDESYNDC--PWDFLNIPVAFGAK-DKY-----KGYKV** : 519
B. cinerea : **DGYTIERFIHGMEEYNDIA-TWNNKDVPAFGAKEGKY-----KLHQI** : 521
C. albicans : **DGYTIERLIHGEKAQYNDIC-PWNNLQLPLFNA--KDY-----ETKRI** : 516
C. glabrata : **DGYTIERLIHGEKAGYNDIC-NWDHLALPTFGA--KDY-----ENHRV** : 512
C. globosum : **EGYTIERYIHGMEAEYNDV--RWQYTDVPTVFGGSDQV-----RKFVI** : 521
C. tropicalis : **DGYTIERLIHGEKAQYNDIC-PWNNLQLPLFNA--KKV-----ETKRI** : 515
F. oxysporum : **DGYTIERFIHGMDAVYNDIN-PWYKDLVSVFGE--KTC-----KTFQI** : 518
K. africana : **DGYTIERLIHGEKAGYNDIC-NWKHLMLPTFGA--KDY-----ENHRV** : 512
K. lactis : **DGYTIERLIHGETAQYNCIC-NWQHLELPTFGA--KDY-----EAVRV** : 512
M. acridum : **EGYTIERFIHGMDATYNDIA-KWNNRALLVDVFGGQ-GKA-----QKFVAV** : 521
M. gypseum : **NGYTIERYIHGWNAAYNDIC-EWKFKEIPSAFGAQPDOF-----TTYQI** : 520
M. thermophila : **GGYTIERFIHGMEAEYNDIT-RWNYIDVPTAFGGSEKQV-----RKFFV** : 521
N. dairenensis : **NGYTIEKLIHGPTAQYNEIC-SWDHLSLPTFGA--KDY-----ENHRV** : 512
N. tetrasperma : -----YRTLH-PW-----YCRGLORY : 490
P. anserina : **EGYTIERCIHGMEAEYNDIR-RWNYTEIPTVFGATDKV-----RKHII** : 523
S. cerevisiae : **DGYTIEKLIHGPKAQYNEIC-GWDHLSLPTFGA--KDY-----ETHRV** : 580
S. cryophilus : **DGYTIERLIHGLHAGYNDINTNWQYQDMLKFFGA--KQY-----RSYRV** : 519
S. japonicus : **NGYTIERLIHGLHAVYNEINTKWQYQELKFFGA--DQS-----RTYKV** : 521
S. macrospora : **EGYTIERYIHGMDADYNDII-RWNYTDIPAVFGAKEGHG-----QKFVI** : 521
S. pombe : **DGYTIERLIHGLHASYNEINTKWQYQIPKFFGAENHF-----RTYCV** : 521
T. blattae : **NGYTIEKLIHGPTAQYNEIC-PWKNLPLPTFGA--KDY-----EALRV** : 512
T. delbrueckii : **DGYTIERLIHGEKAQYNDIC-PWKNLPLPTFGA--KDY-----ETHRV** : 512
T. terrestris : **EGYTIERFIHGMEAEYNNIA-RWQYTEVPAVFGASDKQC-----RKFFV** : 522
U. hordei : **DGYTIERQIHGPERKYNDIP-PYDHSLLDFFGD--OKAGKREGKSHEKDMEPSKKQFVR** : 533
V. polyspora : **DGYTIEKLIHGPTAQYNEIC-PWKNLPLPTFGA--KDY-----EAI RV** : 512
Z. rouxii : **DGYTIERLIHGETAQYNCIC-PWKHLELPTFGA--KDY-----ENHRV** : 512

N. crassa : -----HPLELYRY-----PRGLWRGRPV : 509
U. maydis : **QTKQQLDELLKQSFANPDRIR-----LIEVVMQRGDPAEALKROAET** : 578
A. gossypii : **ATTGEWRALTEKQEFNKNSIR-----MIEIMLDVMDAPSSLIKOAKLT** : 556
A. nidulans : **TTRDELTKLFASEEFSSAPCLQVSLCDLTHYLSANFEQLVEVHMPREDAPASLKITAEAA** : 580
A. niger : **KTRDELRLQLFANQEFASAPYLQ-----LVELHMPRDDAPAAKITAEEA** : 564
A. oryzae : **TTRDELRELAFANQEFASAPCLQ-----LVELHMPRDDCPASLKLTAEAS** : 563
B. cinerea : **KTKDQVNEFLTNKEFNSADCLQ-----FVELYIIPKEDAPRALVLTAEAS** : 565
C. albicans : **STVGEINDLFAKAFVDPKIR-----MVEVMLPTMDAPANLVAQAKLS** : 560
C. glabrata : **ATTGEWDKLTQDKFENKNSKIR-----MIEVMLPVM DAPSSLIEQAKLT** : 556
C. globosum : **KTKDELEKLLTDKFNENAGGLQ-----FVELWMPKDDAPRALKITAEIA** : 565
C. tropicalis : **STVGEINDLFTNKFAVDPDRIR-----MVEIMLPVMDAPANLVAQAKQS** : 559
F. oxysporum : **KTKTELNELLTNKFNAAECLQ-----FVELYMPREDAPRALIMTAEAS** : 562
K. africana : **ATTGEWDALMGDKFENKNSRIR-----MIEVMLPVM DAPSNLVQAKIT** : 556
K. lactis : **STTGEWNLITTDKFNQDNTRIR-----LIEVMLPTMDAPSNLVKQAKLT** : 556
M. acridum : **KTKDELINELLASPSFKAANQLQ-----LVEIYMPKDDAPRLVLTAEAS** : 565
M. gypseum : **RERQELLDLFSNKEFCSAKRLQ-----IVEVYTPQEDAPSTRILTAEAA** : 564
M. thermophila : **KTKDELEELLTDTFNEARGLQ-----FVELWMPKDDAPRALKITAEIA** : 565
N. dairenensis : **ATTGEWIKLTENKEFNQNSRIR-----LIEIMLPVMDAPSNLVAQAKLT** : 556
N. tetrasperma : -----HPLELYRY-----PRGLWRGRPV : 509
P. anserina : **KTKSELEKLLADKDFENDAKGLQ-----LVELWMPKDDAPRALKLTAEQS** : 567
S. cerevisiae : **ATTGEWDKLTQDKSFNDNSKIR-----MIEIMLPVFDAPQNLVEQAKLT** : 624
S. cryophilus : **KTPQELLEKLFNDKQFASADVILQ-----LVEVIMPLMDAPRIIVEQAKLT** : 563
S. japonicus : **QTPSDVEKLFKDKQFASADVILQ-----LVEVMPVTFDAPRIIVEQAKLT** : 565
S. macrospora : **KTKDELEELLKDKDFNEYKGLQ-----FVELWMPKDDAPRALKLTAEIS** : 565
S. pombe : **KTPDVEKLFSDKEFANADVILQ-----VVELVMPMLDAPRIIVEQAKLT** : 565
T. blattae : **ATTGEWIKLITTDKAFQKNSKIR-----MIEIMLPVMDAPSNLVAQAKLT** : 556
T. delbrueckii : **STTGEWNLITTDSEFNKNSKIR-----MIEVMLPVM DAPSSLVQAKLT** : 556


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T.terrestris : KTKDQLEKLLTDKEFNEARGLQ-----FVELWLEKEDAPRALRITTEVA : 566
U.hordei     : QGRKQIDELLESDEFKAPDRIR-----VVELMMERGDAPKALKKRAEAT : 577
V.polyspora  : AATGDFNKLCEDKAFNENSKIR-----MIEIMLEVMDAPSNLVAQAQKLT : 556
Z.rouxii    : STVGEWNKLTQDPKENENSRIR-----MIEVMLEVMDAPSSLVAAQAQKLT : 556

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N.crassa      : SEVRYQDKG-- : 518
U.maydis     : GQANKYE---- : 585
A.gossypii   : AATNAQP---- : 563
A.nidulans   : ASRNK----- : 585
A.niger      : ATRNK----- : 569
A.oryzae     : AERNKSL---- : 570
B.cinerea    : AKNNAKD---- : 572
C.albicans   : EKTNAEQ---- : 567
C.glabrata   : ASTNAKQ---- : 563
C.globosum   : AKNNAKMSD-- : 574
C.tropicalis : AATNAAQE--- : 567
F.oxysporum  : ARNNAKKH--- : 570
K.africana   : AATNAKQD--- : 564
K.lactis     : AATNAKN---- : 563
M.acridum    : AKTNARKE--- : 573
M.gypseum    : AKRNG----- : 569
M.thermophila : ARNNA SMSE-- : 574
N.dairenensis : AKINAKQD--- : 564
N.tetrasperma : SEVRHQDKG-- : 518
P.anserina   : AKNNARME--- : 575
S.cerevisiae : AATNAKQ---- : 631
S.cryophilus : AKINQE----- : 569
S.japonicus  : AKINKQ----- : 571
S.macrospora : AKNNSRNEDEQ : 576
S.pombe      : SKINKQ----- : 571
T.blattae    : AATNAKQ---- : 563
T.delbrueckii : ASINAKN---- : 563
T.terrestris : ARTNARVE--- : 574
U.hordei     : GTANKYE---- : 584
V.polyspora  : ASINAKQ---- : 563
Z.rouxii     : AATNAKQ---- : 563

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S2 Fig. Sequence alignment of homologous pyruvate decarboxylase from 31 fungal species. Black background with yellow font, red background with black fonts and green background with black fonts are representing 100%, 75% and 50% identity of the residues respectively. TPP binding residues are marked in pink stars on the top of the alignment.

S3 Fig.

N. crassa : -----MSSNVFVELKTEVT-GTYKQFTGLFNNNEFVEGVK : 35
U. maydis : -----MPTLNLDLPNGIK---STIQADLFNNKFPALDG : 32
A. delicata : -----MAPTFSYTFDHFYSYSGKVTFFAGVYDGGKYSDCGAE : 36
A. flavus : -----MADLFTTIETPSA--KYEQLGLFINNEFVKAKSG : 33
A. nidulans : -----MSDLFTTITPVI--KYEQLGLFINNEFVKGVG : 33
A. oryzae : MTPDGSRIPEPRDTPFLVYFVSSLTMDLFTTITPFA--KYEQLGLFINNEFVKAKSG : 59
B. cinerea : -----MTSVQLKTELT-GEYTOPFTGLFINNEFVKGVG : 32
C. albicans : -----MFKKALPLVSKLITPKG-IITYNQLGLFINNEFVHPKQK : 38
C. cinerea okayama : -----MPSTFSYQWDTFAYKGTFTFNTEGLFINNQFVDSGNK : 36
C. immitis : -----MALFQKIETPSC--TYEQLGLFINNEFVKGVG : 32
C. tropicalis : -----MFNKALPLVAKLITPKG-LITYNQLGLFINNEFVQPKSN : 38
F. mediterranea : -----MPQFTTYEFNTEVFKGKTSFSTGLYDGGKSDGVG : 36
F. oxysporum : -----MAPLTVELISTPVT-GTYQQLGLFDGGKTEGVG : 34
G. graminis : -----MANLFTLKTPEAT-GAFKQPLGLFINNEFVCGSDK : 34
M. larici-populina : -----MIPEIKIQFPIETNLEPITLKTGLFINNEFVSSSN : 36
P. brasiliensis : -----MALFQQITPTI--TYEQLGLFINNEFVKGVG : 32
P. chrysogenum : -----MSLSTTIQTPNV--TYEQLGLFINNEFVKGVG : 32
P. nodorum : -----MSGLSVKLKTPEQT-GEYDQFTGLFINNEFVKAVDG : 34
P. teres : -----MSETSVKLTPEQT-GEYEQFTGLFINNEFVKSVG : 34
S. commune : -----MTSFSYEFKTEAYTGKVNFTGVFDGKSEGSK : 35
S. japonicus : -----MPAATLREIPAVKIELPNGLT---VDQVGLFINNKFDASAAG : 40
S. pombe : -----MSTKLVHVEITVPTGKT---YIQVGLFINNQHVDSVHG : 37
S. reilianum : -----MPTLNLDLPNGIK---STIQADLFNNKFPALDG : 32
U. hordei : -----MPTLNLDLPNGIK---STVQAELENNKFPALDG : 32
V. alboatrum : -----MGLSALKTEVT-GTYTOPFTGLFINNEFVEGVK : 33

N. crassa : KTFEVINPATEEVICSVHEATEKDVDIWAARAKAF--EGVTRDVTPEQQ-RGIYLLKLAEL : 93
U. maydis : KTFEATINPSTGKEIGQVAEASAKDVIDLAWKAAAEAF--ETTNGENTPGDARGRLILIRLAEL : 91
A. delicata : KLIDVLPNTNGKLVGQIAEGSRADVDRAVEAHRFAF--NNSWGLKVPGFERKLLIRLAEL : 95
A. flavus : RTFFETINPTNEKPTVAVQEADENDVEDAWKAAAEAF--EGEWSKVTPESE-RGRLIVKLAEL : 91
A. nidulans : KTFQVINPNSNEKVTTSVHEATEKDVDAWAARAKAF--EGPWRQVTEPESE-RGILINKLAEL : 91
A. oryzae : RTFFETINPTNEKPTVAVQEADENDVEDAWKAAAEAF--EGEWSKVTPESE-RGRLIVKLAEL : 117
B. cinerea : KTFEVINPTEEVITSVHEATEKDVDIWAARAKAY--EGEWRHVTEPEG-RGKLVKLAEL : 90
C. albicans : KTFEVISSPSTEEKITDVEALEEDIDTAVEAQAAY--HNGWAQGPPEQ--RSKVLKLAEL : 96
C. cinerea okayama : TTIDVINPTNGKVTTSISGVEKDVDAWAARAKAF--ETTNGLNVSGSQSELLVWKLATL : 95
C. immitis : KTFEETINPHNEKPTVAVHEATEKDVDIWAARAKAF--EGEWSKVTPESE-RGRMLNKLAEL : 90
C. tropicalis : KTFEVISSPSTGEKITDVEYSLAEDVDIWAARAKTAY--DNGWASGAPPEQ--RAKLVKLAEL : 96
F. mediterranea : LTYDVINPTTNGKVTTKMCIIGNKIDIDLAWKAAHKAY--NTVWGLNCPGYERKLLMKLAL : 95
F. oxysporum : QKFEVINPSTPEEVITSVCEGTEKIDIDLAVTAAAKAF--EGEWSKVTPESE-RGNYLLKLAEL : 92
G. graminis : KTFEVINPTEEVICSVHEATEKDVDAWAARAKAF--EGEWSKVTPESE-RGILINKLAEL : 92
M. larici-populina : KSIEETINPSTGKPTGKVSIAASKEDVDIWAARAKAEAF--ENHNGTKTEAQKRGRLMLLADA : 95
P. brasiliensis : RTFFETINPSTGKPTGKVSIAASKEDVDIWAARAKAF--KGPWKHVTEPESE-RGRMLNKLAEL : 90
P. chrysogenum : KTFEETINPSTGKPTGKVSIAASKEDVDIWAARAKAF--EGEWSKVTPESE-RGRMLNKLAEL : 90
P. nodorum : KTFEVINPSTPEEVITCQVQEADENDVEDAWKAAAEAF--NCPWRKTEPESE-RGRLIVKLAEL : 92
P. teres : KTFEVINPSTPEEVITCQVQEADENDVEDAWKAAAEAF--NCPWRKTEPESE-RGRLIVKLAEL : 92
S. commune : TTIDVINPTTNGKVTTSVAEATAADVDKAVIAAQAQAF--DTTNGLNAAGSRSELYLKLALL : 94
S. japonicus : GRVIDINPATEKHLCDVVEAREEDIDVAVAAAEAFHPDAPWRKFSSAQ--RGRLISLADK : 100
S. pombe : GRVKYSSPSTFEKLLCEVADNDEEDVDIWAARAKAF--QTDAPWRKFSSAQ--RGRLISRLAD : 97
S. reilianum : KTFEATINPATGKEIGQVAEASAKDVIDLAWKAAAEAF--ETTNGENTPGDARGRLILIRLAEL : 91
U. hordei : KTFEATINPSTGKEIGQVAEASAKDVIDLAWKAAAEAF--ETTNGENTPGDARGRLILIRLAEL : 91
V. alboatrum : KTFEVINPSTPEEVITSVSEATEKDVDIWAARAKAF--NGVWRQVTEPEQQ--RSIYLLKLAEL : 91

N. crassa : LEKLNLLAAVESDNGKSIITMARG-DVAVVGTIRYGGWADKIECKTIIISPDSFHYTR : 153
U. maydis : VEANIDELAAIESDNGKAFSIAASFDAVAANRYGGWADKNHCKVMEVDTKRNLNYTR : 152
A. delicata : IEEHADHLAAIEADNGKAFATARGFDLIVESACFRYYGGWADKLECKVIEINPTKESYTO : 156
A. flavus : FERDSILAAIEADNGKAFATMARG-DVAAAAGCLRYGGWADKIECKTIDINPESLTYTR : 151
A. nidulans : MERDIDTLAAIESDNGKAFATMARG-DLANSIAGCLRYGGWADKIECKTIDINPETLTYTR : 151
A. oryzae : FERDSILAAIEADNGKAFATMARG-DVAAAAGCLRYGGWADKIECKTIDINPESLTYTR : 177
B. cinerea : VEKNADLLAAVESDNGKISMAKRG-DVAVAVAGCLRYGGWADKIECKVIDTDPEYFTYTR : 150
C. albicans : IEENAELLAQIETWONGKSLQNRG-DVALTAYFRSCGGWADKILCSQINNGTHENYTO : 156
C. cinerea okayama : MEQHHEELAAIEADNGKTFAWARGTDVAFSINVIKFAAGWADKIECKTIDDERKLIYTR : 156
C. immitis : LEQHCNLLAAIEADNGKAVSMARV-DVANSAGCIRYGGWADKLECKVVDTDHETFNTR : 150
C. tropicalis : VEEHLETLLAQIETWONGKSLQNRG-DIGFTAYFRSCGGWADKILCSQINNGSNLYTYTO : 156
F. mediterranea : MEEHDELLEAAIEADNGKTFSWARNVDFGSIQIRYGGWADKNECKVIEIDEGKLAYTR : 156
F. oxysporum : AEKLNLLAAVESDNGKSIITMARG-DVAVVGTIRYGGWADKIECKTIIISPDSFHYTR : 152

G. graminis : VEKNDLLAAVESIDNGKAFAMAKG-DVGAVVCLIRYGGWADKIEKTTIIPADMFHYTR : 152
M. larici-populina : IEEENDVLAATESIDNGKAFSIARISFDVPEAAHCLIRYGGWADKNHCKVTEVNDKSMFAFTR : 156
P. brasiliensis : MEQHVDTLAAIEAIDNGKAFYSIARI-DVANAAGCIRYGGWADKIEKVIDTDTDSFNHYTR : 150
P. chrysogenum : FERDMETLAAIEAIDNGKAVTMAKVDIANAAGCLIRYGGWADKITCOTIDTNSSETLSYTR : 150
P. nodorum : FEKNADLLASVEAIDNGKAFNMANKVDVPCVCGCLIRYGGWADKIEKVVDTTPDTEFNHYTR : 153
P. teres : FEKNADLLAAVEAIDNGKAFSMARNVDVPAAGCLIRYGGWADKIEKVVDTSPDTEFNHYTR : 153
S. commune : MEONDELCALEAIDNGKTFGWAANVDVAFAIQTIKFAWADKIEKVVIEDSKLTYTR : 155
S. japonicus : VYEHLELLASVETVDNGKSITLARG-DVRSAAECIRYGGWADKEYCOTIEDTTRFAYTR : 160
S. pombe : IEOLELLEASVETVDNGKSITLARG-DVQAADCFRYGGWADKDYCOTIEDIKRFAYTR : 157
S. reilianum : VEANVDELAATESIDNGKAFSIARISFDVAAVAANLRYGGWADKNHCKVMEVDTSRLNYTR : 152
U. hordei : VEEHVDELAATESIDNGKAFSIARISFDVSAANLRYGGWADKNHCKVTEVDNKRNLNYTR : 152
V. alboatrum : VEKNDLLAAVESIDNGKSITMAKGDVGAVVCLIRYGGWADKIEKTTIIVAPDMFHYTR : 151

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N. crassa : QEPLGVCGQIPWNFPLIMLAWKIGPALATGNTIVVKTAEOPLSALVFAQFVKEAGFPFG : 214
U. maydis : HEPIGVCGQIPWNFPLIMFAWKIGPALATGNTIVVKTAEOPLSALIKMCEIIVEAGFPFG : 213
A. delicata : HEPIGVCGQIPWNFPLIMAAWKIGPALATGNTIVVKPSEFPLSVLYLCOIFSEAGFPFG : 217
A. flavus : HEPIGVCGQIPWNFPLIMWSWKIGPAIAGNVVVKTAEOPLSGLYAAKLIKEAGFPAG : 212
A. nidulans : HEPIGVCGQIPWNFPLIMWSWKIGPAVAGNVVVKTAEOPLSALYAAKLIKEAGFPAG : 212
A. oryzae : HEPIGVCGQIPWNFPLIMWSWKIGPAIAGNVVVKTAEOPLSGLYAAKLIKEAGFPAG : 238
B. cinerea : QEPIGVCGQIPWNFPLIMWSWKIGPAVATGNTIVVKTAEOPLSALVAANLIKEAGFPFG : 211
C. albicans : RVPLVCGQIPWNFPLIMASWKIGPVATGNTIVVKTAESPLSALYLSQLLVEAGMPKG : 216
C. cinerea okayama : HEPIGVCGQIPWNFPLIMLAWKIGPALATGNTIVVKPSEFPLITAIRMCSLIQEAGFPFG : 217
C. immitis : HEAVSVCGQIPWNFPLIMWAWKIGPALATGNTIVVKSAEOPLSALYACQIVKEAGFPFG : 211
C. tropicalis : RVPLVCGQIPWNFPLIMASWKIGPVATGNTIVVKPSEFPLSVLYLCOIFSEAGFPFG : 216
F. mediterranea : HEPIGVCGQIPWNFPLIMLAWKIGPALATGNTIVVKPSEFPLITAIRMVGLEIEAGFPFG : 217
F. oxysporum : SEPIGVCGQIPWNFPLIMLAWKIGPALATGNTIVVKTAEOPLSALVFTQFTEQAGFPAG : 213
G. graminis : QEPIGVCGQIPWNFPLIMFAWKIGPALATGNTIVVKTAEOPLSGLYVMAQFVKEAGFPAG : 213
M. larici-populina : HEPIGVCGQIPWNFPLIMFAWKIGPALATGNTIVVKPAEOPLTALFMAQIISKI-FPPG : 216
P. brasiliensis : HEPIGVCGQIPWNFPLIMWSWKIGPVVATGNTIVVKSAEOPLSALYAAKLIIVEAGFPFG : 211
P. chrysogenum : HEPIGVCGQIPWNFPLIMWAWKIGPAIAGNVVVKTAEOPLSGLYAAKLIIVEAGFPFG : 211
P. nodorum : KEPIGVCGQIPWNFPLIMFAWKIGPALATGNTIVVKTAEOPLSALYIAANLIKEAGFPFG : 214
P. teres : KEPIGVCGQIPWNFPLIMWAWKIGPAIAGNVVVKTAEOPLSGLYAAKLIIVEAGFPFG : 214
S. commune : HEPIGVCGQIPWNFPLIMFAWKIGPALATGNTIVVKPSEFPLITAIRTCLINEAGFPFG : 216
S. japonicus : HEPIGVCAQIPWNFPLIMFAWKIGPALATGNTIVVKTAEOPLSALMLTSFTEECGFPAG : 221
S. pombe : HEPIGVCGQIPWNFPLIMCAWKIGPAVATGNTIVVKTAELPLSALCLTKFVPECCFPFG : 218
S. reilianum : HEPIGVCGQIPWNFPLIMFAWKIGPALATGNTIVVKTAEOPLSALIKFCEIIVEAGFPFG : 213
U. hordei : HEPIGVCGQIPWNFPLIMFAWKIGPALATGNTIVVKTAEOPLSALIKLCEIIVKAGFPFG : 213
V. alboatrum : QEPIGVCGQIPWNFPLIMLAWKIGPALATGNTIVVKTAEOPLSGLYVFANLIKEAGFPFG : 212

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N. crassa : VLNIISGFERIAGALSSEMDIDKVAFTGSTLVGRQIMKAAASNLKQVTTLELGGKSPNII : 275
U. maydis : VVNVISGFERVAGALSSEMDIDKVAFTGSTLVGRNIMKAAASTNLKQVTTLELGGKSPNII : 274
A. delicata : VVNVVNGYENVVGAISSEMHLEKVAFTGSTLVGRAVMKAAASNLKQVTTLELGGKSPNII : 278
A. flavus : VVNVISGFERVAGALSSEMDIDKVAFTGSTLVGRMILQAAAKSNLQVTTLELGGKSPNIV : 273
A. nidulans : VINVISGFERTAGALSSEMDIDKVAFTGSTLVGRTILQAAAKSNLQVTTLELGGKSPNIV : 273
A. oryzae : VVNVISGFERVAGALSSEMDIDKVAFTGSTLVGRMILQAAAKSNLQVTTLELGGKSPNIV : 299
B. cinerea : VVNVISGFERIAGALSSEMDIDKVAFTGSTLVGRQIMKAAASNLKQVTTLELGGKSPNIV : 272
C. albicans : VINIVSGFERVAGALSSEMDIDKVAFTGSTLVGRMILQAAAKSNLQVTTLELGGKSPNIV : 277
C. cinerea okayama : VVNVISGFERVAGALSSEMDIDKVAFTGSTLVGRMILQAAAKSNLQVTTLELGGKSPNIV : 278
C. immitis : VLNVISGFERVAGALSSEMDIDKVAFTGSTLVGRMILQAAAKSNLQVTTLELGGKSPNIV : 272
C. tropicalis : VVNVISGFERVAGALSSEMDIDKVAFTGSTLVGRMILQAAAKSNLQVTTLELGGKSPNIV : 277
F. mediterranea : VVNVISGFERVAGALSSEMDIDKVAFTGSTLVGRMILQAAAKSNLQVTTLELGGKSPNIV : 278
F. oxysporum : VVNVISGFERVAGALSSEMDIDKVAFTGSTLVGRMILQAAAKSNLQVTTLELGGKSPNIV : 274
G. graminis : VLNVISGFERIAGALSSEMDIDKVAFTGSTLVGRMILQAAAKSNLQVTTLELGGKSPNIV : 274
M. larici-populina : VINIVISGFERVAGALSSEMDIDKVAFTGSTLVGRMILQAAAKSNLQVTTLELGGKSPNII : 277
P. brasiliensis : VINIVISGFERVAGALSSEMDIDKVAFTGSTLVGRMILQAAAKSNLQVTTLELGGKSPNIV : 272
P. chrysogenum : VINIVISGFERVAGALSSEMDIDKVAFTGSTLVGRMILQAAAKSNLQVTTLELGGKSPNIV : 272
P. nodorum : VINIVISGFERVAGALSSEMDIDKVAFTGSTLVGRMILQAAAKSNLQVTTLELGGKSPNIV : 275
P. teres : VVNVISGFERVAGALSSEMDIDKVAFTGSTLVGRMILQAAAKSNLQVTTLELGGKSPNIV : 275
S. commune : VVNVISGFERVAGALSSEMDIDKVAFTGSTLVGRMILQAAAKSNLQVTTLELGGKSPNII : 277
S. japonicus : VINIVISGFERVAGALSSEMDIDKVAFTGSTLVGRMILQAAAKSNLQVTTLELGGKSPNIV : 282
S. pombe : VINIVISGFERVAGALSSEMDIDKVAFTGSTLVGRMILQAAAKSNLQVTTLELGGKSPNIV : 279
S. reilianum : VVNVISGFERVAGALSSEMDIDKVAFTGSTLVGRNIMKAAASTNLKQVTTLELGGKSPNII : 274
U. hordei : VVNVISGFERVAGALSSEMDIDKVAFTGSTLVGRNIMKAAASTNLKQVTTLELGGKSPNIV : 274
V. alboatrum : VVNVISGFERVAGALSSEMDIDKVAFTGSTLVGRQIMKAAASNLKQVTTLELGGKSPNIV : 273

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N. crassa      : FNDADLDQAVRWSAFGIMFNHGGCCAGSRVYVOEGYDKFVAAFKQRAQQNKVGPFFHDE : 336
U. maydis     : FKDADLDQAVRWSAFGIMFNHGGCCAGSRVYVEESTYDAFMEKMTAHCKALQVGPFFSAN : 335
A. delicata   : FEDADVDQAVRWSAFGLFFNHGGCCAGSRVYVOESTYDSFVEKFKAHVKTLKVGDPFDKE : 339
A. flavus     : FDDADLDNAISWVNFGIFFNHGGCCAGSRILVOEGYHDKFVARFKERAAANKLGNPFTAD : 334
A. nidulans   : FDDADLDNAISWVNFGIFFNHGGCCAGSRILVOEGYDKFVARFKERAAQKNKVGDPFEQD : 334
A. oryzae     : FDDADLDNAISWVNFGIFFNHGGCCAGSRILVOEGYHDKFVARFKERAAANKLGNPFTAD : 360
B. cinerea    : FNDADLDNAISWVNFGIFFNHGGTCCAGSRVYVQDGIYDKFVESFKARAIANKVGPFFHDE : 333
C. albicans   : FNDADLDKTIQNLIVSIFVNSGEVCCAGSRLLIQSGVYDQVVEKFKAAESVVKVGNPFDEH : 338
C. cinerea okayama : FNDADLDQAVNVAHVHGLFVNHGGCCAGSRIFVQSGIYDEFLEKRFATAKAAAIRVGPFFGVE : 339
C. immitis    : FNDADLDNAISWVNFGIFFNHGGCCAGSRILVOEGYEDFLQRFKERAMKKNKVGDPFNPD : 333
C. tropicalis : FNDADLDKTVHNLVSGVFSNSGETCAAGARVLVQSGVYDEVVARFKAAEAATKVGDPFEED : 338
F. mediterranea : FDDADLDQAVDVAAHGVFWNHGGCCAGSRIFVQEKIYDEFLEKRFATAKQGLKIGDPFGVD : 339
F. oxysporum  : FDDADLDNAISWVNFGIFFNHGGCCAGSRIFVQEGYDKFLAAFKKRAEENKVGDPFNED : 335
G. graminis   : FNDADLDQAVSWSVNFGIFFNHGGCCAGSRVYVOEDIYDKFVAAFKARAENKAVGDPFKDD : 335
M. larici-populina : FDDADLDQAVKNSGFIFFNHGGCCAGSRVYVHEAVYDQFMVKFEYAKSFVGPFFSKN : 338
P. brasiliensis : FNDADLDNAISWVNFGIFFNHGGCCAGSRILVEEGYDFTLERFKARALQNKVGPFFHGD : 333
P. chrysogenum : FEDADLDNAISWVNFGIFFNHGGCCAGSRLLVOESTHDKFVARFKERAAQKNLGNPFEED : 333
P. nodorum    : FADADLDNAISWVNFGIFFNHGGCCAGSRVYVOEETIYDKFIARFRERAAQNAVGDPFSKD : 336
P. teres      : FADADLDNAISWVNFGIFFNHGGTCCAGSRVYVEESTYDKFIERFRERAAQNKVGPFFAAE : 336
S. commune    : FDDADLDQAVNVAAHGVFWNHGGCCAGSRIFVQAGIYDKFLEAFKARTRGKIKVGPFGDN : 338
S. japonicus  : FDDADLDKAVANTYGIFFVNSGEVCCAGSRVYVOEGYDEFVKRMVAKAKTLKVGDPFDED : 343
S. pombe      : FNDADLDNAISWVNFGIFFNHGGCCAGSRVYVOEDVYDEFIKRMVAKAKTLKVGDPFFAAE : 340
S. reilianum  : FKDADLDQAVRWSAFGIMFNHGGCCAGSRVYVEDSTYDAFMEKMTAYCKALQVGPFFAAT : 335
U. hordei     : FKDADLDQAVRWSAFGIMFNHGGCCAGSRVYVEESTYDAFMEKMTAHCKALPVGPFPAAN : 335
V. alboatrum  : FNDADLDQAVSWSVNFGIFFNHGGCCAGSRVYVOEGYDKFLEAFKARAQNKVGPFFHDE : 334

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N. crassa      : TFQGPQVSQIQYDRIMGYIKAGKEEGATVEIGGERHEDKGYFIQPTIFTNVRHDMKIMQEE : 397
U. maydis     : TFQGPQVSQIQYDRIMEYIESGAKD-ANLALGCVRKENEYGFIEPTIFTDVPHDAKIAKEE : 395
A. delicata   : TFQGPQVSQIQYDRIMGYIESGKAGATVEIGGERHENEYGFIEPTIFTNVWPKDMQIVREE : 400
A. flavus     : TFQGPQVSQIQYDRIMEYINHGKQEGATVATGGERHGTGYFIQPTVFTDVHSDMKIAKEE : 395
A. nidulans   : TFQGPQVSQIQYDRIMEYINHGKQAGATVATGGERHENEYGFIEPTVFTDVTSDMKIAQEE : 395
A. oryzae     : TFQGPQVSQIQYDRIMEYINHGKQEGATVATGGERHGTGYFIQPTVFTDVHSDMKIAKEE : 421
B. cinerea    : TFQGPQVSQIQYDRIMGYIDEGKKSAGATVEIGGERHEDKGYFIQPTIFSDVTFEDMKIMQEE : 394
C. albicans   : FFMGAQVSDVQLSKILKVESGASQCATVVTGGARADGKGYFVKPTIFADVKKDMDIVREE : 399
C. cinerea okayama : VDCGPQVSQIQYDRIMGYIDSGKADCATVHLGGERHENEYGFIEPTIFTDVWPKDMKIVREE : 400
C. immitis    : TFQGPQISQIQYDRIMGYIDGKAKAKVEIGGERLGTGYFIQPTIFSNVNEEDMSIVREE : 394
C. tropicalis : FFMGSQVNEIQFSKILEFVESGKEQCATVVTGGERIADKGYFIKPTIFADVKKDMTIFKQEE : 399
F. mediterranea : TYQGPQVSQIQYDRIMGYIESGKQEGATVHTGGERHGTGYFIQPTIFNTRPDMKIVREE : 400
F. oxysporum  : TFQGPQVSQIQYDRIMGYIKAGKDECATVEIGGERLNEKGYFIKPTIFSNVTRPDMKIMQEE : 396
G. graminis   : TFQGPQVSQIQYDRIMEYIKAGKDECATVEIGGERHEDKGYFIQPTIFTNVRNDMKIMQEE : 396
M. larici-populina : TFQGPQVSQIQYDRIMGYIQSGKEDCARCCIIGGNRYENEYGFIEPTIFTDVWPKSMKIMQEE : 399
P. brasiliensis : TFQGPQVSQIQYDRIMGYIREGKAAGATVEIGGERLNEGYFIQPTIFSNVTFEDMKIVREE : 394
P. chrysogenum : TFQGPQVSQIQYDRIMEYINHGKTEGATVATGGERHGTGYFIQPTVFTDVTPDMKIAQEE : 394
P. nodorum    : TFQGPQVSQIQYDRIMGYIEEGKKSAGATVEIGGERHEDKGYFIQPTIFSNVTFEDMTIQEE : 397
P. teres      : TFQGPQVSQIQYDRIMGYIDEGKKECATVEIGGERHEDKGYFIQPTIFSNVTFEDMKIQREE : 397
S. commune    : VDCGPQVSQIQYDRIMSYIDTGKQEGATVCHIIGGNRVGDEGYFIQPTIFTDVWPKDMTIVREE : 399
S. japonicus  : TFQGAQVSKAQYERILSYIDLGLHCAKLEIGKRRHEDRGYFIQPTIFLSNVTEEMAVGQEE : 404
S. pombe      : TFQGAQVSKQYERIVSYIESCIAHCAKLEIGKRRHNLGYFVEPTIFLSNVTFEDMAVGQEE : 401
S. reilianum  : TFQGPQVSQIQYDRIMEYIETGAK-ANLHLGCVRKENEYGFIEPTIFTDAPHESKIAKEE : 395
U. hordei     : TFQGPQVSQIQYDRIMEYIESGAKD-ANLHLGCVRKENEYGFIEPTIFTDVPHDAKIAKEE : 395
V. alboatrum  : TFQGPQVSQIQYDRIMEYIKIGKEEGATVEIGGERHEDKGYFIQPTIFSNVWADMKIMQEE : 395

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N. crassa      : IFGPVCAVAFSTEEVTKLGNDSNYGLAAAVHTKDLNTAIRVSNHFRAGTVWVNTYNALH : 458
U. maydis     : IFGPVVVVSNFKDEKDLIRIANDSIYGLAAAVFSRDISRALETAKHLKAGTVWVNCYNQLI : 456
A. delicata   : IFGPVVVLAKFKDEDDIQAQANDSMYGLAAAVFSQNVSRALSWAORLHAGTVWVNHYNKLE : 461
A. flavus     : IFGPVVTIQKFKDEEEAKIGNS SYGLAAAVHTKNVNTAIRVSNHFRAGTVWVNCYNMIN : 456
A. nidulans   : IFGPVVTIQKFKDEAAATKIGNSTDYGLAAAVHTKNVNTAIRVSNALKAGTVWVNNYNMIS : 456
A. oryzae     : IFGPVVTIQKFKDEEEAKIGNS SYGLAAAVHTKNVNTAIRVSNHFRAGTVWVNCYNMIN : 482
B. cinerea    : IFGPVCSIAKSTEEIIRKIGNSNYGLAAAVHTQNLNTAIRVSNALKAGTVWVNCYNMLE : 455
C. albicans   : IFGPVVTLIKFDTVDEAVELANDSDYGLAAGIHSADVTKCIDVANRVKAGTVWVNTYNDFH : 460
C. cinerea okayama : IFGPVGVLIKFEDEADVTRQANDTVYGLAAAVFSQNIINRALETAKHMQAGTVWVNCANOLE : 461
C. immitis    : IFGPVCSIQKFKTEDEAKIANGTSYGLAAAIHTKDLNTAIRVSNHFRAGTVWVNCYNLIS : 455
C. tropicalis : IFGPVASVIKFDTIDEAIALANDSEYGLAAGVHTTNTINTGLHVANNIKSGSVWVNTYSELE : 460
F. mediterranea : IFGPVGVVIFKHDEEDVTRQANDTVYGLAAAVFSKNIDRALRVAHRLHAGTAVINCANTLN : 461
F. oxysporum  : IFGPVCAISKFKDEKEVIDLAHTAYGLAAAVHTKNLNTAIRVSNALKAGTVWVNCYNMLE : 457
G. graminis   : IFGPVCAIAKFKTEEDVTHMGNDSSYGLAAAVHTKDLNTAIRVSNHFRAGTVWVNCYNMLE : 457
M. larici-populina : IFGPVVAVTKFSSSEDLKLVANGSIYGLAAAVFSKDIQRSIKVANEKAGTVWVNCYNKLE : 460
P. brasiliensis : IFGPVCCVQKFKNDEAICIANNTSYGLAAAVHTDLDNTAIRVSNHFRAGTVWVNNYNMIS : 455
P. chrysogenum : IFGPVIAVTKFKDEADARIGNSTSYGLAAAVHTKNINTAIRVSNALKAGTVWVNNYNMIS : 455
P. nodorum    : IFGPVCTISKFKTKADVTKIGNSTTYGLAAAVHTTNTALEVANALRAGTVWVNTYNALH : 458
P. teres      : IFGPVCTIAKFKTKEVTRIGNAS IYGLAAAVHTTNTALEVANALRAGTVWVNTYNALH : 458
S. commune    : IFGPVGVVIFKHDEEDVTRQANDTVYGLAAAVFTQDINRALETAKHLQAGTAVINCANOLE : 460
S. japonicus  : IFGPVVAIIFKKTIEEARRRANNTSFGLASGVHTRSIDTALQVSNALQAGTVWVNCYNVLE : 465
S. pombe      : IFGPVLAVIKFKTIEEARRRGNSTYGLAAGVHTTNTITNAIKVSNALQAGTVWVNCYNLE : 462
S. reilianum  : IFGPVVVVSNFKDEKDLIRIANDSIYGLAAAVFSRDISRALETAKHLKAGTVWVNCYNQLV : 456
U. hordei     : IFGPVVVVSNFKDEKDLIRIANDSIYGLAAAVFSRDISRALETAKHLKAGTVWVNNYNQLN : 456
V. alboatrum  : IFGPVCAIAKFKDEDEVIALGNDSYGLAAAVHTKDLNTAIRVSNALKAGTVWVNCYNMLE : 456

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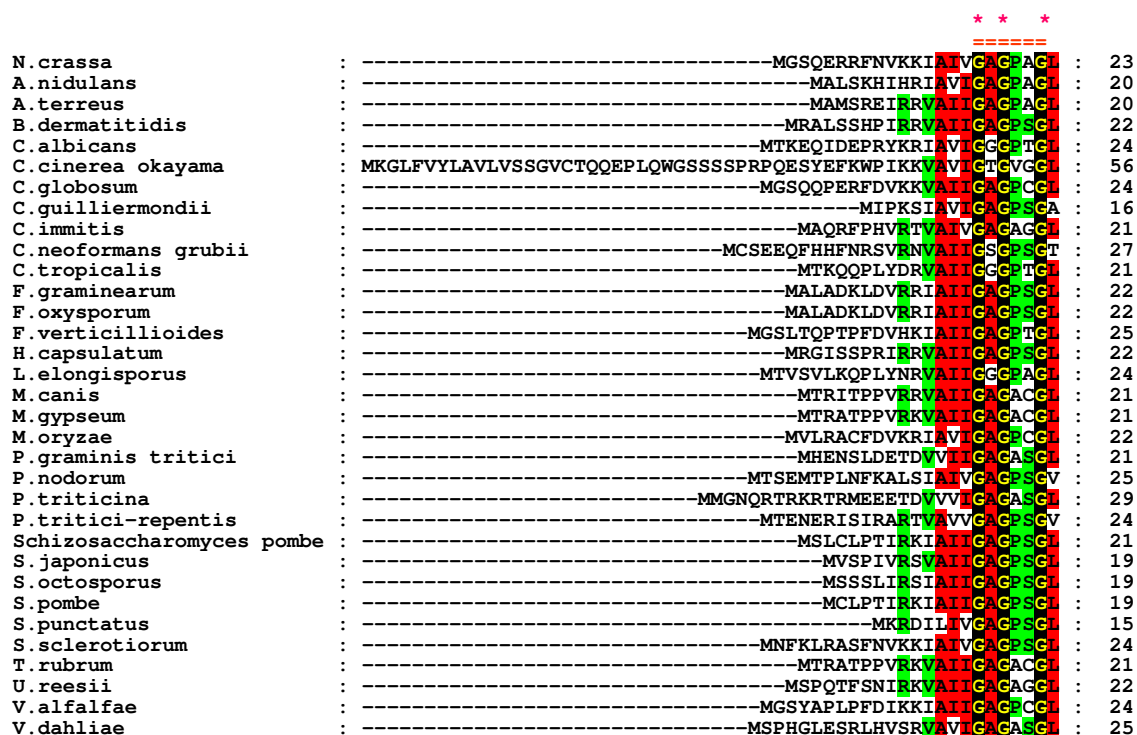
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N. crassa      : HQLPFGGYKESGIGRELGEAALANYTQCKSVAIKLN----- : 494
U. maydis     : PQVPFGGYKASGIGRELGEYALSNYTNIKAVHVNLSPAPI : 497
A. delicata   : PQVPFGGFKQSGIGRELGKYALANYTNVKAVQVNIHDKL-- : 500
A. flavus     : YQAPFGGFKQSGIGRELGSYALENYTOVKTVHYRLGDALFA : 497
A. nidulans   : YQAPFGGFKQSGIGRELGSYALENYTOIKTVHYRLGDALFA : 497
A. oryzae     : YQAPFGGFKQSGIGRELGSYALENYTOVKTVHYRLGDALFA : 523
B. cinerea    : HQVPFGGYKESGIGRELGEAALSNYTQTKSVRIRLGDALFG : 496
C. albicans   : PMVPFGGFKSASGIGREMGEVLEHYTOVRAVRMKNINPPN-- : 499
C. cinerea okayama : ANVPFGGFKQSGIGRELGEYALHNYTNVKAVHVNIQHVM-- : 500
C. immitis    : YQTPFGGFKESGIGRELGEYALDNYTOVKAVRIRLGDAMFG : 496
C. tropicalis : PMVPFGGFKSSGIGREMGEESFKEYTEVRSVRVKLYPDA-- : 499
F. mediterranea : TQIPFGGYKQSGIGRELGEYALANYSAIKAVHVNICMSI-- : 500
F. oxysporum  : HQLPFGGYKESGIGRELGEAALANYTONKSVAIKLY----- : 493
G. graminis   : HQLPFGGFKESGIGRELGEAALANYTQTKSVAIRLGGALF- : 497
M. larici-populina : TQVPFGGFKQSGIGRELGEYALANYTAVKAVHINLGEKL-- : 499
P. brasiliensis : YQTPFGGFKESGIGRELGEYALDNYTOVKAVRIRLGDALFG : 496
P. chrysogenum : YQAPFGGFKESGIGRELGSYALENYTOVKTVHYRLGDALF- : 495
P. nodorum    : WALPFGGYKQSGIGRELGEAALDNYLOTKTVSIRLGDVLF : 499
P. teres      : WQLPFGGYKESGMRELGEAALDNYLOTKTVSIRLGDVIFG : 499
S. commune    : ACVPFGGFKQSGIGRELGEYALENYTNVKAVHVNLRRHM-- : 499
S. japonicus  : HQLPFGGYKESGIGRELGSYGLSNYTQTKAVHINTGMNLPL : 506
S. pombe      : HQLPFGGYKESGIGRELGSYGLTNYTQTKAVHINLGMDSPI : 503
S. reilianum  : PQVPFGGYKASGIGRELGEYALTNYTNIKAVHVNLSPAPI : 497
U. hordei     : PQVPFGGYKASGIGRELGEYALANYTNVKAVHVNLSPAPI : 497
V. alboatrum  : HQVPFGGFKESGIGRELGEAALSNYTONKSVAIRLGGAIY- : 496

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S3 Fig. Multiple sequence alignment of aldehyde dehydrogenase homologs from 25 fungal species. Amino acid residues with black background yellow font are representing 100% sequence identity, 75% identity is shown in red background in black font and 50% sequence identity has been shown in black fonts with green background. NAD serves as cofactor of the enzyme aldehyde dehydrogenase. NAD binding residues are shown with pink star on the top of the residues.

S4 Fig.



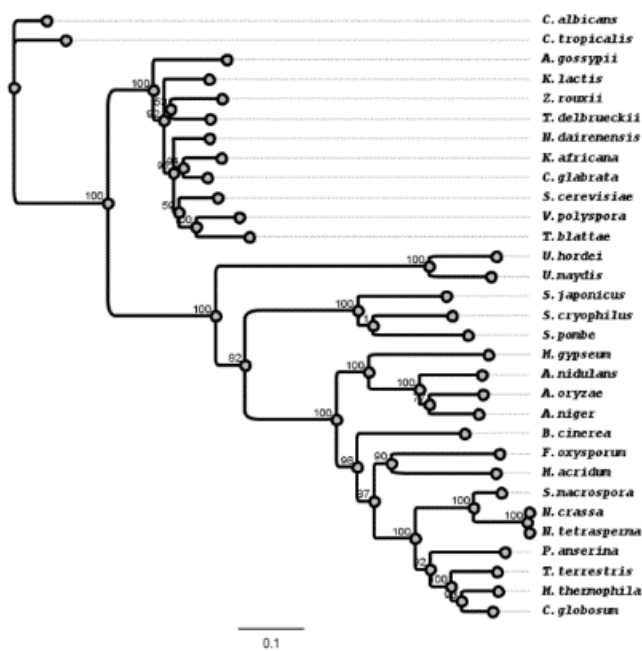
S4 Fig. Multiple sequence alignment of homologous YUC from 33 different fungal species. YUC or flavin monooxygenase is known to bind with FAD through its nucleotide binding motif (GXGXXG). GXGXXG motif has been shown with bar and conserved residues are highlighted with pink stars. Residues with black background and yellow font are 100% identical and residues with red background in black font are 75% identical. **Note:** YUC homolog in *U. mydis* was not found by same BLAST search using same template which was used for other homolog.

S5 Fig.



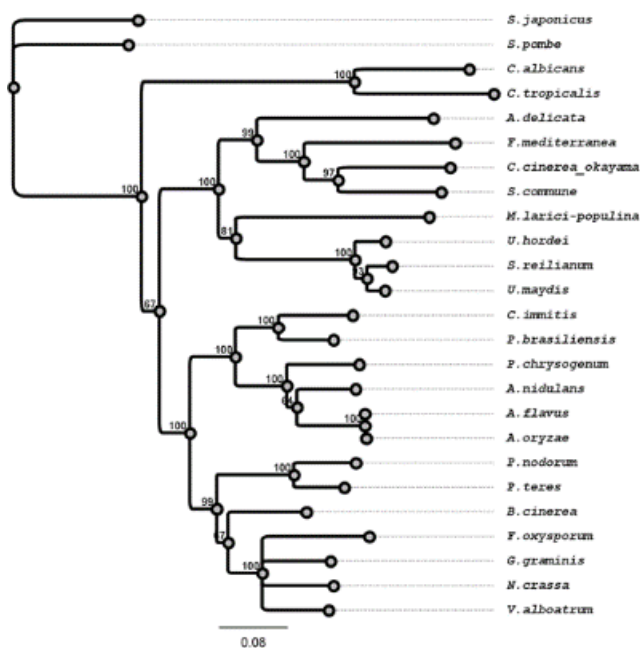
S5 Fig. Phylogenetic analysis of TAM1 from various fungi. n=26

S6 Fig.



S6 Fig. Phylogenetic analysis of IPD from various fungi. n=31

S7 Fig.



S7 Fig. Phylogenetic analysis of IAD1 from various fungi. n=25

S8 Fig.



S8 Fig. Phylogenetic analysis of YUC from various fungi. n=33

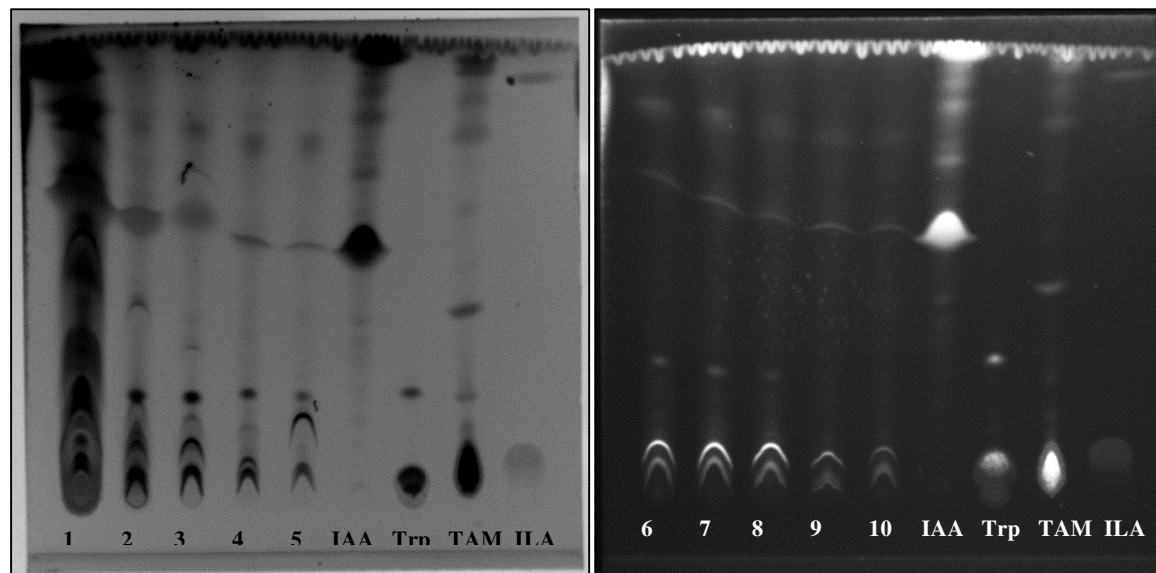
S9 Fig.



S9 Fig. Phylogenetic analysis of “auxin efflux carrier superfamily” in *Neurospora crassa* and its orthologs in different fungal species. n=40

S10 Fig.

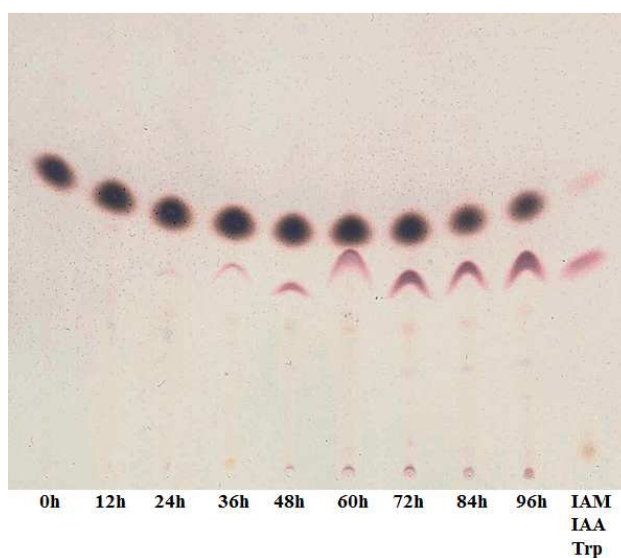
Feeding test using tryptophan at higher concentrations:



Lane No.	Concentration	Total volume	Strain
1.	2 mM	50 ml	Wt
2.	1 mM	50 ml	Wt
3.	0.5 mM	50 ml	Wt
4.	0.25 mM	50 ml	Wt
5.	125 μ M	50 ml	Wt
6.	125 μ M	50 ml	Wt
7.	100 μ M	50 ml	Wt
8.	50 μ M	50 ml	Wt
9.	25 μ M	50 ml	Wt
10.	12.5 μ M	50 ml	Wt

S10 Fig. Indole production by *Neurospora crassa* at different time points with 1 mM indole-3-acetamide (IAM) supplementation into the culture medium. Standard markers IAM, IAA and Trp are spotted on the TLC plate top to bottom respectively.

S11 Fig.



S11 Fig. Indole production by *Neurospora crassa* at different time points with 1 mM indole-3-acetamide (IAM) supplementation into the culture medium. Standard markers IAM, IAA and Trp are spotted on the TLC plate top to bottom respectively.

S12 Fig.



S12 Fig. Effect of histidine supplementation on *N. crassa* in indole production. Fungal culture was supplemented with 2 mM histidine. 1. Fungal culture was supplemented with 1.96 mM tryptophan. 2. Fungal culture was supplemented with 1 mM tryptophan. 3. Fungal culture was supplemented with 2 mM histidine. IAA: indole-3-acetic acid standard.

