

A
DISSERTATION
ON
**Cloning and characterization of dicer like protein
from the ectomycorrhizal fungus *Laccaria bicolor***

Submitted in partial fulfillment of the requirements
for the award of the degree of

MASTER OF TECHNOLOGY
IN
BIOTECHNOLOGY

Submitted
By

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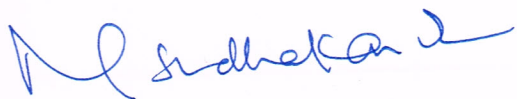
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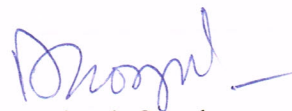
CERTIFICATE

This is to certify that the dissertation entitled, "**Cloning and characterization of dicer like protein from the ectomycorrhizal fungus *Laccaria bicolor***" submitted by Alisha Ishrat in partial fulfillment of the requirement for the award degree of Master of Technology in Biotechnology, Department of Biotechnology, Thapar University, Patiala is an authentic record of student's own work carried out by her under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university.



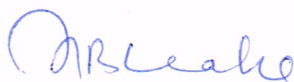
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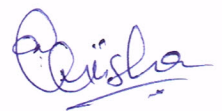


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DECLARATION

I hereby declare that the work being presented in the dissertation entitled "**Cloning and characterization of dicer like protein from the ectomycorrhizal fungus *Laccaria bicolor***" in partial fulfillment of the requirement for the award degree of Master of Technology in Biotechnology, Department of Biotechnology, Thapar University, Patiala, is true and original record of my own independent and original research work carried out during the period of one year from July 2014 to July 2015 under the guidance of Dr. M.S. Reddy, Professor, Thapar University, Patiala. I have not submitted the matter embodied in this dissertation for the award of any other degree or diploma.

I have honored the principles of academic integrity and have upheld the moral student code of academic conduct in the completion of this work.



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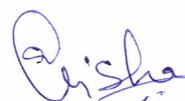
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Alisha Ishrat

ABSTRACT

Laccaria bicolor which is an ectomycorrhizal fungus was used for the present study and the aim was to clone and characterize dicer like protein from it.

The sequence of dicer gene was obtained from the *Laccaria bicolor* genome database. Various bioinformatics' tools were utilized for the analysis of conserved regions, domains throughout the history of evolution. Phylogenetic trees were constructed and their analysis proved that the dicer like proteins are present in various species of plants, mammals, insects and fungi.

Laccaria bicolor was grown on MMN media at 25°C. RNA isolation from the fungus was done and the cDNA was prepared. Primers designed for the gene were used for amplification. The expression vector pET23a was used for the study, it was digested with respective enzymes whose sites were introduced in the primers. The gene was also digested with the same enzymes and further ligated with pET23a in order to proceed with transformation experiments where this recombinant plasmid was put into the *E.coli* DH5a cells and further into the *E.coli* BL21De3 cells. Total protein isolation was done to confirm that protein production takes place via IPTG induction.

Laccaria bicolor was grown on various nitrogen sources, providing stress and the growth of the fungus was monitored, both in terms of diameter and dry weight. Different concentrations of carbon source (glucose) were also used to monitor the growth pattern of the fungus.

Expression of dicer gene in the presence of different glucose concentrations was tested and the level of expression variation was monitored.

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CHAPTER 1

INTRODUCTION

1.1 Mycorrhiza

Mycorrhiza is an association or symbiosis between plants and fungi that colonize the cortical tissue of roots during periods of active plant growth. The term “mycorrhiza”, which literally means “fungus root”, describes a mutualistic relationship between fungi and plant roots. It was introduced by Frank (1885) to describe the long-lived association between plant roots and fungal mycelium.

Mycorrhizal fungi play important roles in nature:

- They may conserve nutrients against leaching by immobilizing elements in tissue (Trappe and Fogel, 1977)
- They help in the uptake of phosphorus by plants (Bolan, 1991)
- They assist the plants in developing resistance against drought (Lehto, 1992)
- They have a protective role for plants present in soils with high metal concentration (Bellion et al., 2006)
- They also play a role in increasing the resistance against the soil parasites.

All of the major taxonomic groups of fungi (Ascomycotina, Basidiomycotina and Zygomycotina) tend to form mycorrhizas. The mycorrhizas have been characterized into various types. This classification is mainly based on fungal associates and structural characteristics of mycorrhizas at the time of maturity (Isaac, 1992). The most ancient and well studied mycorrhizal class is arbuscular mycorrhiza (Smith and Read, 1997)

The most common associations are the arbuscular endomycorrhizas (AM) formed by Zygomycete fungi and the ectomycorrhizas (ECM) formed by Basidiomycetes, Ascomycetes, and a few Zygomycetes. Some other mycorrhizal associations include the orchid, ericoid, arbutoid, monotropoid and ectendo- mycorrhizas (Peterson et al., 2004)

1.1.1 Endomycorrhizas

The endomycorrhizae penetrate cortical cells of most plants with two main types of structure. The vesicles are mainly for storage and the arbuscules are thought to be mainly for nutrient exchange. The fungus receives energy from the plant, and the plant receives minerals (especially Phosphorus) and water from the fungus. Reproductive spores can be formed either in the root or more commonly in the soil (Smith and Read, 1997). Spores produced by fungi forming endomycorrhizal associations are asexual, forming by the differentiation of vegetative hyphae.

1.1.1.1 Arbuscular mycorrhiza

The AM fungi are represented by more than 150 species of the Zygomycota (Morton and Benny, 1990). Work on the phylogeny of AM fungi provided a basis for a new systematics removing these fungi from the polyphyletic Zygomycota and then placing them into a new monophyletic phylum, the Glomeromycota. The AM fungi form intercellular hyphae when they grow into the root cortex and from these hyphae highly branched structures (arbuscules), originate within cortex cells (Fig.1). There are many roles of arbuscules like they are responsible for nutrient exchange between the host and the symbiont, help in transporting carbohydrates from the plant to the fungus and mineral nutrients, especially phosphate, and water from the fungus to the plant (Strack et al., 2003)

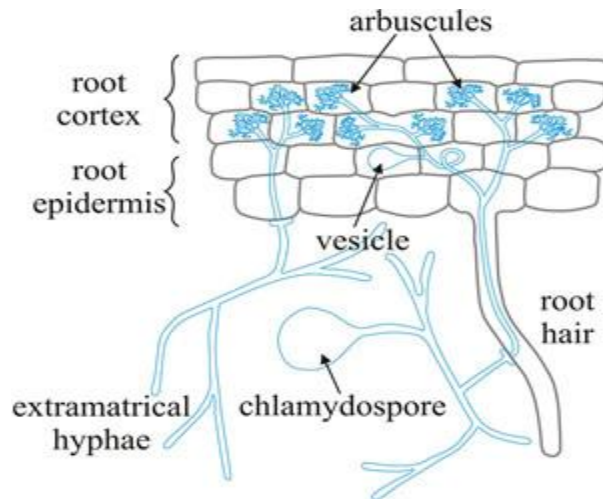


Figure 1: Diagrammatic representation of the main cellular feature of the arbuscular endomycorrhiza (www.davidmoore.org.uk)

1.1.2 Ectomycorrhiza

Ectomycorrhizas (ECMs), the major mycorrhizal symbiosis in boreal, temperate and few tropical forests, are actually formed by 5000-6000 species of ascomycetes and basidiomycetes (Buscot et al., 2000) and hosts that can be either gymnosperms or woody angiosperms (Smith and Read, 1997). Fossil records and molecular clock dating indicate that ECMs evolved around 200 million years ago (Cairney, 2000). The hyphae are intercellular and they form a dense sheath (mantle) around lateral roots. Individual hyphae called rhizomorphs grow out into the soil from this mantle. The mantle varies in thickness, texture, and depending on the respective plant-fungus combination. It increases the surface area of absorbing roots, resulting in root bifurcation and clustering. A very loose network is formed around the root at first but after this; hyphae from the inner zone of the mantle penetrate deep in between the epidermal cells of the root and extend between the outer cortical cells thus forming a network which is called the Hartig net, where nutrients are exchanged (Fig.2). Ectomycorrhizal fungi can influence the host plant in numerous ways:

- It was demonstrated that ectomycorrhizal fungi improve plant nutrition by increasing nitrogen, phosphate and potassium uptake (Harley and Smith, 1983)

- Nutrient exchange between fungus and plant takes place using cell-to-cell contact in ectomycorrhizas, especially in the Hartig net. Ectomycorrhizal root tip, increase the plants access to soil water resources manifold
- Ectomycorrhizas protect host plant roots against root pathogens and also against root herbivorous soil microfauna (Smith and Read, 1997)

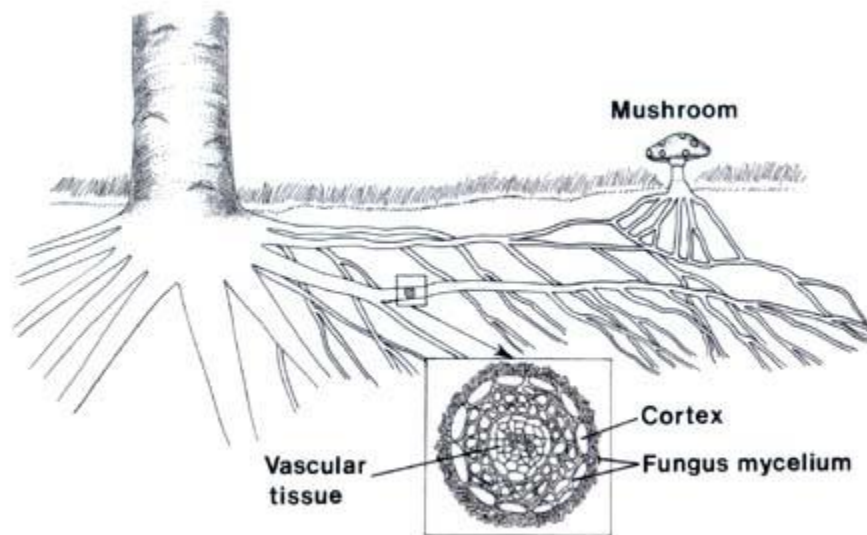


Figure 2: Ectomycorrhizal association pictorial representation (www.apsnet.org)

1.1.2.1 *Laccaria bicolor*: Taxonomy, distribution and host range

Laccaria bicolor is basically a small tan-colored mushroom (Fig.3). It is reported to be eatable, but not palatable, and grows in woods (birch and pine). It is an ectomycorrhizal fungus and can be easily utilized as a soil inoculant. It was described earlier as a subspecies of *Laccaria laccata* by French mycologist Ren   Maire in 1937, after which it was raised to a species rank by Orton PD (Orton, 1960). Its cap is 2–4.5 cm (0.8–1.8 in) across, convex to flat having a central navel. It is mostly incurved at the margin, and is various shades of the color tan, which depends on moisture content. The fibrillose stipe is also tan with a distinct lilac color towards the base. The flesh is white with a slight pink tinge and has no distinctive smell, or taste.

It is found mainly in the temperate zones, in the late summer and autumn (Phillips, 2006) which includes forests of North America and probably Northern Europe (temperate and boreal)

(Mueller et al., 1991). *Laccaria bicolor* is a carnivorous fungus and catches and kills insects, specifically springtails (Klironomos et al., 2001)

It was the first ectomycorrhizal fungus whose genome was sequenced (Martin et al., 2008) which is 65 megabases long and estimated to contain about 20,000 protein coding genes. Further analysis showed a great number of small secreted proteins of unknown function, many of which are only expressed in symbiotic tissues where they initiate symbiosis. It does not have enzymes which degrade plant cell walls but does have enzymes which degrade other polysaccharides, proving how it is able to grow both in soil and also in association with plants (Martin et al., 2008)



Figure 3: Fruiting body of the ectomycorrhizal basidiomycete *Laccaria bicolor* S238N interacting with Douglas fir seedlings (© D Vairelles INRA)

1.2 Dicer

1.2.1 History

Dicer was given its name by Emily Bernstein in 2001- a graduate student in Greg Hannon's lab at Cold Spring Harbor Laboratory, who was working to discover the enzyme responsible for generating small RNA fragments from double-stranded RNA. Dicer had an ability to generate ~22 nucleotide RNA fragments and this was discovered by separating it from the RISC (RNA induced silencing complex) enzyme complex after initiating the RNAi (RNA interference)

pathway with dsRNA transfection. This experiment proved that RISC was actually not responsible for forming the small nucleotide fragments. Further experiments testing the ability of RNase III family enzymes to create RNA fragments brought down the search to *Drosophila* CG4792, which is now called as “Dicer” (Bernstein et al., 2001)

1.2.2 Structural and functional aspects

Human dicer's (also known as hsDicer or DICER1) classification as a Ribonuclease III is because it contains both helicase and PAZ (Piwi/Argonaute/Zwille) domains (Matsuda et al., 2000). hsDicer contains four other functional domains: two RNase III domains and two double stranded RNA binding domains (DUF283 and dsRBD) (Lau et al., 2009)

Current research indicates that PAZ domain binds the 2 nucleotide 3' overhang of dsRNA and the RNase III catalytic domains form a pseudo-dimer around the dsRNA to begin cleavage of the strands. This ends up in a functional shortening of the dsRNA strand. The dsRBD domain binds the dsRNA, but the specific binding site of the domain is not defined. It is quite possible that this domain works as part of a complex with different regulator proteins (TRBP in humans, R2D2, Loqs in *Drosophila*) to position the RNase III domains and control the specificity of the sRNA products (Cenik et al., 2011)

The first Dicer to be found, in terms of crystal structure, was from the protozoan *Giardia intestinalis* (Fig.4). By means of X-ray crystallography, a PAZ domain and two RNase III domains were discovered.

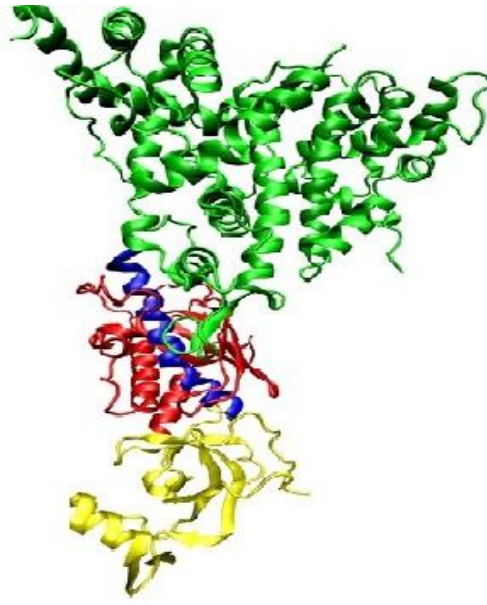


Figure 4: One molecule of the Dicer protein from *Giardia intestinalis*, which catalyzes the cleavage of dsRNA to siRNAs. The RNase III domains are colored green, the PAZ domain yellow, the platform domain red, and the connector helix blue (Macrae et al., 2006)

1.2.3 Roles of dicer

1.2.3.1 RNA Interference

Micro RNA

A process where the breakdown of RNA molecules into miRNA leads to the inhibition of the gene expression of specific mRNA sequences is called RNA interference. Beginning from primary miRNA (pri-miRNA) in the nucleus, miRNA is produced within the cell. These sequences are then cleaved into smaller precursor miRNA (pre-miRNA), which are usually 70 nucleotides with a hairpin structure. Pri-miRNAs are cleaved by Drosha to generate the pre-miRNA. Dicer cleaves these pre-miRNA to form mature miRNA (Merritt et al., 2010)

Small Interfering RNA

Small interfering RNA (siRNA) function in a similar manner to miRNA. RNA-induced silencing complex (RISC) is activated by both miRNAs and siRNAs, which finds the complementary

target mRNA sequence and cleaves using RNase (Vermeulen et al., 2005). This then silences the particular gene by RNA interference (Watson, 2008)

1.2.3.2 Diagnostic and therapeutic applications

- Dicer's role in macular degeneration became clear after it was discovered that affected patients showed decreased levels of Dicer in their Retinal Pigment Epithelium (RPE). However, with insufficient Dicer levels, the accumulation of Alu RNA leads to the degeneration of RPE as a result of inflammation (Meister, 2011; Tarallo et al., 2012)
- Altered miRNA expression profiles in malignant cancers indicate a major role of miRNA and thus dicer in cancer development and prognosis. miRNAs can function as tumor suppressors and thus their altered expression might result in tumorigenesis (Tang and Ren, 2012)
- RNAi mechanisms are also responsible for transposon silencing and in their absence, as in, when Dicer is knocked out/down, can lead to activated transposons that cause DNA damage. Accumulation of DNA damage might result in cells with oncogenic mutations and hence the development of a tumor (Merritt et al., 2010)
- Insects might use Dicer as an antiviral. This finding is important because mosquitoes are responsible for the transmission of many viral diseases including the potentially deadly arboviruses: West Nile virus, dengue fever and yellow fever (NCID)
- Dicer can be used for treating patients by injecting foreign siRNA intravenously to cause gene silencing (Kamlah et al., 2009)

In the present study, dicer like protein was cloned and characterized from the ectomycorrhizal fungus *Laccaria bicolor*.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Mycorrhiza and its importance

It has been observed that mycorrhizal fungi provide linkages among plants that affect plant community development and resiliency. Many fungi are able to form mycorrhizas with varied plant species, including across plant families (Rajak, 2004)

Many mycorrhizal fungi can utilize inorganic forms of Nitrogen (Chalot and Brun, 1998) and Phosphorus (Koide and Kabir, 2000)

Few litter-inhabiting ectomycorrhizal fungi produce proteases and are able to distribute soluble amino compounds via hyphal networks into the root (Read et al., 1989)

There have been many claims that mycorrhizal plants are far more resistant to disease from pathogenic fungi and bacteria than the non mycorrhizal plants. Particularly, mycorrhizal fungi have been reported to provide protection against root invading primary pathogens (Schonbeck, 1979; Duchesne et al., 1989)

2.1.1 Ectomycorrhiza and its roles

It has been observed that some ectomycorrhizal fungi produce oxalic acid which dissolves sparingly soluble calcium phosphates. Iron uptake also might be increased by the release of siderophores (Marschner, 1995)

Ectomycorrhizal tree seedlings have been reported to often resist drought in a better way than non-mycorrhizal seedlings (Bowen, 1973)

Ectomycorrhizal fungi, with their large mycelia strands might increase water flow by filling the gap between the soil and the root (Lamhamedi et al., 1992)

2.2 Quelling

Quelling is defined to be a gene silencing phenomenon which was first described in *Neurospora crassa* in 1992 (Romano and Macino, 1992). The genetic and biochemical features of quelling point towards the fact that quelling belongs to the broad category of RNA-mediated gene silencing mechanisms which includes post-transcriptional gene silencing (PTGS) or co-suppression in plants and RNA interference (RNAi) in animals.

Quelling takes place during the vegetative phase of growth, it has an effect on both transgenes and endogenous genes. It is said to be dominant in heterokaryons with nuclei from “quelled” and wild-type strains, and this indicated that a mobile signal, e.g., RNA, acts in trans mode to cause this silencing phenomenon (Cogoni et al., 1996)

2.2.1 Dicer like proteins and quelling

It was reported that in *Neurospora crassa*, the two dicer-like proteins, DCL-2 and SMS-3 (DCL-1) were redundantly involved in the quelling pathway (Catalanotto et al., 2004). Paralogs of dicer protein, Dcr-1 and Dcr-2, PPD protein, AGO-1 and AGO-2 have been identified in the *Drosophila* genome. *Drosophila* Dcr-1 and Dcr-2 mutants, were characterized and it was shown that mutation in Dcr-1 blocks the processing of miRNA precursors whereas Dcr-2 mutants were found to be defective for processing siRNA precursors (Lee et al., 2004). AGO-1 is dispensable for siRNA-directed target RNA cleavage but is quite essential for mature miRNA production. The miRNA pathway is thus carried out by one set of silencing protein components (Dcr-1 and AGO-1), while another set of proteins (including Dcr-2 and AGO-2) is responsible for the siRNA pathway. In plants, as well, distinct dicer proteins in siRNA and miRNA biogenesis are involved (Xie et al., 2004)

In the model filamentous fungus *Neurospora crassa*, different sets of silencing protein components may also be responsible for two different types of RNA silencing: quelling and meiotic silencing by unpaired DNA (MSUD) (Galagan et al., 2003). A series of studies has indicated that quelling in *Neurospora* is involved in the siRNA silencing pathway in higher eukaryotes (Cogoni and Macino, 2000)

2.3 RNA silencing in different organisms

RNA silencing has been reported in all the four eukaryotic kingdoms—Animalia (Fire et al. 1998), Plantae (Van der Krol et al., 1990), Fungi (Romano and Macino, 1992), and Protista (Ngo et al., 1998)—but not in the prokaryotic kingdom Monera. Therefore, the RNA silencing machinery may have emerged before the divergence of the major eukaryotic lineages.

2.3.1 Gene silencing in fungi

The suppression of gene expression by a dsRNA-expressing plasmid or related-system has been reported in many fungal species including Ascomycota, Basidiomycota, and Zygomycota (Fitzgerald et al., 2004; Hammond et al., 2005; Kadotani et al., 2003; Liu et al., 2002; Mouyna et al., 2004; Nicolas et al., 2003; Rappleye et al., 2004), and also in fungus-like Oomycota (Latijnhouwers et al., 2004)

Involvement of RNA silencing protein components such as **dicer** in the silencing phenomenon was shown in *Aspergillus nidulans*, *Magnaporthe oryzae*, *Neurospora crassa*, and *Schizosaccharomyces pombe* (Cogoni et al., 1999; Catalanotto et al., 2004; Kadotani et al., 2004; Hammond et al., 2005; Volpe et al., 2002), and siRNA biogenesis was detected in *Aspergillus nidulans*, *Magnaporthe oryzae*, *Mucor circinelloides*, *Neurospora crassa* and *Schizosaccharomyces pombe* (Hammond et al., 2005; Kadotani et al., 2003; Nicolas et al., 2003; Volpe et al., 2002; Catalanotto et al., 2002)

There were searches for the typical RNA silencing components in the public fungal genome databases which led to the identification of RdRP, PPD- and dicer-like proteins in various fungi belonging to Ascomycota, Basidiomycota and Zygomycota but was not successful in a subset of fungi like the ascomycetes *Saccharomyces cerevisiae* (budding yeast), *Candida guilliermondii*, and *Candida lusitanae*, and the basidiomycete *Ustilago maydis* (Nakayashiki et al., 2005)

Even *Candida albicans* and *Candida tropicalis* apparently lack dicer- and RdRP proteins in their genomes. Thus, the RNA silencing machinery might have been lost in a portion of fungal species during evolution.

2.3.1.1 Ancient origin of multiple silencing pathways in fungi

Phylogenetic analysis of the RdRP-, Argonaute-, and Dicer-like proteins was performed using fungal species including six ascomycetes (*Aspergillus nidulans*, *Fusarium graminearum*, *Magnaporthe oryzae*, *Neurospora crassa*, *Saccharomyces pombe*, and *Stagonospora nodorum*), three basidiomycetes (*Coprinus cinereus*, *Cryptococcus neoformans*, and *Phanerochaete chrysosporium*), and a zygomycete (*Rhizopus oryzae*). The results indicated that the *Arabidopsis* and *Drosophila* proteins clustered separately from the fungal proteins, which showed that the RNA silencing proteins have been duplicated to establish a new pathway after the divergence of fungi from animals and plants (Nakayashiki et al., 2006) (Fig.5)

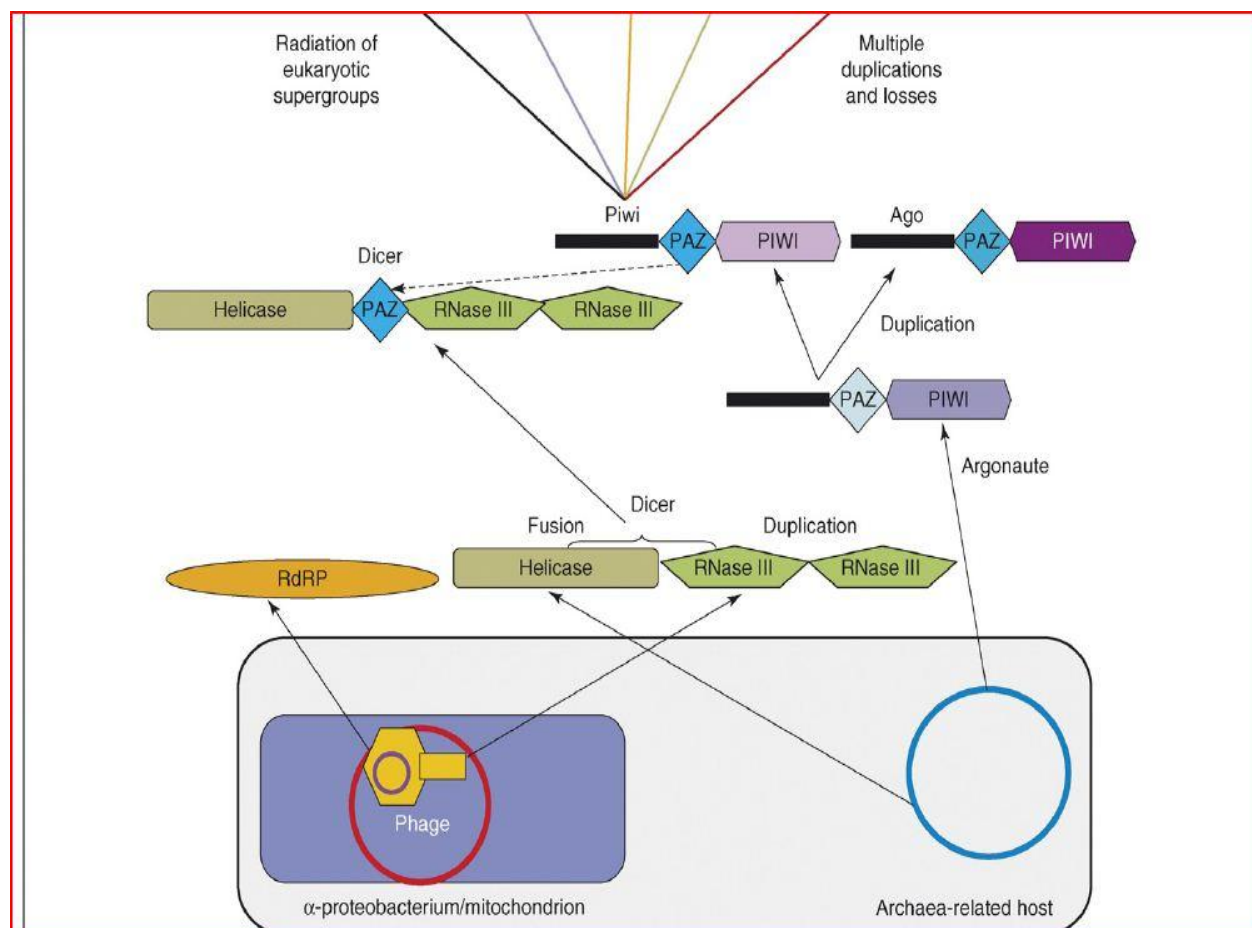


Figure 5: A scenario for the origin and earliest stages of evolution of eukaryotic RNAi protein machinery (Svetlana et al., 2008)

2.4 Small RNAs

The classification of small RNAs is: small interfering RNAs (siRNAs), microRNAs (miRNAs), PIWI-interacting RNAs (piRNAs) and primal small RNAs (priRNAs), and all of this depends on their biogenesis pathways, precursor structures and modes of action (Ghildiyal and Zamore, 2009; Jin and Zhu, 2010). *Neurospora crassa* and various other fungi form small RNAs through at least six different pathways (Li et al., 2010). These small RNAs are proposed to be loaded onto the AGOs or PIWIs (AGO family of effector proteins) to help in a specific regulatory succession which is dependent primarily on sequence homology, e.g. RNA degradation, translational inhibition, or chromatin modification of target transcript RNA or DNA (Jin and Zhu, 2010)

Plant endogenous small interfering RNAs (siRNAs) have been reported as three subcategories: heterochromatic siRNAs (hc-siRNAs), natural antisense transcript-derived siRNAs (nat-siRNAs), and trans-acting siRNAs (ta-siRNAs; TAS).

2.4.1 Roles of sRNA

Small RNAs (sRNAs) are supposed to have key roles in eukaryotic RNA interference phenomena (Baulcombe, 2004). sRNAs are reported to be preferentially sorted into one type of ARGONAUTE (AGO) protein to form RNA-induced silencing complexes (RISCs) and sequence-pairing transcriptionally or post transcriptionally silences transcripts via epigenetic regulation, mRNA destabilization, and translation inhibition (Hutvagner and Simard, 2008; Ghildiyal and Zamore, 2009). Plant microRNAs (miRNAs) are supposed to be transcribed by RNA polymerase II and processed by DICER-LIKE1 (DCL1) protein in the nucleus, then getting associated with AGO1 based on their 5'-terminal uridine nucleotide to form RISCs in cytoplasm and hence perform target mRNA cleavage or translation repression (Voinnet, 2009)

There have been many evidences that demonstrated that small RNAs such as microRNAs (miRNAs) and small interfering RNAs (siRNAs) in eukaryotic organisms have very important roles in developmental regulation (Kidner and Martienssen, 2005), epigenetic modifications (Vaucheret, 2006), tumorigenesis (Murakami et al., 2006), and biotic and abiotic stress responses

(Llave, 2004). There are different types of precursors that lead to the production of non-coding RNAs, miRNAs and siRNAs (Millar and Waterhouse, 2005; Großhans and Filipowicz, 2008)

2.5 RNA interference

The RNAi pathway has been found to be involved in transposon silencing in higher eukaryotes, and that might as well be the same in fungi (Nolan et al., 2005; Murata et al., 2007)

2.5.1 Difference between siRNA and miRNA mediated pathway

There are two major differences between miRNA- and siRNA-mediated systems:

- (i) miRNAs are endogenous RNA molecules that are encoded by their own genes; by contrast, there are no dedicated genes for siRNAs. siRNAs are generated by degradation of exogenous (e.g. viral) double-stranded (ds)RNAs or they are transcribed from transposable elements integrated in the genome or any other types of inverted repeats.
- (ii) siRNAs are completely complementary to their targets, whereas miRNAs show limited complementarity to their recognition sites.

2.5.2 miRNA mediated pathway

Drosha and Dicer, both have been established to be important for the biogenesis of most miRNAs. Biogenesis of miRNA begins with the transcription of a miRNA gene, by RNA polymerase II, generating a long primary (pri)-miRNA transcript which has a short internal stem-loop structure (Kirigin et al., 2012; Lee et al., 2003). This is further recognized by Drosha as a part of a microprocessor complex (Filippov et al., 2000; Gregory et al., 2004). Drosha cleaves the pri-miRNA at the base of the stem-loop, guided by the dsRNA-binding protein Dgcr8 (Denli et al., 2004), thus releasing it from the flanking single-stranded regions (Lee et al., 2002; Zeng et al., 2005). This released stem-loop structure (pre-miRNA) is then exported from the nucleus by Exportin 5 (Lund et al., 2004; Bohnsack et al., 2004; Yi et al., 2003; Zeng et al., 2004). In the cytoplasm the pre-miRNA is cleaved by Dicer, along with another dsRNA-binding protein, Trbp (Bernstein et al., 2001; Chendrimada et al., 2005). The PAZ domain binds the basal portion of the dspre-miRNA, and guides the stem into a cleft which is formed by the dimerization of two RNase III domains (Macrae et al., 2006). Breakage of the RNA removes the loop structure, which leaves a miRNA duplex. The distance between the PAZ domain and the RNase III domain

dimer tells about the length of the RNA product, approximately 22 nt. for miRNAs (Lau et al., 2012). One strand of the miRNA duplex binds with the RNA-induced silencing complex (RISC), which it guides to target mRNAs. Argonaute proteins within the RISC help in this targeting which leads to translational repression and degradation of target mRNAs.

2.5.3 siRNA mediated pathway

Exogenous siRNAs focus on complementary mRNAs for cleavage of transcript and degradation in a process called as post-transcriptional gene silencing (PTGS) (Zamore et al., 2000)

siRNAs have other functions as well like initiation and maintenance of silent chromatin upon recruitment of histone modifying enzymes to the targeted loci (Verdel et al., 2004; Volpe et al., 2002). Effective PTGS results in cleavage of the mRNA by RNA induced silencing complex (RISC) (Martinez et al., 2002). The endonuclease Argonaute 2 (AGO2) is involved in the cleavage mechanism of RISC (Liu et al., 2004). RISC activation involves AGO2-mediated cleavage of the sense/ passenger strand of the double-stranded siRNA (Matranga et al., 2005; Rand et al., 2005), generating the single-stranded antisense strand which guides RISC to complementary sequences. This guide strand is bound in the catalytic RNase H-like PIWI domain of AGO2 at the 5' end (Parker et al., 2005) and a PIWI–Argonaute–Zwille (PAZ) domain recognizes the siRNA at the 3' end (Ma et al., 2004). Cleavage of targeted mRNA is between bases 10 and 11 corresponding to the 5' end of the siRNA guide strand (Elbashir et al., 2001) which leads to the degradation of the cleaved mRNA transcript by cellular exonucleases (Orban et al., 2005). RISC can undergo multiple rounds of mRNA cleavage upon activation by the siRNA guide strand, to demonstrate a good PTGS response against the target gene (Hutvagner et al., 2002). RNA silencing pathways in different organisms are different to each other (Fig.6)

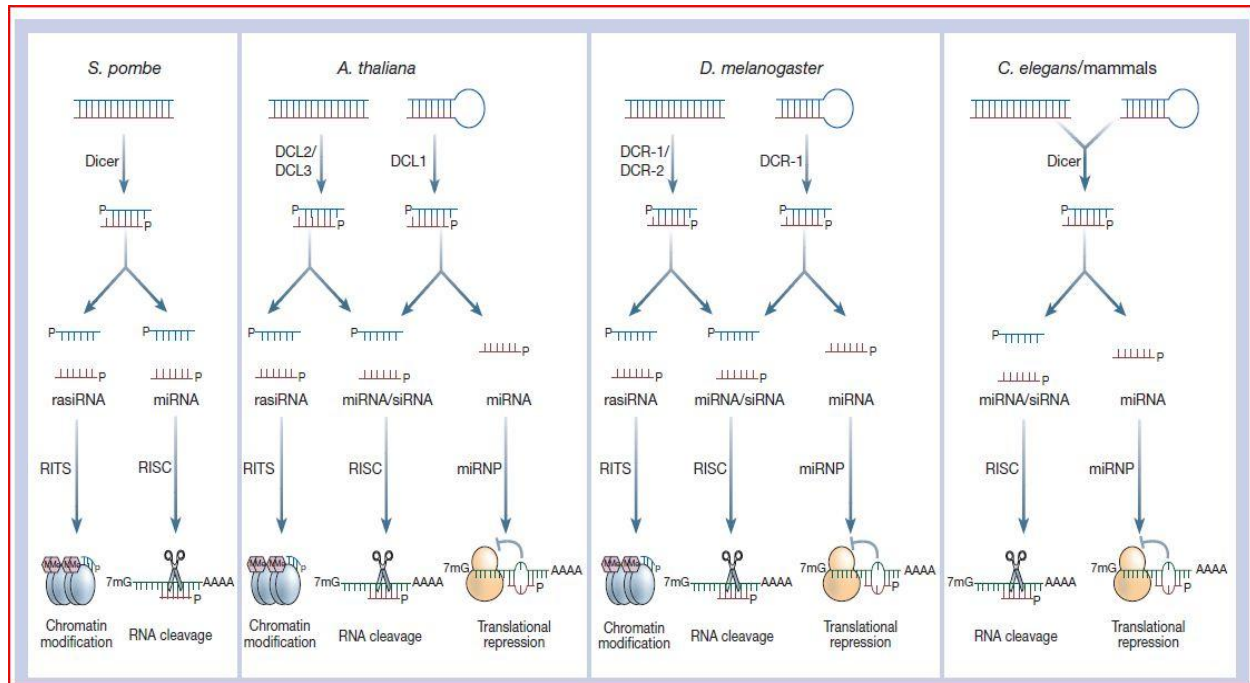


Figure 6: RNA silencing pathways in different organisms (Gunter and Thomas, 2004)

2.5.4 Regulators of RNAi

RdRPs help in amplifying the silencing effect by dsRNA formation of the target mRNAs and its cleavage products (Wassenegger et al., 1998; Sijen et al., 2001).

A homodimer of p19 (viral suppressor protein) binds to the sugar-phosphate backbone of RNA, and positions two α -helices on top of the terminal base pairs of the duplex. This binding permits the p19 protein to differentiate siRNA duplexes from shorter or longer dsRNAs.

The exonuclease domain-containing protein ERI-1 was discovered in genetic screens in *Caenorhabditis elegans* (Kennedy et al., 2004). ERI-1 is expressed in neurons and the loss-of function *eri-1* mutants demonstrate increased RNAi in neurons.

An RdRP-family protein RRF-3 (Simmer et al., 2002) is another negative regulator of RNAi in *C. elegans*. RRF-3 competes for templates/primers of RNA amplification thus inhibiting the production of additional short RNAs.

2.5.5 Applications of RNAi

2.5.5.1 Roles of RNAi in fungi

- **Transposon control in *Neurospora crassa*.** A LINE-1-like transposon, *Tad*, which was found in one African *Neurospora* isolate was able to transpose in *Neurospora* (Kinsey, 1990). By introducing *Tad* into *Neurospora crassa* strains, it was showed that the repression of its transposition requires QDE-2 and Dicer but not QDE-1 or QDE-3 (Nolan et al., 2005). These results suggest that *Tad* transposition may generate inverted repeats, from which dsRNA can be produced without QDE-1 and QDE-3.
- **Viral defense in *Cryphonectria parasitica* and *Aspergillus nidulans*.** The role of fungal RNAi in viral defense was established in the ascomycete filamentous fungus *C. parasitica* (the chestnut blight fungus). It was found that *Cryphonectria* hypovirus 1 (CHV1) alleviates the virulence of the fungal host by expression of a protease, p29. The expression of p29 suppresses RNA-induced silencing in *Cryphonectria parasitica* (Segers et al., 2006). *C. parasitica* has two Dicer-like genes, *dcl-1* and *dcl-2* and the *dcl-2* mutant shows impairment in growth after virus infection, suggesting that DCL-2 is involved in virus suppression (Segers et al., 2007)
Aspergillus nidulans was infected with mycoviruses and it was demonstrated that viral infection diminishes the efficiency of RNAi, suggesting that an RNAi suppressor is encoded by the virus (Hammond et al., 2008)

The application of gene silencing through RNAi has been noticed in over 30 species of filamentous fungi and fungus-like organisms, Oomycota (Kück and Hoff, 2010; Nakayashiki and Nguyen, 2008; Nakayashiki et al., 2005)

Three known methods have been said to trigger RNAi-mediated gene silencing in fungi: expression of a hairpin RNA, generation of dsRNA via convergent transcription from a transgene, and introduction of siRNA or dsRNA directly into the fungal cell (Khatri et al., 2007; Kim et al., 2009)

- **Gene silencing via expression of a long hairpin RNA.** The expression of a hairpin RNA which is specific for the gene of interest needs the creation of some plasmid constructs in which transcription of an inverted repeat (up to 1 kb long) is under the

control of a commonly used promoter (Cheng et al., 2005; Goldoni et al., 2004; Liu et al., 2002). The expression of the inverted repeats in cells confirms the formation of a long hairpin RNA that is recognized and processed by Dicer. This approach has been used in various fungal species (Kadotani et al., 2003; Li et al., 2010; Liu et al., 2002; Namekawa et al., 2005)

- **Dual promoters (convergent transcription).** Here, the gene of interest is flanked by two promoters that drive transcription in opposite directions. Hence, both the sense and antisense RNAs are transcribed at the same time, and dsRNA is formed (Ziv et al., 2010)

2.5.5.2 RNAi and its therapeutic roles

- **Viral diseases.** The therapeutic potential of RNAi has been highlighted in experiments showing the efficacy of this against HIV infection in cultured cells (Lee et al., 2002; Novina et al., 2002; Jacque et al., 2002; Coburn et al., 2002). Viral RNAs and replication intermediates have been quoted as potential targets for RNAi-based therapies, and most of the viral transcripts in cells infected with HIV have already been targeted using RNAi (Rossi et al., 2006)
- **Ocular diseases.** Direct intravitreal injections of siRNAs which targets vascular endothelial growth factor (VEGF) or its receptor (VEGFR1) have been given to test for their safety and efficacy in the eye (Kim and Rossi, 2007)
- **Neurodegenerative diseases.** Treatment of spinocerebellar ataxia type 1 (SCA1) which is a dominantly inherited disease that is part of a group of neurodegenerative disorders that includes Huntington disease; has progressed by delivery of RNAi into the mouse brain (Kim and Rossi, 2007)
- **Cancer.** Oncogenes which are expressed at very high levels are major targets for RNAi-based therapies against cancers (Pai et al., 2006), and these approaches have efficiently inhibited tumour growth *in vivo* in mouse models. A study involved liposomal delivery of siRNAs targeting the tyrosine kinase receptor *EphA2* gene, which tends to be overexpressed in ovarian cancer cells (Landen et al., 2005). Upto 50% reduction in tumour size was observed after biweekly delivery of siRNAs for 4 weeks. The miRNAs miR-15 and miR-16 have been identified in the tumour suppressor locus for *B-CLL* (B-cell chronic lymphocytic leukaemia) (Calin et al., 2002), and down-regulation of miR-

143 and miR-145 has been noticed in advancing colorectal neoplasia (Michael et al., 2003)

2.6 Ribonuclease III

RNase III is a double-stranded RNA (ds-RNA) endonuclease found in bacteria and eukaryotic cells. It was reported to be involved in the processing of a large number of RNA substrates, including precursors of rRNA (Ivakine et al., 2003; Kufel et al., 1999; Wu et al., 2000), snoRNA (Chanfreau et al., 1998; Chanfreau et al., 1998; Ghazal et al., 2005; Giorgi et al., 2001), snRNA (Abou et al., 1998; Chanfreau et al., 1997), mRNA decay (Danin-Kreiselman et al., 2003; Ge et al., 2005) and RNA interference (MacRae et al., 2007)

All members of the RNase III family were reported to contain a ribonuclease domain, which has a highly conserved stretch of nine amino acid residues known as the RNaseIII signature motif. RNase III proteins vary in length, from 200 to 2000 amino acids (Filippov et al., 2000; Nicholson et al., 1999) and have been divided into four classes based on domain composition.

It was reported that Class I is the simplest and the smallest, containing a single ribonuclease domain and a dsRNA binding domain (dsRBD); the bacterial and bacteriophage RNase III (Nicholson et al., 1999) belong to this class.

Class II was identified by the presence of a highly variable N-terminal domain extension and includes the *Saccharomyces cerevisiae* Rnt1 (Allmang et al., 1998) and *Schizosaccharomyces pombe* Pac1 (Rotondo et al., 1996) proteins.

Class III, including Drosha proteins (Filippov et al., 2000), has a dsRBD and two ribonuclease domains.

Class IV, also referred to as Dicer, is the largest and contains two ribonuclease domains, a dsRBD, an N-terminal helicase and a PAZ domain (MacRae et al., 2007; Filippov et al., 2000; Nicholson et al., 1999). In eukaryotic cells, class II and III enzymes are expected in the nucleus and participate in processing of snoRNA and rRNA precursors (Filippov et al., 2000). In addition, it has been shown that nuclear and nucleolar localization of Rnt1 (Catala et al., 2004) and human RNase III (Wu et al., 2000) are cell-cycle regulated. Although RNase III class II has also been implicated in snRNA (Abou et al., 1998; Chanfreau et al., 1997) and mRNA (Danin-

Kreiselman et al., 2003) cleavages, it is not clear where these processes take place. Class IV enzymes are found in the nucleus (Hiraguri et al., 2005; Pontes et al., 2006) and endoplasmic reticulum (Provost et al., 2002) and induce gene silencing. There are different substrates on which dicer can act (Fig.7)

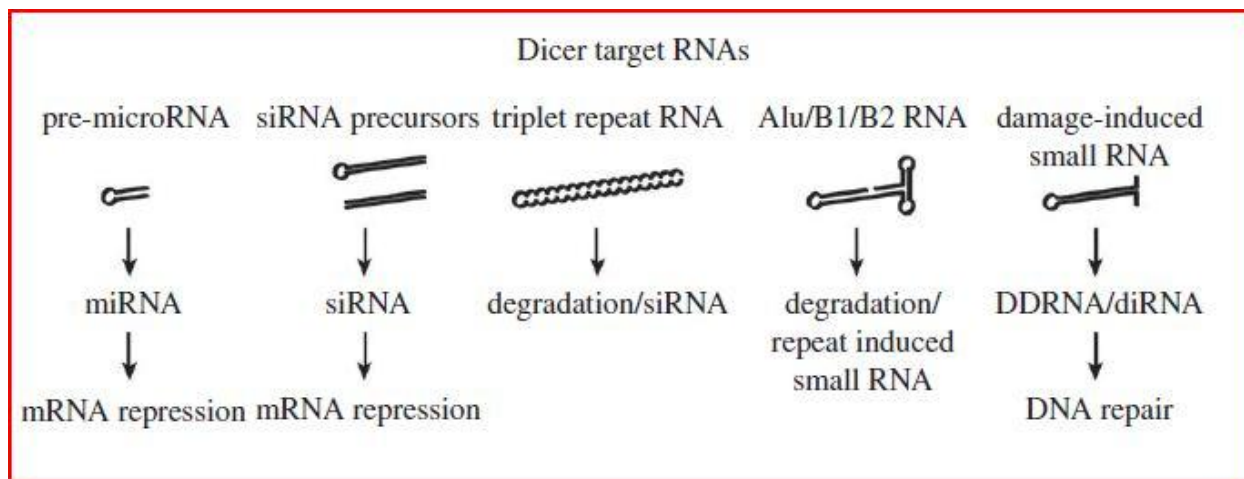


Figure 7: dsRNA substrates of Dicer (Johanson et al., 2013)

2.6.1 Dicer

Most Dicer enzymes have been reported to contain an N-terminal helicase/ATPase domain, and the cleavage activity of some dicers is enhanced or induced by ATP (Bernstein et al., 2001; Hutvagner et al., 2001; Nykanen et al., 2001). *Giardia* Dicer is devoid of an ATPase domain. In vitro, human Dicer is capable of generating siRNA in an ATP- and ATPase domain-independent way (Macrae et al., 2006; Zhang et al., 2004). The dsRNA cleavage activity of human Dicer has been found to be dependent on the PAZ domain and a C-terminally located dsRNA-binding motif (Zhang et al., 2004)

It has been proven that Dicer is capable of multiple-turnover catalysis and that it also behaves as a classic Michaelis–Menten enzyme (Ma et al., 2008). The DExD/ H-box domain was found to inhibit catalysis and it did not affect RNA binding affinity.

2.6.1.1 Domains

Dicer and DCL proteins are proposed to be large multi-domain ribonucleases. Vertebrate, insect, and plant Dicer and DCL proteins have been found to generally contain six types of domains including DEAD box, helicase-C, DUF283, PAZ, RNase III, and dsRBD (Margis et al., 2006). In lower eukaryotes, one or more of these domains might be absent. The PAZ, RNase III, and dsRBD domains are supposed to function in dsRNA binding and cleavage. The PAZ domain of Dicer is directly connected to the RNase IIIa domain by an α helix and can specifically bind the end of dsRNA containing a 3' two base overhang (MacRae et al., 2006). In addition to that, the PAZ domain also plays an important role in binding single-stranded RNAs (Kini and Walton, 2007). It has been suggested that Dicer functions via intramolecular dimerization of its two RNase III domains (Zhang et al., 2004)

The dsRBD domain has been suggested to play a role in carrying out the processes of differentiating between different RNA substrates and the subsequent incorporation of effector complexes (Margis et al., 2006). In higher eukaryotes, the DUF283 domain has been proposed to be involved in siRNA/miRNA strand selection by recognizing the asymmetry in the RNA duplexes directly or by recruiting another dsRBD protein (Dlakić, 2006).

Human Dicer (219 kDa) is nearly three times larger than *Giardia* Dicer (82 kDa). The difference in molecular mass is accounted for by at least five additional protein domains found in most Dicer proteins. These include an N-terminal DExD helicase-like domain (Ma et al., 2008), a TRBP-binding domain (Lee et al., 2006), a putative dsRNA binding domain named DUF283 (Dlakić, 2006), an Ago-binding domain (Sasaki and Shimizu, 2007), and a C-terminal dsRNA binding domain (Provost et al., 2002) (Fig.8). These additional domains were reported to participate in dsRNA processing, regulate dicing activity, and act as molecular scaffolds for managing protein factors involved in the initiation of RNAi (Chendrimada et al., 2005; Gregory et al., 2005; Ma et al., 2008; Maniataki and Mourelatos, 2005; Soifer et al., 2008)

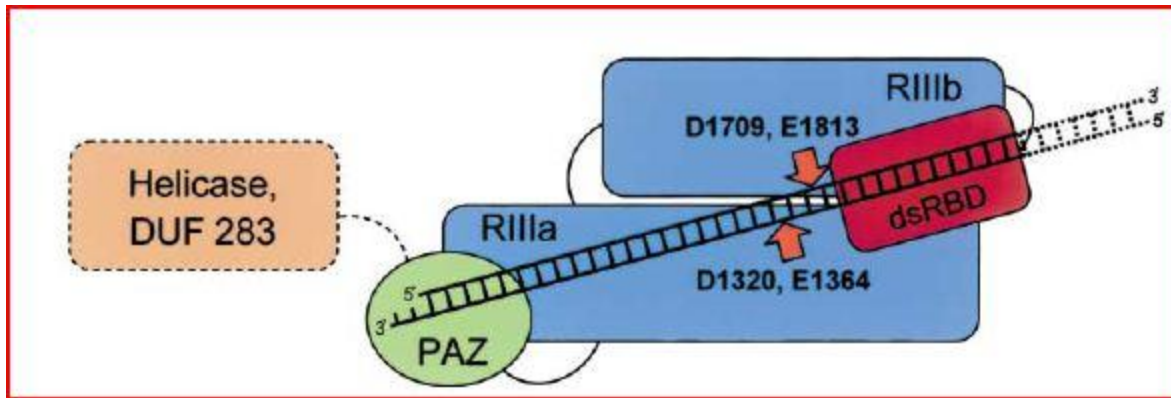


Figure 8: A model of dsRNA processing by Dicer (Zhang et al., 2004)

2.6.1.2 Roles of dicer

- Role of fungal dicer.** The fungal dicers seemed to have a role in more than defense against viruses and transposable elements. In the fission yeast *Schizosaccharomyces pombe*, RNA silencing components were reported to be involved in sequence specific heterochromatin formation through histone modifications (Verdel et al., 2004). RNA silencing dependent transcriptional gene silencing through sequence-specific DNA methylation, as well as histone modifications, was also reported in plants (Zaratiegui et al., 2007). Meiotic silencing by unpaired DNA (MSUD) was identified in *N. crassa* and is a unique RNA silencing pathway that appears to be active for a short period during the sexual phase from an early stage of meiosis after karyogamy till ascospore maturation (Aramayo and Metzenberg, 1996; Shiu et al., 2001)
- Role of dicer in lower eukaryotes.** Mutagenesis studies have indicated that Dicer is absolutely crucial to normal germline development for *Caenorhabditis elegans* (Knight and Bass, 2001) and maintaining the two types of stem cells (GSCs and SSCs) in the *Drosophila* ovary (Jin and Xie, 2007). Knockout of Dicer in mouse oocytes resulted in an inability to carry on through first meiotic division due to disorganized spindles and chromosome congression defects (Murchison et al., 2007). Dicer has also been found to play major roles in embryogenesis (Yang et al., 2005), lung epithelium morphogenesis (Harris et al., 2006), apoptosis (Matskevich and Moelling, 2008), and limb development (Harfe et al., 2005). In *Drosophila*, there are two Dicers and they have distinct but related

roles (Lee et al., 2004): Dicer-1 processes miRNA precursors, whereas Dicer-2 is required for processing siRNA precursors. Both Dicer-1 and Dicer-2 are imperative for siRNA-directed mRNA cleavage, and a role of Dicer to protect host against virus infection has also been established (Millar and Waterhouse, 2005). Flies with a loss-of-function of Dicer-2 were found to be more susceptible to infection by flock house virus than the wild type, thus proving the importance of Dicer-2 in virus defense (Galiana-Arnoux et al., 2006)

A recent report proved that the efficiency of DNA damage repair was greatly reduced in *Arabidopsis* with mutations in Dicer-like genes (*dcl2*, *dcl3*, and *dcl4*) and *ago2* alleles and that knockdown of *Dicer* and *Ago2* in human cells inhibited DNA damage repair (Wei et al., 2012)

- **Role of dicer in mammals.** In mammals, the absence of Dicer has been reported to lead to an increase of virus production and enhanced apoptosis of influenza A virus-infected cells (Matskevich and Moelling, 2007)

2.7 Dicer like proteins (DCL)

Dicer or Dicer-like (DCL) proteins are supposed to be major components in the miRNA and siRNA biogenesis pathways in processing long double stranded RNAs into mature small RNAs (Millar and Waterhouse, 2005; Chapman and Carrington, 2007; Großhans and Filipowicz, 2008). Dicer or DCLs form a small gene family composed of two, four, or five members in higher plants, insects-*Drosophila melanogaster* (two), protozoa, and some fungi such as *Neurospora crassa* (two) and *Magnaporthe oryzae* (two), whereas in vertebrates, nematodes, *Schizosaccharomyces pombe*, and green alga *Chlamydomonas reinhardtii* only one Dicer protein is found. In *Arabidopsis thaliana*, four Dicer-like proteins (DCL1–DCL4) with different roles were found (Xie et al., 2004; Dunoyer et al., 2005; Moissiard et al., 2007; Mlotshwa et al., 2008); DCL1 was found to be associated with miRNA production as well as with the production of small RNAs from endogenous inverted repeats. Genome sequencing projects have proved that Dicer like (DCL) proteins are evolutionarily conserved in a diverse range of eukaryotic genomes. The number of DCL proteins in the genome varies among distinct organisms (Schauer et al., 2002). In RNA silencing pathways, different domains and motifs of dicers are involved, which play important roles (Table 1)

Table 1: Dicer or dicer-like proteins involved in RNAi (Gunter and Thomas, 2004) Hs: *Homo sapiens*; Ce: *C.elegans*; Dm: *Drosophila melanogaster*; At: *Arabidopsis thaliana*

Protein	Domains and motifs	Function	Species
Dicer and Dicer-like proteins			
Dicer	RNase III, DEAD, PAZ,dsRBD	Long dsRNA processing	Hs
DCR-1	RNase III, DEAD, PAZ,dsRBD	Long dsRNA processing	Ce
DCR-1	RNase III, HELICc, PAZ,dsRBD	Long dsRNA processing, RISC assembly	Dm
DCR-2	RNase III, DEAD, dsRBD	Long dsRNA processing, RISC assembly	Dm
DCL2	RNase III, DEAD, PAZ,dsRBD	Long dsRNA processing	At
DCL3	RNase III, DEAD, PAZ	Long dsRNA processing	At

In a recent research (Kidwell et al., 2014) two related Dicer enzymes (dicer like proteins) from a thermophilic fungus *Sporotrichum thermophile* were expressed and purified and further analyses was done. Some important results are-

- Stdicer-1 and stdicer-2 display distinct cleavage preferences
- Stdicer-1 is a thermostable protein that cleaves pre miRNAs
- Stdicer-2 displays robust ATP hydrolysis in the presence of dsRNA
- Helicase domain inhibits RNA binding and cleavage for Stdicer-2

2.8 Rationale

According to review of literature, till date no work has been reported on cloning and characterization of dicer-like proteins in any ectomycorrhizal fungus.

Therefore the present study is aimed at cloning and characterization of dicer-like protein from the ectomycorrhizal fungus *Laccaria bicolor*.

2.9 Objectives

- Cloning and characterization of dicer-like protein from the ectomycorrhizal fungus *Laccaria bicolor*
- Expression studies in response to different glucose concentrations

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Biological materials

- Ectomycorrhizal fungus: *Laccaria bicolor*, maintained at 25°C in the dark on Modified Melin-Norkrans medium (Appendix) (supplemented with Heller's micronutrients on EDTA processed cellophane discs (Melin, 1953)
- Bacterial strains: *E.coli* DH5a and BL21De3
- Carbon source: Glucose
- Nitrogen sources: Ammonium hydrogen phosphate, ammonium sulphate, ammonium nitrate and urea

3.2 Methodology

Growth of *Laccaria bicolor* in carbon and nitrogen stress

MMN medium was prepared by changing concentrations of glucose (0.3%, 0.2%, 0.1% and 0.01%). Agar was added to the media (15 g/l) and media was autoclaved and then poured in 90 mm petri plates. Cellophane discs were placed on the MMN agar plates. The mycelium discs (7 mm) were cut from freshly grown culture and placed in the centre of the cellophane disc. The plates were kept at 25°C in dark for 30 days. After 30 days, the mycelium was harvested and crushed using liquid nitrogen and stored at -80°C.

Similarly, to check the effect of different nitrogen sources, MMN media was prepared by changing nitrogen source from ammonium hydrogen phosphate to ammonium nitrate, ammonium sulphate and urea.

The mycelium was harvested after 30 days of growth at 25°C, for biomass measurement and for RNA isolations.

3.2.1 Bioinformatic's analysis

The nucleotide sequence of *dicer* gene was retrieved from the *Laccaria bicolor* genome (genome.jgi.psf.org). **ORF finder** (www.ncbi.nlm.nih.gov/projects/gorf/) at NCBI (National Centre for Biotechnology Information) was used to find the translated DNA sequence. The **BLASTp** (Basic Local Alignment Search Tool) was used for obtaining various homologous sequences. The conserved domains were noted. The protein parameters were noted via ProtParam at **ExPASy** (www.expasy.org).

MULTALIN (www.mutalin.toulouse.inra.fr/mutalin/) was used to align the sequences. **CLUSTALW2** at **EBI** (European Bioinformatics Institute www.ebi.ac.uk) was used for multiple sequence alignment. Phylogenetic trees were constructed using MEGA6 software by Neighbor Joining method with 1000 bootstrap replicates. Similarly *dicer* like proteins were searched in insects, mammals and plants and used for comparison via phylogenetic tree construction.

3.3 Molecular Methods

3.3.1 Isolation of nucleic acids

3.3.1.1 Total RNA isolation

Laccaria bicolor was grown on cellophane discs which were pre processed and placed on MMN agar medium replaced by different concentrations of glucose for 21 days at 25°C. The mycelia was then crushed into fine powder using liquid nitrogen and stored at -80°C. RNA was isolated from the mycelia powder using the TRI® reagent (Molecular Research Centre, Inc, USA) method as per the manufacturer's instructions.

Procedure for total RNA isolation

1. ~100 mg of the crushed mycelium was taken in an eppendorf tube; 1 ml of TRI® reagent was added to it and pipetted vigorously for homogenization.
2. The sample was then incubated at 15°C for 5 min. and centrifuged at 12,000 g at 4°C for 10 min.
3. 0.2 ml of chloroform was added and the tube vigorously shook for 15 sec., incubated at 15°C for 2 to 3 min. and centrifuged at 12,000 g at 4°C for 15 min.
4. The upper aqueous layer was transferred into a fresh tube. Then the RNA was precipitated by mixing with 0.5 ml isopropyl alcohol. Then the sample was incubated at -20°C for 20-30 min.
5. Centrifugation was carried out at 12,000 g at 4°C for 10 min. and supernatant was discarded
6. RNA pellet was washed with 1 ml of 75% ice cold ethanol and the sample mixed by vortexing.
7. Centrifuged the sample at 7500 g at 4°C for 5 min. and air dried the pellet.
8. The pellet was dissolved in 30-50 µl of DEPC (RNase free water) and stored at -80°C.

3.3.2 Molecular analysis of nucleic acids

3.3.2.1 Electrophoresis of nucleic acids on agarose gel

Nucleic acids were loaded on agarose gels (1%) prepared in 0.5 X TBE, pH 8.0 (Appendix) using a 6X loading dye (Appendix). Ethidium Bromide (0.5 µg/ml) was added to stain the gel prior to pouring. Samples were then eletrophoresed at 70 Volts for 60-80 minutes and visualised on a U.V. transilluminator. Equipment used for RNA samples was washed in detergent and rinsed with distilled water to eliminate ribonuclease contamination prior to use.

3.3.3 Nucleic acid quantification

DNA and RNA was quantified using Nanodrop 1000 spectrophotometer. The purity of the sample from contaminating polysaccharides and proteins was evaluated by the ratio between OD_{260/230} nm and OD_{260/280} nm respectively. Pure samples were indicated by a value closer to or

higher than 1.8 for DNA and 2.0 for RNA ($OD_{260/280}$ nm) and the expected values of RNA and DNA at $OD_{260/230}$ nm are commonly in the range of 2-2.2

3.3.4 Amplification of mRNA by reverse transcription PCR (RT-PCR)

From all the different samples, cDNAs were synthesized from total RNA by reverse transcription PCR method (The reverse AID™ First Strand cDNA Synthesis Kit, Fermentas Life Sciences, USA). 5 µg of total RNA was denatured at 65°C for 5 min. in the presence of 1 µl of oligo (dT)₁₈ primer and DEPC water and it was immediately cooled on ice. The first strand cDNA was synthesized in the presence of 5X reaction buffer: 4 µl; Ribolock RNase: 1 µl; 10 mM dNTP mix: 2 µl and reverse transcriptase: 1 µl. Then incubation was done at 42°C for 60 min and then at 70°C for 5 min.

3.3.4.1 Amplification of cDNA using specific primers

Gene specific primers were designed for the dicer gene retrieved from NCBI (National Centre for Biotechnology Information). Restriction sites were added to both the forward and reverse primers (EcoRI site to forward primers and Not1 site to reverse primers).

Gene specific primers (Table 2) were also designed for actin, tubulin and dicer genes for the purpose of Real-Time PCR.

Table 2: List of primers used in the study

Primer	Sequence
LBDLP-F1	5'- ATGGACCTTCCCTCATCCACACCAG -3'
LBDLP-R1	5'- GAGTTCCGTGGTCATGATGTGATCAAC -3'
LbdicF	5'- CAGTATTGGGTGCGAGCTTC -3'
LbdicR	5'- CAAACAATGCAGAGACGGCT -3'
LtubF	5'- CCCACGTTGCCATTTTCTCA -3'
LtubR	5'- CATGCGGTTCTTGGGTTGAA -3'
LactF	5'- TGGAAGCGTAATGGGGAAGT -3'
LactR	5'- CAGTGTTGGTGATGTGCGAA -3'

cDNA was prepared and used for the amplification of dicer gene. The amplification was performed using **LBDLP-F1** and **LBDLP-R1** primers. PCR reactions were carried out in a final volume of 25 µl containing 10X reaction buffer, 1.5 mM MgCl₂, 200 µM of each dNTPs, 0.5 µM of each primer, 1µl of cDNA and 1.25 U of Taq DNA polymerase. The PCR program was carried as follows- Initial denaturation at 94°C for 3 min. followed by 35 cycles for 1 min. at 94°C, 1.5 min. at 64°C of annealing temperature, 3.5 min. at 72°C and final extension at 72°C for 8 min. Amplified products were visualized on a 1% (w/v) agarose gel.

Similarly all cDNAs were amplified with their respective primers.

3.3.4.2 qPCR (Real-Time PCR)

Expression analysis of dicer gene in presence of various percentages of glucose was done by the method of quantitative or Real-Time PCR. For one reaction the SYBR mix used was 4.5 µl, forward and reverse primers 1µl each, template used 0.5µl and ultrapure water to make up volume 9 µl. Depending on the number of reactions and the different primers used, the calculations were done.

The PCR program was carried as follows- Initial denaturation at 95°C for 2 min. followed by 40 cycles for 15 seconds at 95°C, 15 seconds at 55°C of annealing temperature and extension at 68°C for 20 seconds.

3.3.5 Double digestion of the PCR product and plasmid vector (pET 23a)

The PCR product obtained after the amplification of cDNA using gene specific primers and the plasmid vector pET 23a were digested by staggered end cutting restriction enzymes EcoRI and Not1 (Fermentas).

1. Two labeled tubes were taken and the following reaction components were added for the respective reaction.

Component	PCR product	pET vector
Sample	30 µl	40 µl
Digestion buffer 2X	4 µl	5 µl
Not1	1.5 µl	2 µl
EcoRI	1.5 µl	2 µl
Deionized water	3 µl	1 µl

2. Serial digestion was performed by first adding Not1 to the samples and kept at 37°C for 1hr., followed by addition of EcoRI and incubation at 37°C for 2hrs.
3. Heat inactivation of the reaction was carried out at 65°C for 10 min.

3.3.5.1 Purification of DNA

Purification of DNA was done by using Thermo Scientific GeneJET Gel Extraction Kit.

1. The gel slice containing the DNA fragment was excised using a clean scalpel or razor blade and placed it into a preweighed 1.5 ml eppendorf tube and weighed.
2. 1:1 volume Binding Buffer (Appendix) was added to the gel slice (volume:weight).
3. The gel mixture was incubated at 50-60°C for 10 min. or until the gel slice was completely dissolved. Mixed the tube by inversion every few min. to facilitate the melting process.
4. Up to 800 µl of the solubilized gel solution was transferred to the GeneJET purification column, centrifuged for 1 min at 13,000 rpm. The flowthrough was discarded and placed the column back into the same collection tube.
5. 700 µl of Wash buffer (Appendix) was added to the GeneJET purification column, centrifuged for 1 min at 13,000 rpm. The flowthrough was discarded and placed the column back into the same collection tube.
6. The empty GeneJET purification column was centrifuged for an additional 1 min. to completely remove residual wash buffer.

7. The GeneJET purification column was transferred to a clean 1.5 ml eppendorf. 20-30 µl of Elution Buffer was added to the centre of the purification column membrane and centrifuged for 1 min.
8. The GeneJET purification column was discarded and the purified DNA was stored at -20°C.

The quantification of both PCR product and digested plasmid was done using Nanodrop 1000 spectrophotometer

3.3.6 Ligation

1. One labeled tube was taken and the following reaction components were added for the ligation reaction. The ligation formula used was:

$$\left\{ \frac{\text{nanogram of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of insert} = \text{nanogram of insert} \right\}$$

vector

The insert to vector ratio used was 3:1

Components	Volume
Buffer	1 µl
Insert	4.8 µl
Vector	1.7 µl
Deionized Water	1.5 µl
T4 ligase	1 µl

2. After adding the following components, the mixture was gently tapped and incubated at 4°C overnight.

3.3.7 Cloning of dicer gene products

The PCR product was ligated with pET23a vector and transformed into *E.coli* DH5α competent cells. From the transformed cells, the plasmid with insert was isolated and further used to transform *E.coli* BL21De3 cells.

3.3.7.1 Transformation of *E.coli* DH5α by heat shock method

1. A single colony of *E.coli* DH5α was picked from a freshly grown plate and transferred into 20 ml of Luria Broth (LB) (Appendix). The culture was incubated for 16-20 hours at 37°C with vigorous shaking (200-250 cycles/minute in a rotary shaker).
2. 500µl of the above saturated culture was transferred into 50 ml of fresh LB. The culture was incubated at 37°C with vigorous shaking for 2-3 hours ($OD_{590} \approx 0.5$).
3. The above culture was transferred to sterile, disposable, ice cold polypropylene tubes. The culture was cooled to 0°C by storing the tubes on ice for 10 min.
4. The cells were recovered by centrifugation at 5000 rpm for 10 min. at 4°C. The media was decanted from the cell pellet. 10 ml ice cold 0.1 M $CaCl_2$ was added to the pellet which was dissolved in it and stored on ice for 10-15 min.
5. The cells were recovered by centrifugation at 5000 rpm for 10 min. at 4°C, fluid was decanted from the cell pellet and it was resuspended in 1 ml of ice cold 0.1 M $CaCl_2$. The cells in this stage may be stored on ice for 12-24 hours.
6. 100 µl each of the suspension of competent cells was transferred to sterile and prechilled eppendorfs. Plasmid DNA was added in a volume of 5 µl or less in one eppendorf, mixed gently and stored on ice for 30 min.
7. The cells were given heat shock for exactly 2 min. in a still water bath preheated to 42°C and rapidly transferred on ice and chilled for 1-2 min.
8. 1 ml of LB + Ampicillin (50 µg/ml) was added to the cells and were incubated at 37°C for 45-60 min. to allow the bacteria to recover and express the antibiotic resistance marker encoded by the plasmid.
9. The cells were then centrifuged at 8000 rpm for 5 min. Some of the supernatant was discarded and in the rest the pellet was dissolved.
10. 100 µl of this solution was spread on Luria Agar (LA) + Ampicillin plates and were incubated overnight at 37°C.
11. They were checked the next day for the appearance of colonies and screened for the presence of the plasmid.

3.3.7.2 Colony PCR

A pin-point amount of bacterial cells was added in 5 µl of ultrapure water, mixed and incubated at 95°C (in PCR) for 10 min. The tubes were then quick chilled in ice and vortexed for 5 seconds. Then they were quick centrifuged and 3 µl of supernatant was transferred in fresh tube. PCR mastermix was added in the tube. The PCR was performed with primers **LbDic-F:** and **LbDic-R** (Table 2). The PCR program was carried out as follows: initial denaturation at 94°C for 3 minutes, followed by 30 cycles for 1min, at 94°C, 1 min. at 55°C annealing temperature, 2 min. at 72°C and final extension at 72°C for 7 min. Amplified products were visualized on a 1.5% (w/v) agarose gel.

3.3.7.3 (a) Bacterial plasmid DNA isolation

1. The colonies were picked up from the plates with the help of autoclaved toothpicks and patches were made on LA + Amp. Plates (Appendix) and also inoculation was done in LB + Amp. (Appendix) and incubated overnight at 37°C.
2. 1.5-2 ml culture was taken per eppendorf tube and centrifuged at 8000 rpm for 5 min. The supernatant was discarded.
3. 200 µl of Solution 1 (Appendix) was added to each tube and with vigorous shaking/vortexing the pellet was dissolved and kept at room temperature for 5 min.
4. 400 µl of freshly prepared Solution 2 (Appendix) was added, mixed by gentle inversion 10-20 times and kept on ice for 3 min.
5. 300 µl of Solution 3 (Appendix) was added, mixed by gentle inversion 10-20 times and kept on ice for 15 min.
6. 400 µl of phenol:chloroform:isoamylalcohol (25:24:1) was added to the contents, mixed by inversion and centrifuged at 12,000 rpm for 10 min.
7. The upper aqueous layer was transferred to a fresh tube and added equal volume of ice cold isopropanol and precipitated at -20°C for 20-30 min.
8. This was then centrifuged at 10,000 rpm for 10 min. for precipitation.
9. The supernatant was discarded; the pellet was washed with 100 µl of 70% ethanol and centrifuged at 8000 rpm for 5 min.

10. The supernatant was removed and the pellet was dissolved in 30 µl of autoclaved Milli Q water.

3.3.7.3 (b) Bacterial plasmid DNA isolation via Qiagen Miniprep Kit

1. 1-5 ml bacterial overnight culture was pelleted by centrifugation at 12,000 rpm for 1 min.
2. Pelleted bacterial cells were resuspended in 250 µl Buffer P1 (Appendix) and transferred to a microfuge tube.
3. 250 µl Buffer P2 (Appendix) was added and mixed thoroughly by inverting the tube 4-6 times until the solution becomes clear.
4. 350 µl Buffer N3 (Appendix) was added and mixed immediately and thoroughly by inversion.
5. Centrifugation was done at 13,000 rpm and applied the supernatant to the QIAprep spin column by decanting or pipetting. Again centrifugation was carried out at 13,000 rpm for 1 min. and flow-through was discarded.
6. The QIAprep spin column was washed by adding 500 µl Buffer PB (Appendix), centrifuged at 13,000 rpm for 1 min. and flow-through was discarded.
7. 1 min. centrifugation was carried out to remove residual wash buffer.
8. The QIAprep column was placed in a clean 1.5 ml microcentrifuge.
9. To elute DNA, 30 µl Buffer EB (Appendix) was added to the centre of the QIAprep spin column, kept for 1 min. and centrifuged for 1 min. at 13,000 rpm.

3.3.7.4 Transformation of *E.coli* BL21De3 by heat shock method

The same protocol of bacterial transformation was followed as for DH5α. Along with Ampicillin (50 µg/ml), chloramphenicol (34 µg/ml) was also added at the respective steps.

3.3.7.5 Screening for positive clones

The plasmid DNA of the recombinant bacterial cells was isolated to confirm the presence of the plasmid in the cells after a positive colony PCR result. These plasmids were used for PCR reactions by insert specific primers to confirm that the plasmid DNA contained the insert.

3.3.8 Calibration of protein production via IPTG induction

1. Inoculation of the BL21De3 cells having the vector with insert as well as without insert as control was done in LB+Ampicillin+Chloramphenicol and was kept overnight at 37°C.
2. After 16-18 hours 250 µl culture from both flasks was transferred into fresh medium and kept at 37°C.
3. OD₆₀₀ was taken after 3.5 hrs which was 1.0 (ideal OD for good yield of protein is 0.8-1, after which IPTG (1mM) was added to both flasks.
4. Four time points were taken namely 3, 6, 9 and 12 hours. IPTG induction was checked at these time intervals.
5. Centrifugation of the cultures at 8000 rpm for 3 min. at each time interval was done and the pellets (with some supernatant) were stored at 4°C overnight.
6. The following day, all the eppendorfs were centrifuged again and supernatant was discarded.
7. Cracking buffer (Appendix) was added to the pellets and they were dissolved and then kept at 95°C for 10 min.
8. Centrifugation was carried out at 5000 rpm for 2min. and the supernatants were loaded on the SDS PAGE gel.

3.3.8.1 Polyacrylamide gel electrophoresis

Resolving (12%) and stacking (4%) gels were prepared and poured in the cast apparatus for PAGE. Samples were loaded and electrophoresed in Tank buffer (Appendix) at 90 Volts at first and then at 120 Volts. Staining and destaining of the gel was done after electrophoresis to visualize the bands.

CHAPTER 4

RESULTS AND DISCUSSION

Results

4.1 *In silico* characterization of dicer like protein

4.1.1 Sequence retrieval and translation

The dicer gene sequence (Fig.9) was retrieved from the *Laccaria bicolor* genome (genome.jgi.psf.org) and was then translated for protein sequence using ORF finder (Fig.11). Translated DNA sequence showed 1122 amino acids containing both start and stop codon.

5'ATGCTATCACTCGCTATAAATTCATATACCACCCCGTCAAACCTATGAGCTCCTGGA
TCTCTCAACTCCAGAGCTATCCTCTCCAACGGACGTCCTAGTCAAAGTTCATGCAGC
AAGCATCAATCCCGGCGACGTCAAAACGGCTGCAGGCGCAACGAGGGTCATGATGC
CCCTTACTTTTCCATATAAACTCGGATTGGATCTGAGCGGGACAGTCGTGCGCGTTG
GGGAAGCAGTCACCGAGTTTGCTGAGGGCGACGAGGTATTTGGGATGCTGCCTTTA
ACAACACGAGGTGGTTCCGTGGCGGAATATGCCTTGATATCCTCAACAAGGCTCGTA
CATAAACCAACGTCCATTTTCGCACACCGAAGCAGCTTCCCTTCCTTGTGTTGCCGTC
ACCGCTTTACAATCCCTCGAAAGGGCTAACACCGTCATCGAAGGGGGCCTGTCCGG
AAAAACAGTCTTCATCCCGGCTGGTCTGAGCGGGACGGGCTCCATCGCAGTTCAACT
AGCCAAGCGGGTATATGGTGCAGGGAAAGTGATTACCACCGTATCAACGTCGAAGA
TCGGTAAGGTGGACGAATTTCTGGGCGCCGGTGTGTCGATCAAGTGATTGACTATA
CGAAGGAGGACGTCTTGAAGCTTGTGGAGAAAGGTAGCGTAGATTTTTTCTATGATA
CTGCGGGGGCGAGCATGCAGTACATCTCGGTGATGAAGAAGGGCGGGTTAATTATT
TCGATCACGACGATGCCTTCGGGAAAAGAGGTCGCCAACTTATGCCTGACGCGCC
GTATAGCATTCTCCGCGTGCTTGATGTCTTGGAAGTGGGTTAATCGATGGAGAGTTTG

GTGGTGGGGTGCTCGGTTAGAAGCTCGGGTGGAGACGGTGGACACAAGTGGTGTG
 CTCGAGTATCGCAGCTGGTTGAGGAAGGCAAAGTTAAGGCTGTTGTTGGTCCGACC
 GCGGATCTGAATGATTTGCAGGGGATAAAGGACGCTTGTGCAGAGATAAAGGCGGG
 CAAAGGAGGCCTCATGGCTAACGTTACTCTAACCTGCCAACAACTGGAACTCTGTT
 ACCACGTCGGTATCAGGAGGAAGTTTTTGCACGAGCACAGAAAGGGAACATCATCG
 CGGCTCTCAACACAGGTTTCAGGAAAGACCCTAATCAGTCTCCTTCTCATCAAATGGA
 CAATAGCCAAGGAACAGTCAAAGGGAAAGGTTATCATCTTCTTGGTCCCGAAAGTA
 ACGCTCGTGGAGCAACAGAGCCTTTACATTGATAAGAACAACCTCCTGATAGCATTCGG
 GTTATGAAACTCCACGGCGCTTTGGATATTAAGTTGGGCGACAGAGCAGGTTGGAA
 GAAGCGATTTCGAGGAACATGAAGTGCTCGTCATGACTGCTCAGATCTTCCTCAACAT
 ATTGACGCATTCAGTGTGGAGCATCGACAAAGTTTCACTGATGATCTTTGACGAATG
 TCACCATGCGAGGAAGAATCATCCGTACAATGGGATCATGCGCGAATATTTTACGTC
 CCCTTCCTCGCGAAGACCAAAGATTTTTGGAATGACTGCCTCTCCTATCTGGAACAT
 AAAAGATCCGGCGGGATCTTTGGCGACCCTCGAGCAAAACATGGATGCCAAGGTGA
 TTGGCGTTCGAGATCATATCGAGGAATTGGTGGATCATTTCTCCAAAGCCTATCGAGA
 TTATAGAAGAATATCCATCGCCCCCAGAAGAGTACGACTATCTTTTCCCTACAATCT
 ATATGTGCCTTCAAATATTCGAGAAGACACTCTGGGATCAATTAGACATTCCGTGGA
 GTAAAATAGAAATGCGGTATTTTTCTACTCTTCATAACCTGGGCCCATACTGCGCTTC
 CCTTTTCTTTACCTGGAGATCCAGCATCACCTCACCAACCTGTTCGCCGAATGCCA
 AAAGTATTTATACCCAGTCGATGGGGACATGGCCATC 3'

Figure 9: Dicer gene sequence of *Laccaria bicolor*

The translated protein sequence (Fig.10) of the Dicer gene is-

MDLPSSTPAKRIPLEIFDIDDIVSDFRSFFSSVDCPAQIPIPIQLAWCSPKVDSL VKVLLKYN
 SPTFQGIIFVEQRQVALTLGRLLLEAVPELQGTIRCAHLVGQGVNSEGVSRHSDRYQGDA
 VKLFRDKEINILIATSVAAEGLDFPASHIVVTL SVYPDVLFNQACDLVVRFPDFQHLVGY
 VQSRGRARNKASTFIIMI QKDDLAQLTRYKALQEGEPEINRAYQTRHLNTDDDLDDED
 DDINDLVDLAERERYVVPSTGAILS YDNAINLIHHL CALIPRDAFTPPHQPTFTGYFQSTM
 RLPPSLPLPPRHLSYLGPPKRSKKEAKRAVAFC AVKRLCKLDVFDDYLLPISSQVHEEDK
 QRVIRVRDVP AIMTVSVRDPWCMG TKLWIHPILIGGCQVAGLVTG TKLPPVDVLAASSH
 VRTGLAELVFHEDSEFEERKIMHDFTKLGIYYRITSSPFVSATSLFIPLTSDNQPDFHTMA

RLVDDPWGREDWDSVTEDDYDRRVIMSTYEKGRTYLLKKIREDLSPMSVPPRDSLQAA
FPTYHEYFVSKWTRKRKVIVPVNGPLLEAAIHPKSHLGSYSLYPTSVDTIAASSNVQVRL
LPQGCTRWIDLSFDLCRAFEALPVICRRITDVYRIQCGRFDLGLPLITENLLIEAFTLPSSKP
SCNNQRMETLGDAVLQLCTTVHLLNAYPNRHEGQLTDLRQQFVSNNYLLSRAKDVGLE
HFLTSEVETVHKWRYTLPDDGNAEGLRDPPKRYAEREYPRRSLQDCMEAVLGASFLTG
GISMALATGTALGLAFGGPHPWRRYPPSALPTAVSALFEGLETSLG YKFHHNHLILEA
VTHPSFTSSSGGPSYQRLEFLGDALLDLVVIKYLYDKFPSATSHELAFPRTRTICAQTLAH
VAVKRLGLHNVMLVNCPALTMAINGYVPMLKAASSEEIVNKGWKYDPPKALSDVFES
VMGAIFVDTGYDYDKTASVIEYVMEDVLSVLSPSLTKDPVSDLLEWAGKAGCMKVVF
GRKVKSTGKLQAEGVTVSVHGNIMIGPIVSTSLPVAKYAAAERAMAMLRDGETDKSLA
HLCDCGKHMEIDSTGPALVEDGAKLDAVFDEALDAEEAAEVDHIMTTELS

Figure 10: Translated DNA sequence of dicer gene of *Laccaria bicolor*

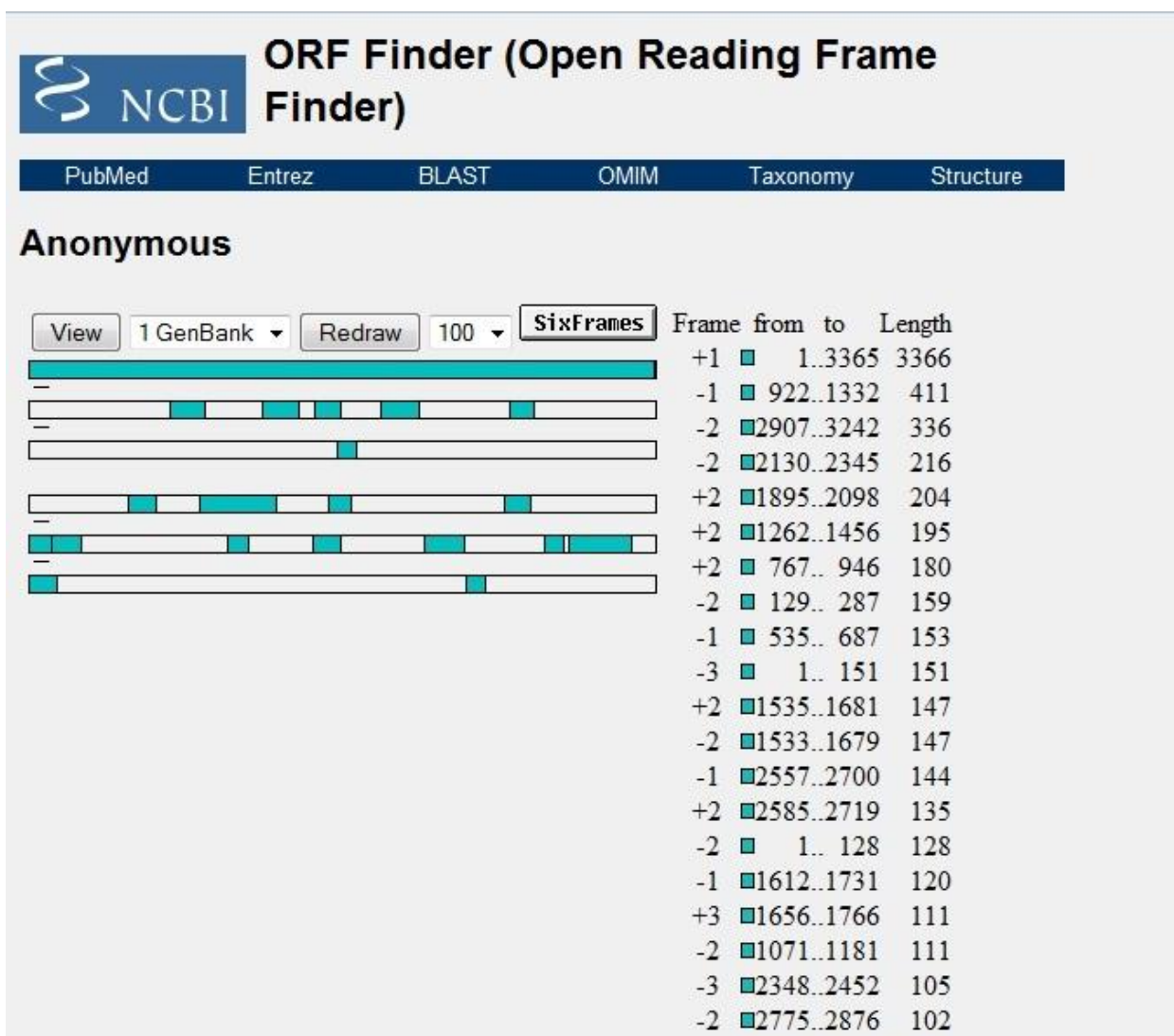


Figure 11: ORF finder of dicer like gene retrieved from the *Laccaria bicolor* genome

4.1.2 BLASTp and conserved domains analysis

ORF finder provided the reading frame which was further used for BLAST analysis (Fig.12) and obtaining various homologous sequences or sequences producing significant alignments, from the database.

Sequences producing significant alignments:

Select: [All](#) [None](#) Selected:0

Alignments	Download	GenPept	Graphics	Distance tree of results	Multiple alignment							
Description							Max score	Total score	Query cover	E value	Ident	Accession
	hypothetical protein K443DRAFT_652072 [Laccaria amethystina LaAM-08-1]						2011	2011	100%	0.0	90%	KIK03791.1
	hypothetical protein GAIMADRAFT_59401 [Galerina marginata CBS 339.88]						1219	1219	99%	0.0	55%	KDR81905.1
	hypothetical protein M413DRAFT_438720 [Hebeloma cylindrosporum h7]						1187	1187	96%	0.0	55%	KIM49536.1
	hypothetical protein HYP5UDRAFT_207469 [Hypholoma sublateritium FD-334 SS-4]						1173	1173	96%	0.0	53%	KJA15967.1
	hypothetical protein PILCRDRAFT_814967 [Piloderma croceum F 1598]						1057	1057	96%	0.0	49%	KIM87443.1
	type III restriction enzyme [Coprinopsis cinerea okavama7#130]						1055	1055	96%	0.0	49%	XP_002911949.1
	hypothetical protein AGAB1DRAFT_125544 [Agaricus bisporus var. burnettii JB137-S8]						1053	1053	99%	0.0	49%	XP_007326912.1
	hypothetical protein AGAB2DRAFT_64825 [Agaricus bisporus var. bisporus H97]						1051	1051	99%	0.0	48%	XP_006458539.1
	hypothetical protein HYDPIIDRAFT_113252 [Hydnomerulius pinastri MD-312]						1029	1029	96%	0.0	48%	KJ63284.1
	hypothetical protein SERLADRAFT_441040 [Serpula lacrymans var. lacrymans S7.9]						1025	1025	94%	0.0	49%	XP_007321595.1
	hypothetical protein SERLA73DRAFT_112480 [Serpula lacrymans var. lacrymans S7.3]						1025	1025	94%	0.0	49%	EGN96270.1
	hypothetical protein PLICRDRAFT_91716 [Plicaturopsis crispa FD-325 SS-3]						1020	1020	95%	0.0	50%	KII89314.1
	hypothetical protein GYMLUDRAFT_48671 [Gymnopus luxurians FD-317 M1]						1015	1015	95%	0.0	49%	KIK54544.1
	hypothetical protein CY34DRAFT_805907 [Suillus luteus UH-Slu-Lm8-n1]						1009	1009	97%	0.0	48%	KIK41537.1
	hypothetical protein PAXINDRAFT_130762 [Paxillus involutus ATCC 200175]						1005	1005	99%	0.0	46%	KJ17159.1

Figure 12: Some aligned sequences with the translated dicer gene sequence of *Laccaria bicolor* obtained via BLAST

The conserved domains (Fig.13) obtained were as follows-

List of domain hits				
Name	Accession	Description	Interval	E-value
RIBOc	cd00593	RIBOc. Ribonuclease III C terminal domain. This group consists of eukaryotic, bacterial and ...	835-969	1.17e-30
RIBOc	cd00593	RIBOc. Ribonuclease III C terminal domain. This group consists of eukaryotic, bacterial and ...	652-787	2.44e-26
HELICc	cd00079	Helicase superfamily c-terminal domain; associated with DEXDc-, DEAD-, and DEAH-box proteins, ...	49-189	4.86e-10
RIBOc	smart00535	Ribonuclease III family;	835-970	8.15e-27
RIBOc	smart00535	Ribonuclease III family;	651-785	2.06e-24
Ribonuclease_3	pfam00636	Ribonuclease III domain;	669-781	5.31e-19
Ribonuclease_3	pfam00636	Ribonuclease III domain;	855-967	2.07e-18
Dicer_dimer	pfam03368	Dicer dimerization domain; This domain is found in members of the Dicer protein family which ...	270-353	6.91e-11
Helicase_C	pfam00271	Helicase conserved C-terminal domain; The Prosite family is restricted to DEAD/H helicases, ...	114-189	1.16e-08
HELICc	smart00490	helicase superfamily c-terminal domain;	121-189	5.74e-07
Rnc	COG0571	dsRNA-specific ribonuclease [Transcription];	818-1062	8.83e-31
RNaseIII	TIGR02191	ribonuclease III, bacterial; This family consists of bacterial examples of ribonuclease III. ...	822-1062	2.08e-30
rnc	PRK00102	ribonuclease III; Reviewed	818-1004	7.20e-29
Rnc	COG0571	dsRNA-specific ribonuclease [Transcription];	643-783	2.16e-17
RNaseIII	TIGR02191	ribonuclease III, bacterial; This family consists of bacterial examples of ribonuclease III. ...	643-785	1.12e-16
rnc	PRK00102	ribonuclease III; Reviewed	650-782	3.17e-14
MPH1	COG1111	ERCC4-related helicase [Replication, recombination and repair];	50-204	6.60e-13
PRK13766	PRK13766	Hef nuclease; Provisional	70-203	4.43e-11

Figure 13: List of domain hits with the translated dicer gene sequence

This result showed that there are conserved domains matching the present study. The Ribonuclease domain and the helicase domains which are present in most dicers were also observed. This indicated that the domains have been conserved throughout the span of evolution.

4.1.3 Protein parameters

The ProtParam tool at ExPasy was used which allows computation of various physical and chemical parameters (Table 3) for a given protein sequence.

Table 3: Parameters for the assessment of protein

Parameters	Values
Number of amino acids	1122
Molecular weight	125053.1
Theoretical pI	5.71
Number of positively charged amino acids	115
Number of negatively charged amino acids	142
Instability index	43.28
Aliphatic index	93.24
GRAVY value	-0.129

These were the values for different parameters of protein. The instability index indicated the protein to be unstable. The aliphatic index is the relative volume occupied by the aliphatic side chains. GRAVY (grand average of hydropathy) value is the sum of hydropathy values of all amino acids divided by the number of residues in the sequence.

4.1.4 Multiple Sequence Alignment

CLUSTALW2 was used for multiple sequence alignment of the translated DNA sequences which had a significant match with each other.

CLUSTAL 2.1 multiple sequence alignment

```

Hebeloma      MAMLDRAAIIHSDSVMPHGLGGLVPHQYQSEVVFVRAQKANVIAALPTGSGKTLISVLLMR 60
Hypholoma     --MAQLVNEWNEASPIPN----DVPRGYQTEVFERAQKENIIAALNTGSGKTFISLLLIK 54
Laccaria      -----
Serpula       ----MTVAQIPQVIVSES----ILPRRYQEEIFRRAQNSNVIAALDTGSGKTFISILLIK 52
Plicaturopsis -----
Coprinopsis   --MTQAIFTNPFKSTATG----EVARKYQEEIFRQAQKGNVIAYLRTGLGKTLISVLLMR 54
Agaricus      ---MTIIDPIANTTSELR-----PRKYQEEIFERAQKENVITALNTGSGKTLIGLLLIK 51

Hebeloma      WIASQEQSKGRIVAFVLVFKVTLVEQQWRYIQKTSLRVAKLHGALD--LDLSDRSGWKKR 118
Hypholoma     WITAQSSSNGKLVFLVFKVTLVEQQYEYLGRAALRIKKFHGTTDTMPSFTDRPRWKHV 114
Laccaria      -----
Serpula       WIVLQEKAKRKVIVFLVFKVALVKQADSIAAHTPLRVKMFHGSLD--LDLTDRAWWKS 110
Plicaturopsis -----
Coprinopsis   WMCTQEKAKDKAIIIFLVFKVALVEQQANYIHKQTGLRTCKIHGALN--LSFADRDGWKKR 112
Agaricus      WMSALESSGDKLIIIFLVPRVALVEQQAKYLRDNTPLRTEKLYGALD--ISLSDRMQWKKK 109

Hebeloma      FETHDVFVMTAQIFLNILTHSLWKMDKVSIMIFDECHHARKNHPYNGILREYFQLDPSDK 178
Hypholoma     FDSTDVLVMTAQIFLNLLTHSLWSFDRVSLVVFDECHHARKNHPYNGIMREYFQVADISK 174
Laccaria      -----
Serpula       FEQSDLTVAQAQIFLNILTHSHWSVEKVSLLIFDECHHTRKNHAYNGIMHEYFQTIPS-D 169
Plicaturopsis -----MTAQIFLDVLTHSHWSIEKVSLLILDECHHTQKNHPYNGIMREYQWTTTH-L 51
Coprinopsis   FADHDVFVTTTQMFLEAITHAVWKIEQCSLIIIFDECHHARKNHPYNGIMREYFQVQPLNA 172
Agaricus      LGKCDVIVMTPIILVNFITHSIWSLRQIALLVFDECHHARKNHPYNIIMQEYFYIKEVED 169

Hebeloma      RPKIFGMTASPIWNVRNPLGSLSDLESMMNAKVIAVVEHVQELNEHSFPAKEIIKEYFLA 238
Hypholoma     RPKIFGMTASPVWNTKRNPLISIAATLEQMMNAKVISMENIKELLESFPAKEEMKYYPAP 234
Laccaria      -----
Serpula       RPKVFGMTASPIWNPKDAEGSLATLEKNLDATVIAVREHVEELQENSPPRPFVEVIEKFSPP 229
Plicaturopsis RPKVFGMTASPIWNPRDAQGSLAILERNLNATVIGVRDHADLSKHSFKPKKEMIKIYPPP 111
Coprinopsis   RPKIFGMTASPYWNQKNPALSLSTLEANLDAKIIIGVHEHVEELQSHYSKLNVEVIEKYPYP 232
Agaricus      RPKIFGMTASPVWNTKDFVGSLLTLETNLDSVIISVREHVDLAVHTPRPSELITIFPAP 229

Hebeloma      PEEYNYSSPTIYQCLKVVDNSV---WDDLEIFWRQIDNRYWVTFYNLGPYCASMFLFHEI 295
Hypholoma     PENFNYPSPPTIYDCLKIIDQCT---WDQLDLFWAKVAARYSSTLLNLGPYCASMFLFLEL 291
Laccaria      -----
Serpula       SDTYDYLYPTLWDCLSLFTNAV-----EINIPVDKLMKYQMTRNSLGAFSADLFLYTDI 284
Plicaturopsis PQAYDYPSPTLLDALGILDLP-----EADFPWSKVRMRYAVALNSLGPYCAELYLYGYL 166
Coprinopsis   PETYNFPEPTLYQCLSVFYASDPTSFDAWDIPWTKLAMRHNMTLGNIGPYCASLYLFSSEM 292
Agaricus      PDNYDFHDPNFLRCLSVISPAT---WEHLEVAWSSLVMRHDATLYNLGPYCASFLLYLEV 286

```


Hebeloma	--VDLAARERYVVPSTGAVLSYDNLSLLNLHLCSLIPRDAFTPIHKPKYTGEFEQTLHLP	609
Hypholoma	YQADLRERERYVEPSTGAMITYDNALNLLSYLCIIIPRDAFTPTHRAKYTEGFQTSVVLP	609
Laccaria	-LVDLAERERYVVPSTGAILSYDNAINLIHHLICALIPRDAFTPPHQPTFTGYFQSTMRLP	305
Serpula	--ADVAVRERYIVPSTGAVLTYDSAVNLLNLHLCSLIPHDAYTPPQLPKYSGDYAVMLELP	616
Plicaturopsis	--DDLKERERYVVESTGAILTYDSSISLLAYLCSLIPRDSFTSVHLPKYTGFTSTIQLP	474
Coprinopsis	--MDLAERERFVVPSTGAVLTYDNALSLINHLICALIPRDSFTEAYKPPQFIGDFQSRMLP	606
Agaricus	--LDLASRERYVVPSTEAVLSYDSAIGLLNYLCALIPCDAYTPPHKPKFTGDFQSTVQLP	599
	*: ***: ** *::**::*: :*:** *:*	
Hebeloma	RALPLSPQDLTFTGPIRRSKREAKRAAAFMAVKRLKELDVDFEYLLPLAKYADGEEEEVR	669
Hypholoma	RALPLESADLSYMGPTKRSYKEAKRAVAFMAVKRLRELDVDFEYLLPVFTGSDDFEGSLQ	669
Laccaria	PSLPLPFRHLSYLGPPKRSKKEAKRAVAFCAVKRLCKLDVFDYLLPISQVHEEDK---	362
Serpula	SSLPLPIDRLFFSGPERRSKREAKRAVAFVAVKELHRLDVFDYLLPATSSKGRGTEDAD	676
Plicaturopsis	STIPLPPSKLVYTGPVKRSKKEAKRAAFVAVKHLHALKVFDHLLPVSSSTRGRGTEDAD	534
Coprinopsis	ASLPLQPRDLVYLGYPKRSKQEAKRSAVAFQAVKRLKLVFDYLLPVSSER-PGDFDD	665
Agaricus	LSIPLPFDQLTYTGPKLCSKKEAKRAVAFVAVKRLHELDIFDQYLLPTPGSTLGEEMAQ	659
	::** * : * : * :****: ** ***. * *:***:***	
Hebeloma	SITGDGKLKRQIPPPIMSAHVDRDPWYIGQTLWSHTILVENAAIASLITSTVLPPQDFVIE	729
Hypholoma	NLRGQ-KAKRKNIPMMTVSVDRDFWCMGDRLWLHPIVIDQILAGLVGTALAPEMMNIT	728
Laccaria	----QRVIRVRDVPAMTVSVDRDFWCMGTKLWIHPILIGGCQVAGLVGTCLPPVDVLA	418
Serpula	---GRGILDFSDVPDLMVLRDPWTILGAKLYLHIVTIDDQDVGGVLVTGTCLPPVEFKSD	733
Plicaturopsis	---GRPLGDIRGIKIMDVNVRDPWTDGRNLWVHVVDGKRLAGIVTGTMLPPVAUVCN	591
Coprinopsis	---AYLHRGNKLPPDVMEVHVDRDPWYMGPKLWLHPIVLDSETTAGLVGTSLPFTDILVD	722
Agaricus	---TWLGLESRDTPVMNVDPKYFWTIGEKLWLHPIYLGGEPTGLVSGTPLPSVELR-S	715
	:* . *: ** *: * : : . :...* *..	
Hebeloma	GSNVQMLPAALLEFDADQHEQRLAMHQFTRCGVWYMNISRPFSSPFSFYLVPIITPDRLP	789
Hypholoma	NKHVKTLPAKLLEFDTESEHELRAEMKEFTRLAVWYNVTSRPFSSSLSFYVVPITETWDP	788
Laccaria	SSHVRTG-LAEVLFHEDSEFEERKIMHDFTKLGIYRITSPPFVSATSLFIIPLTSDNQF	477
Serpula	GSTIQLRYDRVLLFDEDEEWDQRRMLSEFMRMGLWFCVTGRPAELPFSSFLVPLLPNKHI	793
Plicaturopsis	GRVVRHTANFVRFDAVDERQQRAILHDYMKLAVWFCNTGRPITLPLTLFLVPLTRHRQP	651
Coprinopsis	QMDCKLLPGTLLAFDPEDEREQRRIMQDFTHLGVWARNTAKPIIHDLSSFFLVPLTSTHQF	782
Agaricus	DILVRLGHPEAFVLDSEEEVELRRTMHEYTRLGIYRVVTGSPLAEPGVYLVPLTDTLEV	775
	: . : . * : * : : : : . * . :...:	
Hebeloma	DFAIQCLLSYPEGNGDWSKISEEDYDKLMVICRPQFGSIHILRRIREDLTPLSTPLPGS	849
Hypholoma	DFVEIRSLNPNRGSTDWTQISEGHYDKLIILSRYRFGSTYLLRNIRHDLSPMSSPLPGS	848
Laccaria	DFHTMARLVDDFPWGREDWDSVTEDDYDRVIMSTYERGRYLLKKIREDLSPMSVPPRDS	537
Serpula	DFEVVERVASEPYGSYDWSGASEKDYMHLMVMNSNQYGRALVLRNIRYDLTFLSTTPPGS	853
Plicaturopsis	DFDEMIRLVTCPLGSYDWAGVGPSDYGRLLILGNNGEWGRTIRLMGIRHDLSPLSATIPGS	711
Coprinopsis	DFAWMQHIVDGQKGSHDWSNITEKDYGTWVFNTNQIGRIYQLQCIRNDLALDSRPPQGS	842
Agaricus	DFHQIRRVVDNPRGIASLDDIYGDEKLLVLNKFESGRTRVLQKIRSDLSFMSIPPIS	835
	** : : * . : : . * * ***: * . *	

Hebeloma	PAPAALEENLNKYFRHNHLVME ^{SL} THP ST FTS-DVPSYQRLEFLGDAVL ^{DL} FVVKYLF ^{DK}	1189
Hypholoma	ALFSSLEQTLGKYFRHNHLLLES ^{LS} HP ^{SV} TY ^S -EAPSYQRLEFLGDAILD ^{LV} VVKYLY ^{DK}	1186
Laccaria	ALFEGLETSLGYKFHHNHLILEAV TH PS ^{FT} SSSGG ^{PSY} QRLEFLGDALL ^{DL} VVIKYLY ^{DK}	876
Serpula	HLFADLQESLCYEFHRGDL ^{VEA} ATH ^{PS} FAT ^S -A ^{SS} SYQRLEFLGDAVID ^{LV} LVNLY ^{YRR}	1192
Plicaturopsis	PLFDRLEESLGYTFHRGELLLEAL TH PS ^{FT} S-SNPSYQRLEFLGDALID ^{LV} VVKYLY ^{DK}	1052
Coprinopsis	HLFDTLQERLG ^{YTF} HSN ^{SL} VEAV TH PS ^{FAT} D-G-PSYQRLEFLGDAILD ^{LV} VVHYLY ^{EK}	1176
Agaricus	ALVAGLEKEIGYVFENPELLVEAV TH PS ^{LE} FS-STPSYQRLEFLGDSVIK ^{ML} VT ^{EY} LY ^{HK}	1188

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Hebeloma	EIVKNGWKYDPPKALSDFESVVGAVLVDSGYDYERTAAVLEYVMEDVLEVLSPSLAKDP	1309
Hypholoma	EIVRLGWKFDPPKALSDFESLLGAVLVDSGYDYERTAAVVEYVMEDVLEALSPDVAKDP	1306
Laccaria	EIVNKGWKYDPPKALSDFESVMGAIFVDTGYDYDKTASVIEYVMEDVLSVLSPSLTKDP	996
Serpula	DIINKGWKYDPPKALSDFESVVGAI FVDSAYNFEKVASVVELIMSDVLELLSPNLPKDP	1312
Plicaturopsis	DIVRKGWKLDPKKAISDFESLVGAVLVDSAWNYEKAEAVVEEVMWDVLEVLSPDL PQDP	1172
Coprinopsis	EIARSYYKFDPKKAISDFESLVGAVLVDSGYNYDKTSAVVLDIMSKVLDQLSLDMAKDP	1296
Agaricus	EIVRRGWRYDPPKAISDAFESVVGAVFVDVG YDLDR TGGIVCRIMEDILEVLNPSIPKDP	1308

[illegible]

Hebeloma	HGKLVVGPPIVSSSLNIARFAAAERALTKLRDPASEMNLANLDCALAMSIDGCPFA3PFV	1406
Hypholoma	HEQVIVGPPIVSTSLTVAKFLAAERALTALRDSGSSKHLALLCDCGMSMDAIS3QLPG---	1400
Laccaria	HGNIMIGPIVSTSLPVAKYAAAERAMAMLRDGETDKSLAHLCDGKHEIDSTGPAL---	1090
Serpula	HDVVVAGPITASNL3LSKGLAAERARILLDDHSSDQCLTSLCDCSMAMKPQVQV3L---	1406
Plicaturopsis	HDTLVAGPITGSSMLVSKGLAAERAHHILNDITSNHS3LQRLCNCPGAHLAPKIPLPQT	1269
Coprinopsis	HGKQVVGPIVASSPGVAKFIASERALSILTDSENELALS3KLCDCENRQNMEIDVDAALA	1390
Agaricus	HGOIVAGPIVSN3KAVSRFAVAERAFMALR-ASDEELFKKLCDCONOMITDEVTGSGEVD	1427

Hebeloma	VDS-----	1409
Hypholoma	-----	
Laccaria	-----	
Serpula	-----	
Plicaturopsis	NAT-----	1272
Coprinopsis	EMK-----	1393
Agaricus	DLDTLDEVEE3EVANMLMVEDKYKERWLKHKVESDYTNLAVL3ERLGIYDGCRTCTCKFSI	1487
Hebeloma	-----TEPTGDISSTDDDAEDIDEV	1430
Hypholoma	-----ASAIATDDL3DCDDTEDI	1418
Laccaria	-----VEDGAKLDAVFDEALDAEEA	1110
Serpula	-----AIPDEND-ISKPV3DE-NEE	1424
Plicaturopsis	-----PEVPDLSE-LEEAPPDDGTED	1292
Coprinopsis	-----VVTPEFVAPPEVSGSEDGEIE	1414
Agaricus	AFEGVVP3GASVRRCYCVVLANFWYIPAVVAYSASNKPEAVPKVFEYALQELKANKATEA	1547
Hebeloma	AEFLIEDTTEDDQEKVAI-----	1448
Hypholoma	LKLDVQEQQVDMEL-----	1432
Laccaria	AEVDHIMTELS-----	1122
Serpula	GFATIAQRKLDETR-----TVNPEECEGEVDAEIVVHEGPE-----	1460
Plicaturopsis	GFALLAQMTLLALEGPAVLDTAGLELADKDQDNSYAGEAAVENEEREVAMLLTEV-----	1347
Coprinopsis	SDEEEDMRRINDMLAGRIQAGDGSFPDMVDEDTDIASSDDEMD-----	1457
Agaricus	EQFLLAQKMRREALLKAGLTSGYPRAINSLKALHDVMPEELKAKSPERHYEKIGKFFEDI	1607
Hebeloma	-----	
Hypholoma	-----	
Laccaria	-----	
Serpula	-----	
Plicaturopsis	-----	
Coprinopsis	-----	
Agaricus	YGGTAKSVQTLDDIYPDMGQVSLVIGYGLIYGH3KVVPALET3YTLVASLIAGDTPLQI	1667
Hebeloma	-----	
Hypholoma	-----	
Laccaria	-----	
Serpula	-----	
Plicaturopsis	-----	
Coprinopsis	-----	
Agaricus	GWHLNGARRGGATLEQVRAVREISIEVAKRCGVVW33SVPEVAEKLPEDE	1717

Figure 14: CLUSTALW2 alignment of dicer like protein sequence from *Laccaria bicolor* with closely related other fungal dicer like proteins

Dicer like protein sequences of *Hebeloma cylindrosporum*, *Hypholoma sublateritium*, *Agaricus bisporus*, *Coprinopsis cinerea*, *Serpula lacrymans*, *Plicaturopsis crispa* were aligned with the sequence under consideration (Fig.14) (dicer like protein from *Laccaria bicolor*). The sequence was found to be majorly conserved among these species. *Hebeloma cylindrosporum* was found to be the closest (alignment score 53.57)

4.1.5 Phylogenetic tree generation

MultAlin software was used to align all the sequences to be further utilized for tree generation. Phylogenetic tree generation was done using MEGA6 software. Neighbour Joining method was used with 1000 bootstrap replicates.

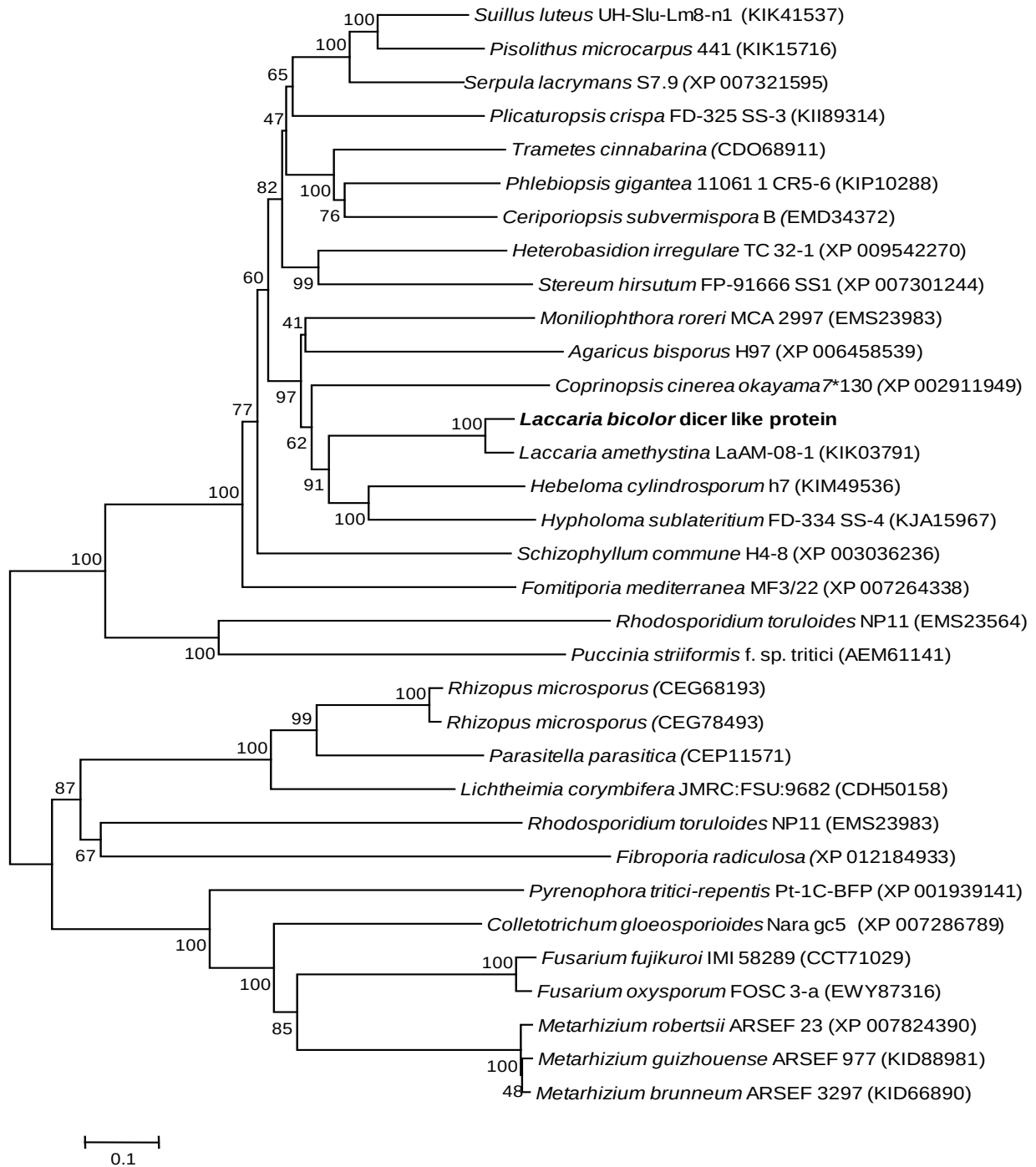


Figure 15: Phylogenetic tree of dicers in fungi. *Laccaria bicolor* falls in cluster with *Laccaria amethystina*, *Hebeloma cylindrosporum*, *Hypholoma sublateritium*

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 10.30702395 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 33 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 791 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. The value 0.1 denotes the length of the branch that represents an amount genetic change of 0.1

Laccaria bicolor dicer like protein has maximum similarity with *Laccaria amethystina*, followed by similarity with *Hebeloma cylindrosporum* and *Hypholoma sublateritium*. They form the same cluster with *Laccaria bicolor* and are ectomycorrhizal fungi (Fig.15)

Similarly dicer like proteins from different plants/animals and insects were searched from NCBI database. Same tools were used till tree generation for comparison with the dicer like protein from *Laccaria bicolor*. The tree obtained was-

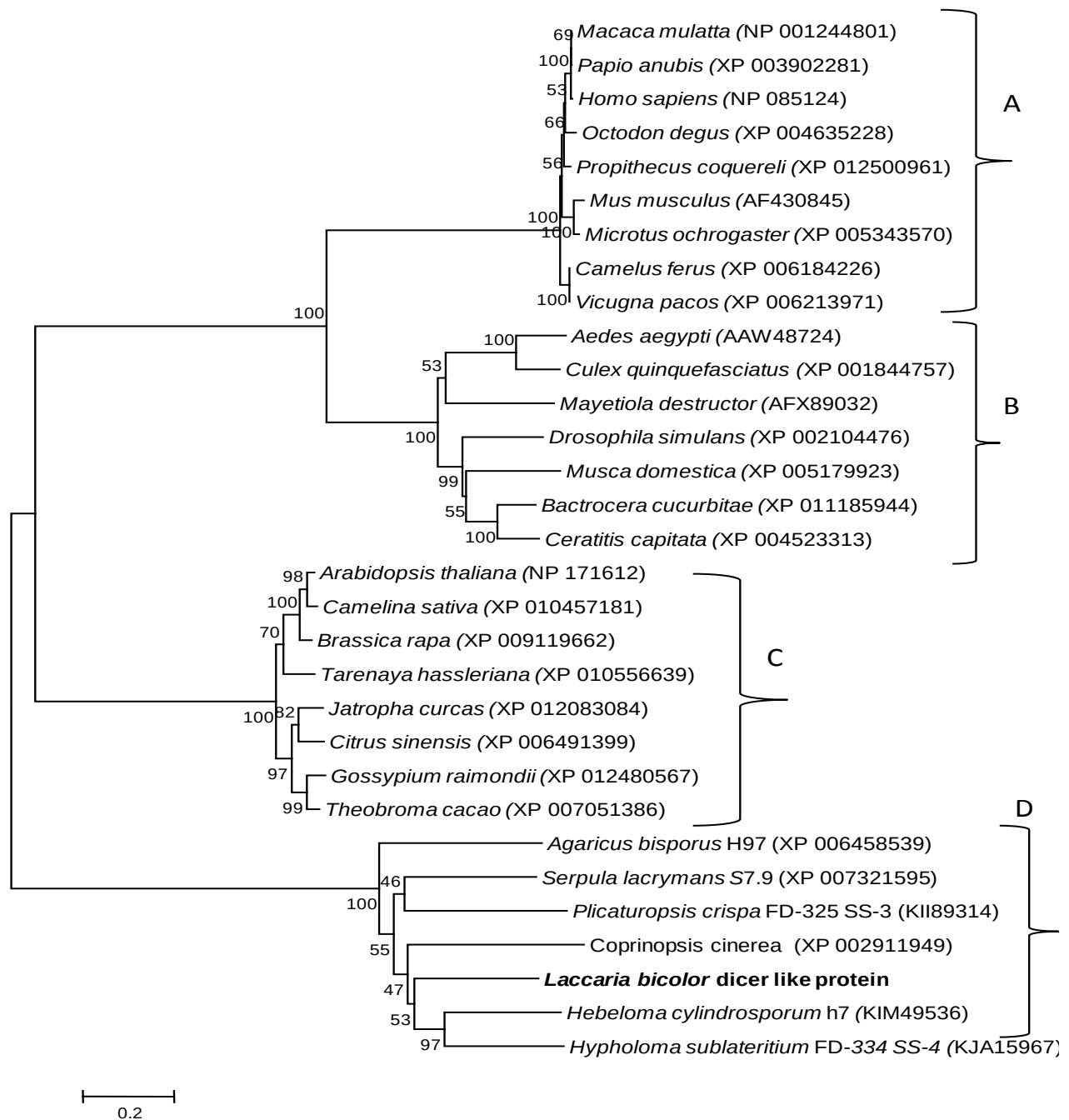


Figure 16: Phylogenetic tree of dicers in fungi, plants, insects and mammals. The dicer like proteins are distributed as per their nature. Cluster A: mammals; Cluster B: insects; Cluster C: plants; Cluster D: fungi

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 7.02157128 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 31 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 758 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. The value 0.2 denotes the length of the branch that represents an amount genetic change of 0.2.

Four clusters were formed in the phylogenetic tree (Fig.16). Cluster A was of mammals, cluster B of insects, C of plants and D of fungi. *Laccaria bicolor* (dicer like protein) falls in the cluster of all fungi i.e cluster D (Fig.16). Since our protein of interest falls in the cluster of fungi, it is conserved and has maximum similarity with these; whereas the dicer like proteins found in plants, mammals and insects have maximum similarity with their respective clusters.

4.2 Expression analysis of dicer

4.2.1 Growth kinetics

4.2.1.1 The effect of different concentrations of glucose on the growth of *Laccaria bicolor*

The response of *Laccaria bicolor* was assessed by growing mycelial cultures on pre processed cellophane discs placed on MMN agar medium supplemented with different concentrations of glucose (0.01%, 0.1%, 0.2% and 0.3%) and the biomass was harvested after 30 days of incubation at 25°C and the diameter and dry weight were recorded. The assessment of fungal biomass is used as a measurement of growth.

According to the results there was a variation in the diameter of the growth of fungus in different concentrations of glucose. The mean diameters suggested that when 0.3% glucose was used in the medium then the growth was best as compared to the other percentages. As the concentration of carbon source decreased, so did the diameter (Table 4 Fig.17).

Even the dry weight indicates that the growth is best in a higher percentage of carbon source. Thus it can be concluded that with increasing concentration of carbon source, the growth of *Laccaria bicolor* also increased (Table 5 Fig.18).

Table 4: Effect of different concentrations of glucose on the mycelial growth of *Laccaria bicolor*

Glucose (%)	Diameter (cm)
0.01	3.267 ± 0.115
0.10	4.133 ± 0.115
0.20	4.400 ± 0.200
0.30	4.867 ± 0.115

Values are Mean ± SD (n=3)

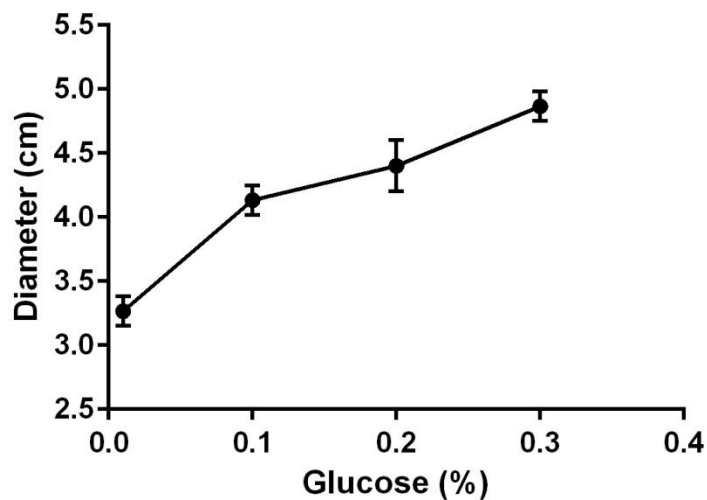


Figure 17: Effect of glucose concentrations on the mycelial growth of *Laccaria bicolor*

Table 5: Effect of different concentrations of glucose on the dry weight of *Laccaria bicolor*

Glucose (%)	Dry weight (mg)
0.01	29.333 $\pm$ 0.503
0.10	46.300 $\pm$ 0.458
0.20	49.533 $\pm$ 0.666
0.30	59.300 $\pm$ 0.954

Values are Mean $\pm$ SD (n=3)

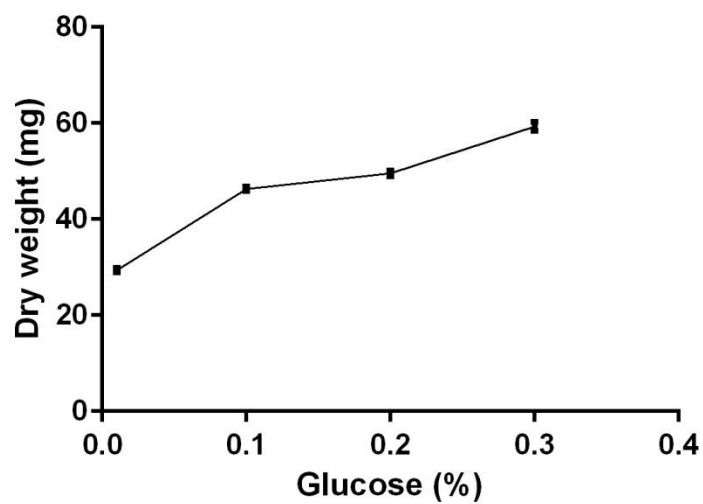


Figure 18: Effect of different glucose concentrations on the dry weight of *Laccaria bicolor*

4.2.1.2 The effect of different nitrogen sources on the growth of *Laccaria bicolor*

The response of *Laccaria bicolor* was assessed by growing mycelial cultures on pre processed cellophane discs placed on MMN agar medium supplemented with various nitrogen sources (ammonium hydrogen phosphate, ammonium nitrate, ammonium sulphate, urea). The mycelium was grown for 30 days at 25°C. After 30 days the diameter and dry weight were recorded. The assessment of fungal biomass is used as a measurement of growth.

According to the results the maximum diameter was observed when the ‘ammonium hydrogen phosphate’ was used in MMN medium as a nitrogen source (Table 6 Fig.19). The second best growth was observed on ‘ammonium nitrate’, then ‘ammonium sulphate’ and the least growth was observed when ‘urea’ was used. This indicated that *Laccaria bicolor* can utilize different nitrogen sources (organic or inorganic) but the best growth was in ‘ammonium hydrogen phosphate’ and the least when urea was used which means the fungus had a difficulty in the uptake of this source as compared to others.

According to the biomass measured (mg) (Table 7 Fig.20) it was clear that the growth is optimum in ammonium hydrogen phosphate as compared to other sources. This proved that the fungus can utilize ammonium hydrogen phosphate the most.

Table 6: Effect of different nitrogen sources on the mycelial growth of *Laccaria bicolor*

Nitrogen sources	Diameter (cm)
Ammonium Hydrogen Phosphate	5.133 $\pm$ 0.115
Ammonium Nitrate	4.400 $\pm$ 0.200
Ammonium Sulphate	4.333 $\pm$ 0.231
Urea	4.000 $\pm$ 0.000

Values are Mean $\pm$ SD (n=3)

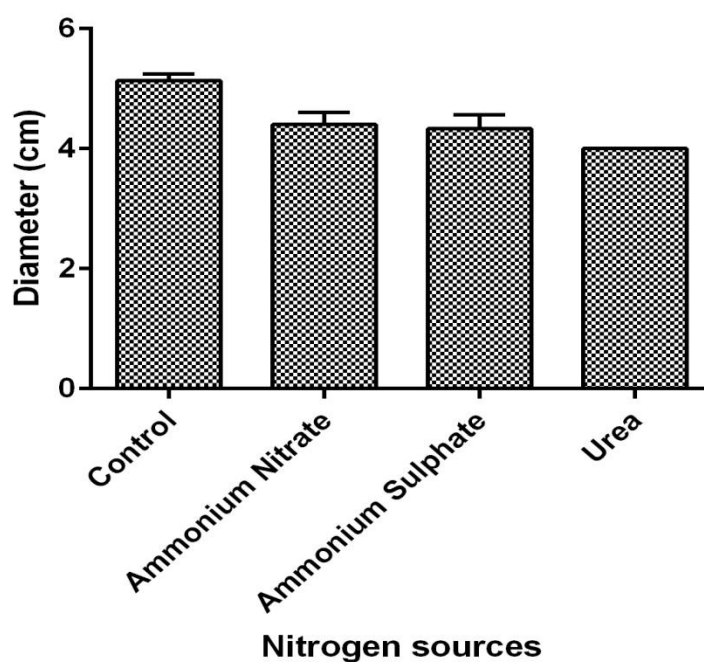


Figure 19: Effect of various nitrogen sources on the mycelial growth of *Laccaria bicolor*

Table 7: Effect of different nitrogen sources on the dry weight of *Laccaria bicolor*

Nitrogen sources	Dry weight (mg)
Control (Ammonium Hydrogen Phosphate)	57.633 ± 2.011
Ammonium Nitrate	48.300 ± 1.212
Ammonium Sulphate	46.900 ± 0.361
Urea	42.833 ± 0.551

Values are Mean ± SD (n=3)

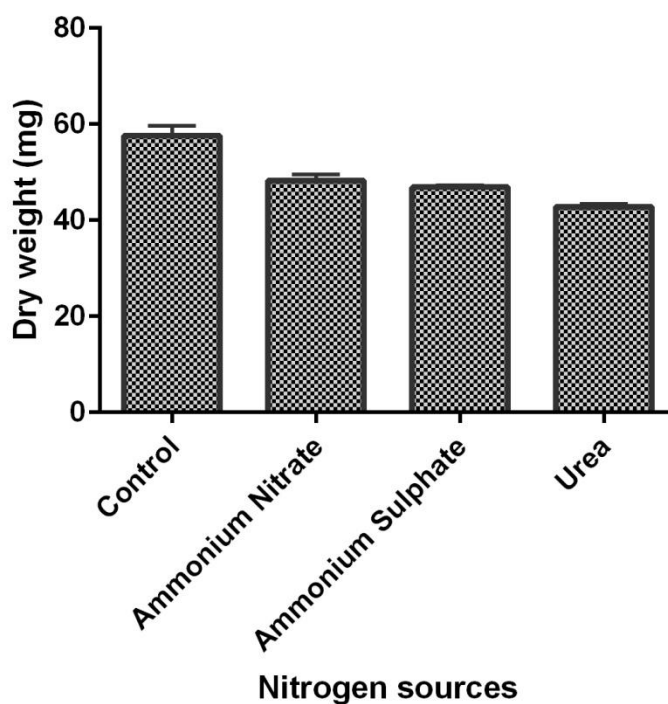


Figure 20: Effect of various nitrogen sources on the dry weight of *Laccaria bicolor*

4.2.2 RNA isolations and cDNA synthesis

The mycelium grown on MMN medium with different concentrations of glucose was scrapped after 30 days of incubation at 25°C and crushed in liquid nitrogen. Total RNA isolation (Fig.21) was carried out from the crushed mycelium (*Laccaria bicolor*) via TRIzol method. cDNAs were prepared using 5 µg RNA sample using the reverse AID™ First Strand cDNA Synthesis Kit, Fermentas Life Sciences, USA.

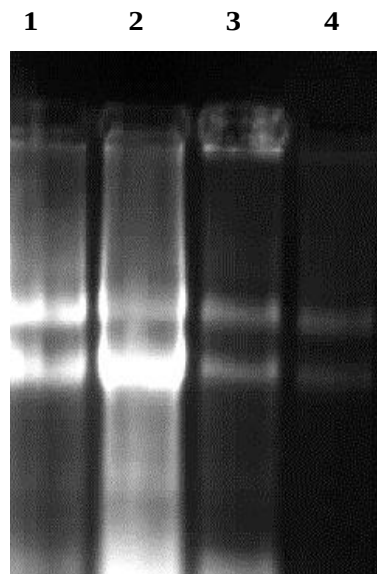


Figure 21: Total RNA isolation from *Laccaria bicolor* grown on different concentrations of glucose. Lanes 1, 2, 3 and 4: Total RNA from mycelium grown on 0.01, 0.1, 0.2 and 0.3% glucose respectively

Real time PCR primers were designed and the cDNA prepared (Fig.22) was checked by amplifying the dicer gene using the qPCR primers LbdicF (Table 2) and LbdicR (Table 2).

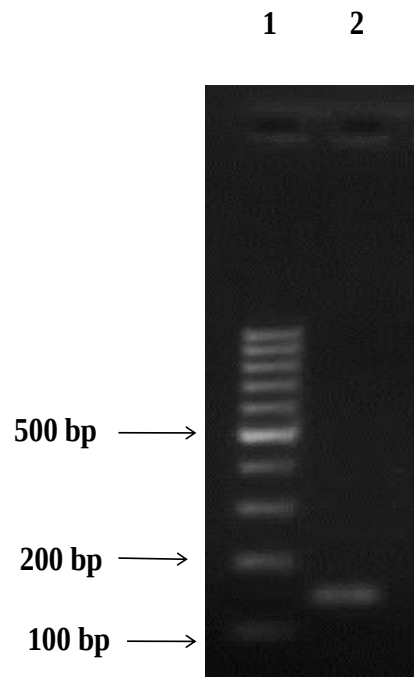


Figure 22: Amplification of cDNA of *Laccaria bicolor* with LbdicF and LbdicR primers. Lane 1: 100 bp ladder; Lane 2: amplified cDNA

4.2.3 qPCR (Real-Time PCR)

Real-Time PCR of various cDNA synthesized from mycelium of *Laccaria bicolor* at different concentrations of glucose was done.

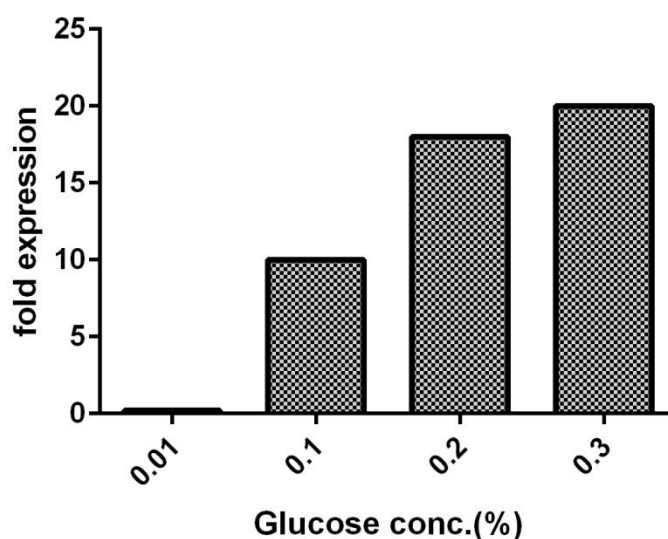


Figure 23: Real-Time PCR graph showing fold increase with respect to increasing glucose concentrations

There is a directly proportional relation between increase in the glucose concentration and the level of expression (Fig.23). There is a gradual increase in the level of expression of dicer gene with increasing percentages of glucose. This proves that there is a maximum level of expression when the concentration of glucose was maximum.

4.3 Cloning of dicer

4.3.1 RNA isolation and cDNA preparation

Total RNA isolation (Fig.24) was done from the crushed mycelium (*Laccaria bicolor*) grown on MMN agar medium for 30 days at 25°C. cDNA was synthesized with 5 µg of RNA.

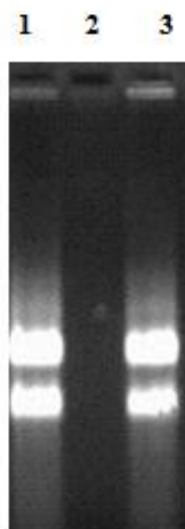


Figure 24: Total RNA isolation from *Laccaria bicolor* grown on MMN agar medium. Lanes 1 and 3: Total RNA isolated from *Laccaria bicolor*

4.3.1.1 Amplification of cDNA

Amplification of cDNA using gene specific primers LBDLPF1 and LBDLPR1 was carried out in a PCR. The amplicon obtained was of 3.5 kb (Fig.25).

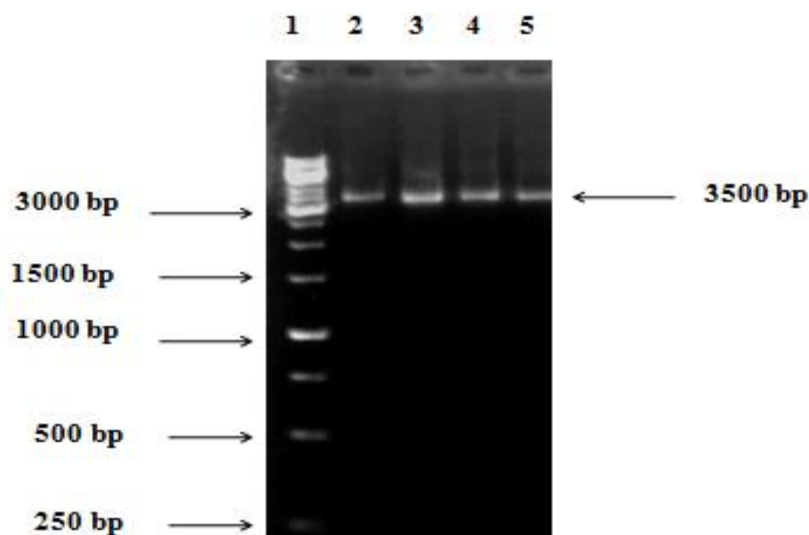


Figure 25: Amplification of cDNA of *Laccaria bicolor* with LBDLPF1 and LBDLPR1 primers. Lane 1: 1 kb ladder; Lanes 2, 3, 4 and 5: amplified cDNA

4.3.2 Transformation of DH5α with pET23a

The plasmid expression vector pET23a was introduced into the competent cells of DH5α by heat shock method. The colonies observed were screened for the plasmid.

4.3.2.1 Plasmid isolation

The colonies were screened for the presence of the plasmid by the method of plasmid isolation. The band observed after gel electrophoresis was observed around 3 kb (Fig.26) which is slightly less than the original size of the vector (3.6 kb) which might be due to supercoiling. This led to the confirmation of the presence of plasmid.

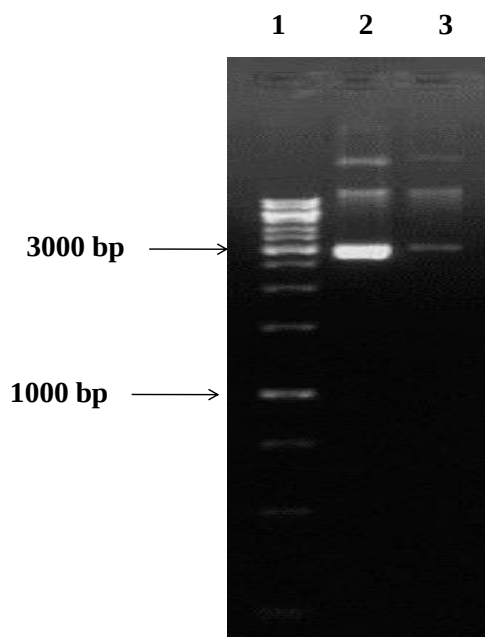


Figure 26: Plasmid (pET23a) isolated from DH5α cells. Lane 1: 1kb ladder; Lanes 2 and 3: pET23a

4.3.3 Transformation of *E.coli* DH5α with pET23a+insert

The amplicon of 3.5 kb obtained from *Laccaria bicolor* was digested along with the plasmid (3.6 kb) and they were eluted from the gel and ligated in order to be introduced in the DH5α cells. Thus transformation was carried out by heat shock method and the colonies observed the next day were screened for the presence of the recombinant plasmid.

4.3.3.1 Colony PCR and Plasmid isolation

Colony PCR was carried out with gene specific primers (LbdicF and LbdicR) to check the presence of recombinant plasmid in the colonies. The bands observed after electrophoresis were around 100 bp (Fig.27) thus confirming the presence of plasmid. The positive clones were further used for isolation of recombinant plasmid.

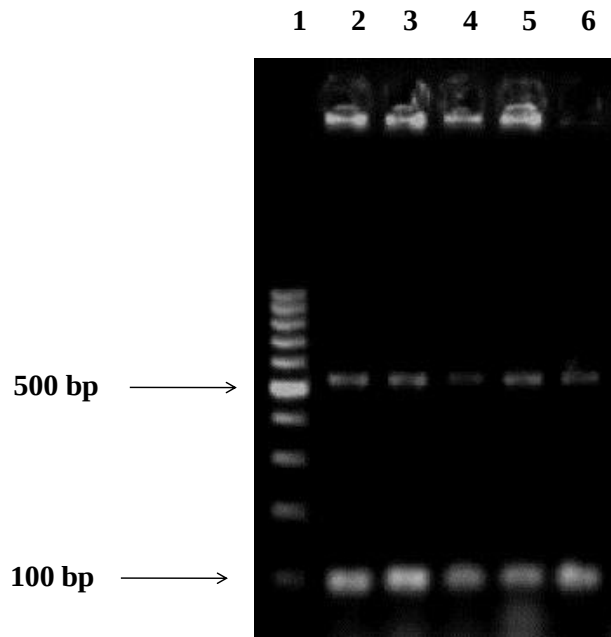


Figure 27: Positive colonies observed after colony PCR on agarose gel. Lane 1: 100 bp ladder; Lanes 2, 3, 4, 5 and 6: positive colonies for plasmid

4.3.3.2 Plasmid amplification to confirm insert in *E.coli* DH5a

Gene specific primers were used to confirm the presence of insert in the plasmid after isolation. By using LBDLPF1 and LBDLPR1, it was confirmed that the insert was present in the plasmid by normal gene amplification cycle. A band at 3.5 kb was observed indicating the size and the presence of amplicon (Fig.28). The internal primers (LbdicF and LbDicR) were used for testing as well and the band observed was slightly above 100 bp, thus confirming the presence of insert.

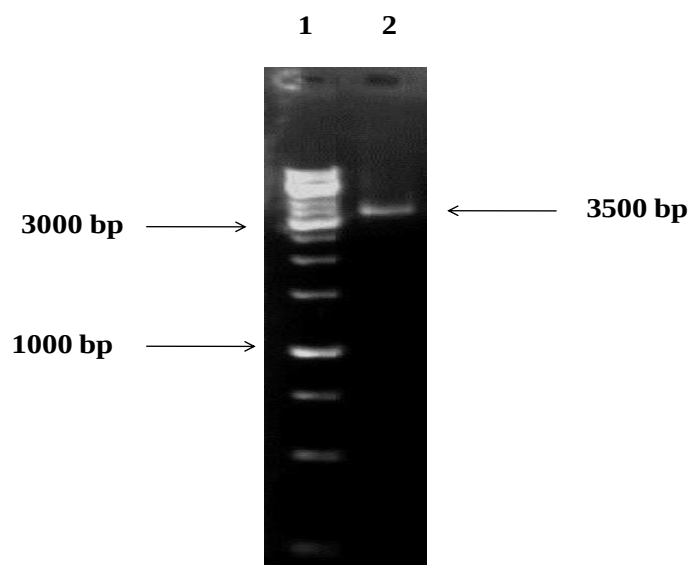


Figure 28: Plasmid amplification to confirm the gene present using LBDLPF1 and LBDLPR1 primers. Lane 1: 1 kb ladder; Lane 2: insert in the recombinant plasmid

4.3.4 Transformation of BL21De3 cells with pET23a+insert, colony PCR and plasmid isolation

The recombinant plasmid isolated from DH5α cells was further used to transform BL21De3 cells by heat shock method and the colonies observed the next day after overnight incubation at 37°C were screened for the presence of recombinant plasmid. Colony PCR was done to get positive clones and those clones were further used for plasmid isolation (Fig.29)

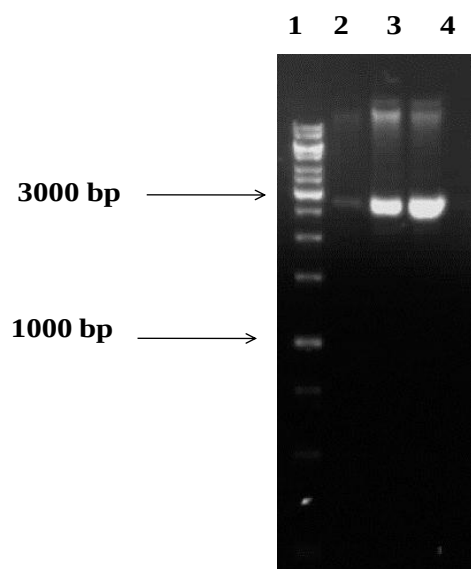


Figure 29: Recombinant plasmid (pET23a) with insert isolated from BL21De3 cells. Lane 1: 1kb ladder; Lanes 2, 3 and 4: recombinant plasmid

4.3.4.1 Plasmid amplification to confirm insert in *E.coli* BL21De3

Amplification reactions with gene specific primers (LBDLPF1, LBDLPR1, LbdicF and LbdicR) were carried out to confirm the presence of insert in the plasmid isolated from BL21De3 which was positive thus confirming the presence of insert.

4.3.5 Protein production and SDS PAGE analysis

From the positive clone of BL21De3, calibration or optimization of protein production via IPTG induction was done. SDS PAGE was carried out to check the presence of total protein. The bands were observed both in control and test sample at different time intervals after IPTG induction. The time points taken after addition of IPTG were- 3, 6, 9 and 12 hours.

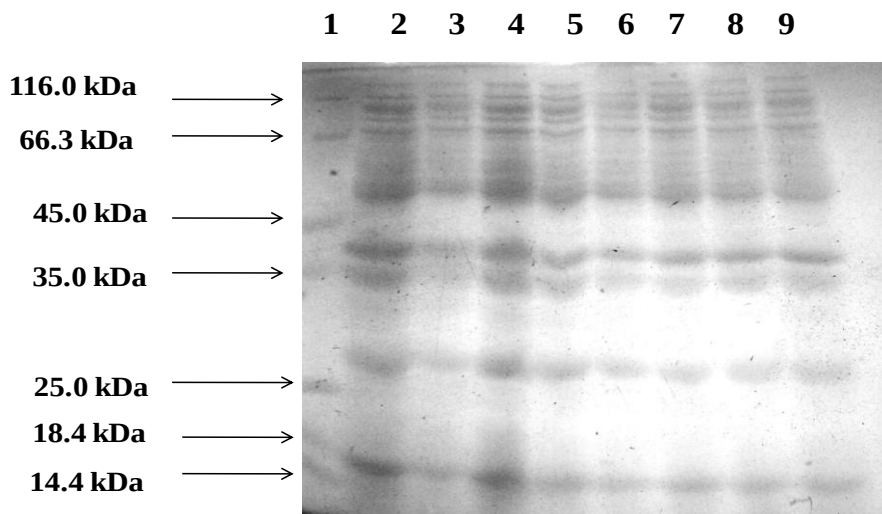


Figure 30: Total protein isolation after IPTG induction. Lane 1: protein molecular weight marker (Fermentas); Lanes 2, 4, 6 and 8: Controls (pET23a in BL21De3) at 3, 6, 9 and 12 hrs respectively; Lanes 3, 5, 7 and 9: Test (recombinant plasmid with gene of interest) at 3, 6, 9 and 12 hrs.

From the gel picture, it is evident that protein production occurred after IPTG induction at all the time intervals. No significant difference could be made out as far as the level of protein production is concerned in control and test.

Discussion

The present study was aimed at cloning and characterization of dicer like protein from ectomycorrhizal fungus *Laccaria bicolor*. The dicer gene sequence was retrieved from *Laccaria bicolor* genome and was translated using ORF finder and the protein contained 1122 amino acids sequence was obtained.

Sequence analysis was performed with BLAST program. The results showed that the dicer gene sequence retrieved from *Laccaria bicolor* genome had similarity with other fungal dicer like protein sequences.

Ectomycorrhizal fungi improve plant nutrition by increasing nitrogen, phosphate and potassium uptake (Harley and Smith, 1983). Ectomycorrhizas protect host plant roots against root pathogens and also against root herbivorous soil microfauna (Smith and Read, 1997). Nitrogen availability has been a primary factor which limits forest growth. Ectomycorrhizal fungi have been presumed to contribute to the nitrogen nutrition of the host plants and the increased surface area of ectomycorrhizal root systems (Bowen, 1973) helps in increasing the efficiency of absorption and translocation of inorganic nitrogen to the root (France and Reid, 1983). The capacity of ectomycorrhizal fungi has been reported to be low, where organically bound forms of nitrogen are concerned (Lundeberg and Read, 1990)

The effect of different nitrogen sources (organic/inorganic) on the growth of *Laccaria bicolor* was observed by growing the fungus on MMN agar media at 25°C in dark for 30 days. According to the diameter of the mycelium and the dry weight, it was observed that the maximum growth was in the presence of ammonium hydrogen phosphate and minimum growth was in presence of urea which is an organic source.

D-Glucose has been reported to be used more by fungi as compared to other sugars (Lilly and Barnett, 1951)

The effect of different concentrations of glucose on the growth of the fungus was also observed. Based on the dry weight and the mycelial diameter it was evident that the maximum growth was in the maximum concentration (0.3%) of glucose.

RNA isolation was performed, cDNAs were prepared and the expression levels were checked using Real-Time PCR, and the dicer gene showed maximum expression when the concentration of glucose was maximum. This showed that during stress the level of gene expression goes down.

CHAPTER 5

CONCLUSION

In the present study, the sequence for dicer gene was obtained from the database. Various bioinformatics tools were utilized for the analysis of conserved regions, domains throughout the history of evolution. Phylogenetic trees were constructed and their analysis proved that the dicer like proteins are present throughout various species of plants, mammals, insects and fungi.

RNA isolation from *Laccaria bicolor* which is an ectomycorrhizal fungus was done and the cDNA was prepared. The primers designed for the targeted gene were used for amplification and hence the desired gene was amplified. This was further used to for restriction digestion, along with the expression vector pET23a and ligation. Cloning experiments were successful and the positive clone from BL21De3 cells was used to calibrate protein production via IPTG induction, which was successful as there was protein production.

Thus by the present study, it is clear that *Laccaria bicolor* does have dicer like protein and this could be further isolated, purified and used for dicing assays.

The expression analysis of the dicer gene in presence of different glucose concentrations led to the conclusion that the gene expression is increased when the fungus gets a good concentration of carbon source via the medium and is less when there is starvation.

CHAPTER 6

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APPENDIX

TBE Buffer (10X)

Tris-HCl 0.09 M (pH 8)

Boric acid 0.9 M

EDTA 0.02 M (pH 8)

Agarose gel loading dye (6X)

Bromophenol blue 0.25%

Xylene cyanol FF 0.25%

Glycerol in water 30.0%

Luria-Bertani (LB) Medium

Ingredient Quantity (g/l)

NaCl 10.0

Beef extract 5.0

Tryptone 10.0

pH adjusted to 8.0 with 1N NaOH, sterilized by autoclaving at 15 lbs pressure (121 °C) for 15 min.

LB+ Amp.agar plates

Prepared LB broth as above. Agar (15 g/l) was added, media was autoclaved, and cooled to 50°C. Ampicillin 50 µg/ml was added. Poured plates and stored at 4°C.

LB+ Amp.+ Chloramphenicol agar plates

Prepared LB broth as above. Agar (15 g/l) was added, media was autoclaved, and cooled to 50°C. Ampicillin 50 µg/ml and chloramphenicol (34 µg/ml) was added. Poured plates and stored at 4°C.

Plasmid extraction solution I (10X)

Tris-HCl 25 mM (pH 8.0)

Glucose 50 mM

Na₂EDTA 10mM

Plasmid extraction solution II

NaOH 5M

SDS 10%

Plasmid extraction solution III

5.0 M K-acetate (pH 4.5)

Modified Melin-Norkrans medium

(NH₄)₂HPO₄ 250 mg/L

KH₂PO₄ 500 mg/L

MgSO₄·7H₂O 150 mg/L

CaCl₂·H₂O 50 mg/L

NaCl 25 mg/L

1 % (w/v) FeCl₃ 1.2 ml/L

Thiamine HCl 40 mg/L

Biotine 0.4 mg/L

Glucose 2500 mg/L

Heller's micronutrient (100x) 10 ml/L

Agar 15 g/L

Heller's Micronutrient solution

FeCl₃·6H₂O 100 mg/L

ZnSO₄·7H₂O 100 mg/L

H₃BO₃ 100 mg/L

MnSO₄·4H₂O 10 mg/L

CuSO₄ ·5H₂O 3 mg/L

AlCl₃ 3 mg/L

NiCl₃.6H₂O 3 mg/L

KI 1 mg/L

pH adjusted to 5.5-5.7; autoclaved for 10 minutes at 121°C for 15 minutes.

IPTG stock solution (0.1M)

1.2 g IPTG

Added water to make up 50 ml final volume. Filter sterilized and stored at 4°C.

X-Gal (2ml)

100 mg 5-bromo-4-chloro-3-indolyl-D-galactoside dissolved in 2ml N,N'- dimethylformamide.

Covered with aluminum foil and stored at 20°C.

Buffer P1 (resuspension buffer)

50 mM Tris·Cl, pH 8.0

10 mM EDTA

100 µg/ml RNase A

Buffer P2 (lysis buffer)

200 mM NaOH

1% SDS (w/v)

Buffer N3 (neutralization buffer)

3.0 M potassium acetate pH 5.5

Buffer PB (wash buffer) 1.0 M NaCl

50 mM MOPS, pH 7.0

15% isopropanol (v/v)

Buffer EB (elution buffer) 1.25 M NaCl

50 mM Tris·Cl, pH 8.5

15% isopropanol (v/v)

Cracking buffer

0.5 M Tris (pH 6.8)

SDS (10%)

Glycerol (50%)

β -mercaptoethanol 0.8 ml

Bromophenol blue 0.3 g

Resolving gel (12%)

Distilled water 3.33 ml

1.5M Tris pH 8.8 2.5 ml

Acrylamide solution 2.5 ml

SDS (10%) 100 μ l

TEMED 10 μ l

APS (10%) 70 μ l

Stacking gel (4%)

Distilled water 3 ml

1M Tris pH 6.8 1.25 ml

Acrylamide solution 0.8 ml

SDS (10%) 25 μ l

TEMED 10 μ l

APS (10%) 70 μ l

Tank Buffer (1 liter)

Tris 6.05 g

Glycine 28.8 g

SDS (10%) 10 ml

Distilled water to make up the volume to 1 liter

Staining Solution

Coomassie Blue R250 0.3 g

Methanol 80 ml

Glacial acetic acid 20 ml

Distilled water 100 ml

Destaining Solution

It's the same as staining solution except that there is no dye in it.