Computational Tools: RNA Interference in Fungal Therapeutics

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Abstract

There is steady rise in the number of immunocompromised population due to increased use of potent immunosuppression therapies. This is associated with increased risk of acquiring fungal opportunistic infections in immunocompromised patients which account for high morbidity and mortality rates, if left untreated. The conventional antifungal drugs to treat fungal diseases (mycoses) are increasingly becoming inadequate due to observed varied susceptibility of fungi and their recurrent resistance. RNA interference (RNAi), sequence-specific gene silencing, is emerging as a promising new therapeutic approach. This chapter discusses various aspects of RNAi, viz., the fundamental RNAi machinery present in fungi, in silico siRNA features, designing guidelines and tools, siRNA delivery, and validation of gene knockdown for therapeutics against mycoses. Target gene identification is a crucial step in designing of gene-specific siRNA in addition to efficient delivery strategies to bring about effective inhibition of fungi. Subsequently, designed siRNA can be delivered effectively in vitro either by soaking fungi with siRNA or by transforming inverted repeat transgene containing plasmid into fungi, which ultimately generates siRNA(s). Finally, fungal inhibition can be verified at the RNA and protein levels by blotting techniques, fluorescence imaging, and biochemical assays. Despite challenges, several such in vitro studies have spawned optimism around RNAi as a revolutionary new class of therapeutics against mycoses. But, pharmacokinetic parameters need to be evaluated from in vivo studies and clinical trials to recognize RNAi as a novel treatment approach for mycoses.

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12.1   Introduction

The last two decades have seen a rising trend in the immunocompromised patients (Richardson 1991; Krcmery 1996; Warnock and Richardson 1991; Ribes et al. 2000), who are at a heightened risk for "opportunistic" fungal infections (mycoses). The fungal infections can affect any part of the body and cause superficial mycoses (affecting only the outermost layers of the skin and hair), cutaneous mycoses (extending deeper into the layers of the skin, hair, and nail), subcutaneous mycoses (involve the dermis, subcutaneous tissues, muscle, and fascia), or systemic mycoses (dissemination in the body). The fungal diseases (listed in Table 12.1) are classified according to the three important phyla of the fungal taxonomy – Ascomycetes, Basidiomycetes, and Zygomycetes. These diseases turn out to be severe if disseminated, which is common in the immunosuppressed. Candidiasis is one of the highly frequent fungal infections in 90% of untreated, advanced HIV cases suffering from oropharyngeal candidiasis according to World Health Organization (WHO) 2010 statistics. Another study reported overall mortality rate of approximately 80% for zygomycosis (Ribes et al. 2000) and 80–90% in invasive aspergillosis of high-risk leukemia patients and allogeneic bone marrow transplant patients (Chamilos and Kontoyiannis 2005).

The existing fungal treatments require parallel approaches involving surgical intervention and antifungal therapy (Ribes et al. 2000). Surgical intervention has shown improving survival rates for many patients, but surgery is not the only solution in treating fungal diseases. The currently available antifungal drugs comprise polyenes, macrolides, azole drugs, and echinocandins (Mukherjee et al. 2005). Among them, the polyene amphotericin B is the first-line drug of choice for invasive mycoses (Ribes et al. 2000). These drugs have drawbacks such as nephrotoxicity and hepatotoxicity as exhibited by liposomal amphotericin B, narrow spectrum of activity as shown by fluconazole, and itraconazole causes problems with absorption (Pauw and Picazo 2008). Moreover, the fungi are acquiring resistance recurrently against novel antifungal agents (Rogers 2008) either due to defects in drug import and efflux of drugs, variations in intracellular drug processing, alterations in the enzymes of a target-specific biosynthetic pathway, or its competitive inhibition (Chamilos and Kontoyiannis 2005; Bhanderi et al. 2009).

The existing treatments are not effective due to varied susceptibility of fungi toward the drugs; hence, newer safe and effective therapeutic interventions are desired. There have been reports of the use of gene therapy, antisense oligonucleotides, ribozymes, DNAzymes, and RNAi therapy. RNAi scores several

Table 12.1 Fungal diseases associated with immunocompromised patients

Disease	Causal fungal agent	Symptoms	Treatment
Ascomycetes			
Candidiasis	*Candida albicans*, *C. tropicalis*	Localized infection of the skin or mucosal membranes like the vagina: itching, burning, soreness, irritation, and a whitish discharge	Clotrimazole, nystatin, amphotericin B, fluconazole, caspofungins
Aspergillosis	*Aspergillus fumigatus* (causes more than 95% of cases), *A. flavus*	Fungus balls in lungs, coughing up blood; fever, chest pain, difficulty breathing; aspergillosis of the ear canal causes itching and occasionally pain and fluid draining	Amphotericin B, voriconazole, caspofungin
Histoplasmosis	*Histoplasma capsulatum*, *H.duboisii* (causes disease restricted to Africa and Madagascar)	Acute respiratory infection leads to respiratory symptoms, malaise, fever, chest pains, dry cough, flu-like illness, fever, cough, headache	Fluconazole, itraconazole, amphotericin B
Coccidioidomycosis	*Coccidioides immitis*	Only 40% cases symptomatic, flu-like symptoms, fever, cough, headache, rash, myalgias, chest pain, fatigue, arthralgia	Fluconazole, amphotericin B, voriconazole, posaconazole
Blastomycosis	*Blastomyces dermatitidis*	Fever, chills, myalgia, arthralgia, pleuritic chest pain, cough, difficulty breathing can become disseminated to mostly skin, bones, and genitourinary tract	Amphotericin B, itraconazole, voriconazole
Penicilliosis	*Penicillium marneffei*	Fever, weight loss, anemia, pneumonitis, skin lesions, pharyngeal, and palatal lesions	Amphotericin B, itraconazole
Basidiomycetes			
Cryptococcosis	*Cryptococcus neoformans*, *C.grubii*	Headache, fever, visual loss, osteolytic bony lesions, meningitis, calcification in lungs	Fluconazole, amphotericin B

(continued)

Table 12.1 (continued)

Disease	Causal fungal agent	Symptoms	Treatment
Zygomycetes			
Zygomycosis	*Rhizopus oryzae, R. rhizopodiformis, Rhizomucor pusillus, Absidia corymbifera, Apophysomyces elegans, Mucor circinelloides*	Facial pain, headache, nausea, fever, blood or pus draining from nose, lethargy, impaired vision, bulging eyes, convulsions, ulcers in roof of mouth, may become disseminated	Amphotericin B, posaconazole

benefits over other techniques, for instance, its effective delivery into various organs at low concentrations, thereby increasing safety, its cost-effectiveness as compared to protein/enzymes, and its non-immunogenicity bypassing the interferon pathway (Ruddon 2007). Subsequently, it was marked the "Breakthrough of the Year" in 2002 by *Science magazine*. One of the recent developments in this field has been the FDA approval of siRNA (short interfering RNA) drug DGFi in 2008, developed by Quark Pharmaceuticals, Inc., for use in kidney transplantation. Another siRNA-based therapy, Sirna-027 originally developed by Sirna Therapeutics for the treatment of acute macular degeneration, is moving forward into phase II clinical trials. Hence, the vital relevance of innovative treatments like the siRNA therapy can be envisaged for antifungal infections. RNAi application has also been identified in functional genomics and therapeutics, viz., cancer, neurodegenerative diseases, and multiple sclerosis besides fungal infection. This chapter presents the prospects of RNAi technology in fungal infection.

12.2 RNA Interference in Fungi

Gene cosuppression (RNAi-type phenomenon), which is more commonly known as "quelling" in fungi, was first discovered in the filamentous fungus *Neurospora crassa* by Romano and Macino (1992). The significant aspect of RNAi was established in 1998 when Fire and Mello found out that dsRNA is 10–100 times more effective in triggering silencing as compared to ssRNA in *Caenorhabditis elegans* (Fire et al. 1998) for which they shared the Nobel Prize in Medicine and Physiology in 2006.

RNAi naturally occurring "sequence-specific gene silencing" phenomenon and evolutionarily highly conserved mechanism are well developed in eukaryotic organisms, which is induced by double-stranded small noncoding RNA (dsRNA). These RNA-mediated gene silencing pathways have been comparatively thoroughly known and established in plant and animal system and are widely investigated in a variety of fungi, few of them are reported on opportunistic

pathogenic fungi in human. The RNAi machinery in fungi shares similar mechanistic principles with other organisms where RNAi has been discovered which consists of dsRNA precursors, Dicer enzyme, RISC (RNA-induced silencing complex), and the target mRNA. The dsRNA is processed into short interfering RNA (siRNA) by two steps – the initiator step and the effector step. In the *initiator step*, the dsRNA is cleaved by the enzyme Dicer of the RNase III family (Jinek and Doudna 2009) into specific lengths of 21–25 nucleotides. In the *effector step*, the siRNA must segregate into "competent" single strands (the guide strands) which are guided through a ribonucleotide protein complex called the RISC (Siomi and Siomi 2009), a member of the Argonaute family of proteins (Song et al. 2004a, b; Leuschner et al. 2006; Fulci and Macino 2007). Both Dicer and RISC complex require ATP for energy. The Argonaute has a N-terminal domain, PAZ domain, a middle domain, and a PIWI domain. It is the "guide strand" which is incorporated into RISC; the non-incorporated strand known as the "passenger strand" is also crucial for proper target mRNA cleavage. Until the passenger strand is not cleaved at ten nucleotides from the 5′ phosphate of the guide strand, target mRNA cleavage is severely impaired (Leuschner et al. 2006). The cleaved target mRNA is utilized by RNA-dependent RNA polymerase (RdRp) generating more dsRNA that is further recognized and cleaved by Dicer to increase the number of siRNA molecules (Schepers 2005). Genes responsible for RNAi have been discovered in fungi such as *qde-1* (*quelling deficient-1*) which was the first RNAi gene discovered in *N. crassa* which encodes a cellular RdRp. Simultaneously, *qde-2* was cloned and found out to encode Argonaute protein. Further, partially redundant Dicer proteins DCL-1 (Dicer-like-1) and DCL-2 have been characterized from *N. crassa* by reverse genetics (Li et al. 2010).

So far, RNAi mechanism has not been observed in *Saccharomyces cerevisiae*, *Candida guilliermondii*, *C. lusitaniae*, *C. tropicalis*, and *Ustilago maydis* (Nakayashiki 2005; Münsterkötter and Mannhaupt 2008). In *S.cerevisiae*, it has been attributed to the absence of conserved components like Dicer-like RNases, Argonaute or PIWI-like components, and RNA-dependent RNA polymerases. However, RNAi was very recently discovered in *S. castellii*, which is closely related to *S.cerevisiae*, and also in *C. albicans*, a common human pathogen, by David P. Bartel's lab [22] (Drinnenberg et al. 2009). They found out that these species with noncanonical Dicer activity have RNase III domain containing gene to generate siRNA(s). This gene is orthologous to RNase III domains of other Argonaute-containing budding yeasts and is mostly enriched in long inverted repeats and transposable elements.

12.3   siRNA Design and Computational Tools

A significant facet of siRNA design involves identification and characterization of gene target which plays a key role in the survival of the organism such that suppression of the gene by inhibiting translation should limit the growth of the fungus. A particular fungal disease is often caused by more than one fungus, for

instance, zygomycosis is caused by *Rhizopus*, *Absidia*, *Mucor*, and *Rhizomucor*. siRNA can be designed taking into consideration a conserved antifungal drug target present ubiquitously in the species, so that the drug discovery process becomes less intricate; but a crucial point to be taken care of is that the protein and its corresponding gene should not have similarities with the human genome, such that the human processes are not affected. For instance, cell wall is a very promising target in disease-causing fungi. The pathway for formation of cell wall is readily accessible (Moussian 2008), and the KEGG database (http://www.genome.jp/kegg/) can be referred. Silencing a gene coding for a significant component of the cell wall biosynthesis pathway can delimit the formation of cell wall and hence restrain the growth of the pathogenic fungus. For instance, a study reported loss of a cell wall polysaccharide, α-(1,3) glucan synthase from the cell walls of *Histoplasma capsulatum* which led to decreased virulence and pathogenesis of the fungi (Rappleye et al. 2004). Apart from the cell wall, targeting can be carried out against lipid biosynthesis pathways or at the translational or posttranslational levels. Previous studies have led to the confirmation of N-myristoyl transferase, lanosterol 14-α demethylase, and geranylgeranyl transferase I as potential drug targets against which antifungal drugs benzofurans, azoles, and azaphilones have been designed, respectively (Kawasaki et al. 2003; Song et al. 2004; Singh et al. 2005). Furthermore, orthologous and paralogous studies can be performed to check the presence of the target gene in other related organisms, which can highlight the evolutionary history of the gene, and information about gene conservation can acquaint us with the vital relevance of the gene.

siRNA should be a perfect complementary match to its target mRNA, and hence, it needs to be cautiously designed. Various computational tools are freely available online for designing siRNA. For instance, the Ambion's siRNA Target Finder, Eurofins MWG Operon's free online siMAX™ Design Tool, the BLOCK-iT™ RNAi Designer from Invitrogen, the SVM RNAi 3.6, and the siDESIGN Center by Dharmacon can be used for siRNA designing against fungal genes. The designing of siRNA molecule is purely dependent on suitable selection of various siRNA features, viz., sequence, motif, GC content, and thermodynamic features, etc. Moreover, various studies have documented the comparison among few siRNA design tools (Yiu et al. 2005; Matveeva et al. 2007). The tools mainly follow the Reynolds et al. (2004) or Tuschl et al. (1999) guidelines for rational siRNA design of usually 21 nucleotides in length. Few guidelines are described in onward section. According to these guidelines, regions within 50–100 bp of the start codon and the termination codon; intronic regions, stretches of 4 or more bases such as AAAA and CCCC; and regions with GC content less than 30% or more than 60%, single-nucleotide polymorphisms, repeats, and low complex sequences should be avoided.

12.3.1 General Guidelines for siRNA Designing

Many guidelines to design siRNA were proposed by different groups which are mentioned below.

12.3.1.1 MPI (Max-Planck-Institute) Rule Set

Tuschl et al. (1999) have provided a set of guidelines (commonly known as the MPI principles) on how to design effective siRNA.

Initially an empirical rules (based on GC content and symmetric 3′ TT overhangs) for effective siRNA designing were established by Tuschl et al. (1999), which have been found to show significant proportion of ineffective siRNAs as described by Yiu et al. (2005) and identify the need of understanding the structural features of sequences (Yiu et al. 2005). Therefore, the new rules were suggested with advancement of technology for effective designing of siRNA, (1) where select targeted region from a given cDNA sequence beginning 50–100 nt. downstream of start codon (2) afterward finds 23-nt sequence motif AA (N$_{19}$). If no suitable sequence is found, then find for 23-nt sequence motif NA(N$_{21}$) and convert the 3′ end of the sense siRNA to TT or search for [N (any nucleotide base pair)A (adenine) R (adenine or guanine (purines)) (N$_{17}$)Y(thymine or cytosine (pyrimidines)) NN], and the GC content must be around 50% in target sequence.

12.3.1.2 Rational siRNA Design

In the designing of efficient siRNA molecule, a rational rule sets (total of eight criteria with weight values) were developed after the experiential analysis of the silencing efficiency of 180 siRNAs targeting the mRNA of two genes, by Reynolds et al. (2004) at Dharmacon, Inc.; these rules/criteria cover the different compositional properties such as sequential features of individual siRNAs. These characteristics are used by rational siRNA design algorithm to evaluate potential targeted sequences and assign scores to them (Reynolds et al. 2004). The higher value or score of these sequences reflects the higher chance of success of RNAi experiment.

The tools offered by Dharmacon, Inc. deploy the eight criteria-based (rational siRNA design rules) guideline, while Maurice Ho et al. have developed the Excel-based template on similar guidelines and available at http://boz094.ust.hk/RNAi/siRNA.

It is clear from Fig. 12.1 that different base pair shows differential stability with respect to their position (H = A, C or U).

12.3.1.3 Challenges

Despite several in silico siRNA design cases being reported in fungal systems, there are challenges that need to be addressed. These include optimal siRNA designing, off targeting, and efficient delivery systems apart from the genomic architecture and levels of organizational complexity. For example, whole-genome duplication events are commonly observed in fungi (Wapinski et al. 2007; Dujon et al. 2004; Ibrahim et al. 2009) such as in *Rhizopus oryzae*, giving rise to paralogous genes might not have 100% nucleotide sequence similarity with each other, which are actively expressed to code for an enzyme. Such constraints are often envisaged while designing effective siRNA against a fungal enzyme, as targeting only one out of many genes for a given enzyme target doesn't result in a very high efficacy for
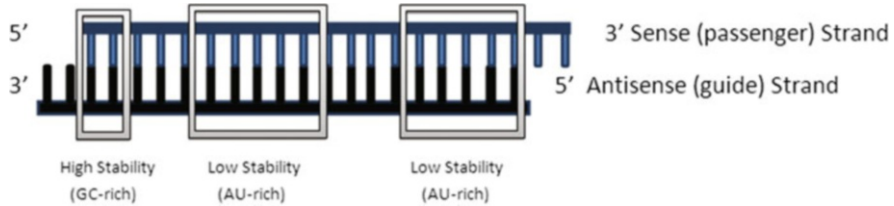
Fig. 12.1 Relative stability of both ends of the siRNA (Adopted from Reynolds et al. 2004)

silencing (Yamada et al. 2007) silenced only one out of the three α-amylase genes in *Aspergillus oryzae* and found merely 10% silencing, which is not sufficient to enter the therapeutic phase. Hence, designing common siRNA for all the genes targeting an enzyme becomes a very arduous task. Chitin synthase is a well-accepted antifungal drug target, yet it cannot be used to design siRNA(s) for *R. oryzae* because genome level analysis has revealed that 23 genes can encode chitin synthase (Ibrahim et al. 2009). The prospects of selecting the conserved regions of an enzyme are lucrative, as a single siRNA might be effective against several fungi causing a particular disease, but the enzyme should be present exclusively in fungi. Hence, target selection is critical toward its gene nature and diversity.

12.3.1.4 Resources and Tools
There are several tools available to design the siRNA, few of them are described in Table 12.2.

siVirus It is an effective siRNA designing tool for several divergent viral genomes such as human immunodeficiency virus (HIV), *hepatitis C virus* (HCV), influenza virus, and *SARS coronavirus*, based on various siRNA designing guidelines such as Ui-Tei et al. (2004), Reynolds et al. (2004), and Amarzguioui and Prydz (2004), while the off-targeting siRNA sequences were identified using siDirect tool. Out of 35 predicted siRNAs, 31 sequences of siRNA against conserved region of HIV-1 genome have shown inhibition of viral replication, which conclusively supports the designing capacity of these tools (Naito et al. 2006).

RNA Rule Set 1.0 It is a Java-based program to predict the efficient siRNA design. Basically siRNArules 1.0 operates with two scoring sums: one for the positive rules deduced from the set of the best siRNAs and one for the negative rules deduced from the set of the worst siRNAs. The rank is calculated by a simple sum of these two values. How these rules should be weighted to obtain the most efficient algorithm is a challenge for the open-source community (Holen 2006).

DSIR This tool was developed by Vert et al. (2006) which uses only two parameters for designing of siRNA, i.e., position-specific thermodynamic stability and non-position-specific motif formation. Their composite score decides which

Table 12.2 List of selected siRNA design tools

S. no	siRNA designing tool	Website
1	OligoWalk	http://rna.urmc.rochester.edu/cgi-bin/server_exe/oligowalk/oligowalk_form.cgi
2	BLOCK-iT™ RNAi designer	https://rnaidesigner.thermofisher.com/rnaiexpress/rnaiExpress.jsp
3	RNAi design tool	http://eu.idtdna.com/Scitools/Applications/RNAi/RNAi.aspx?source=menu
4	siDESIGN center	http://dharmacon.gelifesciences.com/design-center/
5	siRNA at whitehead	http://sirna.wi.mit.edu/
6	TROD	http://www.unige.ch/sciences/biologie/bicel/websoft/RNAi.html
7	AsiDesigner	http://sysbio.kribb.re.kr:8080/AsiDesigner/menuDesigner.jsf
8	GenScript's siRNA design center	http://www.genscript.com/design_center.html
9	SiDirect	http://sidirect2.rnai.jp/
10	Side	http://predictor.nchu.edu.tw/side/about_siDE.php
11	siVirus	http://sivirus.rnai.jp/
12	DSIR	http://biodev.extra.cea.fr/DSIR
13	SiSearch	http://www.biolabprotocols.com/details/2985/siSearch.html

siRNA is better through lasso regression technique. It also provides the options to design 19–21 bp siRNA. The effectiveness of this tool is comparable and evaluated as one of the best tools by Matveeva et al. (2007) while comparing with other tools.

12.4   RNAi Delivery

In silico siRNA designing is followed by its efficient delivery into an organism of choice for knockdown of the target gene. siRNA delivery strategies employed in fungi mainly comprise of soaking approach using chemically synthesized siRNA or inserting inverted repeat transgenes (IRT) into the desired plasmid using long-hairpin RNA (lhRNA). Table 12.3 lists some of the fungal species in which these strategies have been performed.

12.4.1 Soaking

This technique employs chemically synthesized siRNA(s) which are absorbed by the organism. Briefly, it involves annealing of the siRNA strands, quantification of the siRNA duplex, and soaking of the organism with these double-stranded siRNA (s). This protocol has been followed in protozoan parasite *Entamoeba histolytica* (Solis and Guillén 2008) and schistosomes (Ndegwa et al. 2007). In the model

Table 12.3 Silencing by exogenous siRNA

Presence of RNAi machinery in fungi	Gene targeted	Strategy	Inhibition	Refs.
Ascomycetes				
Aspergillus nidulans	ODC – fungal ornithine decarboxylase gene	Soaking method	Inhibition of 10%, 26%, 33%, 33% observed with 10 nM, 15 nM, 20 nM, 25 nM siRNA, respectively	Khatri and Rajam (2007)
Aspergillus nidulans	Bristle brlAβ – developmental regulatory gene	Inverted repeat of alcA (alcohol dehydrogenase) promoters flanking 498 bp brlAβ upstream	Expression was 3–4 fold less abundant on threonine than that on glucose	Barton and Prade (2008)
Aspergillus oryzae	brlA and α-amylase genes	Hairpin RNA cassette of 556 bp brlA, 1656 bp, and 750 bp α-amylase	Decreased signal for brlA gene, reduction in α-amylase activity	Ibrahim et al. (2009)
Aspergillus fumigatus	ALB1/PKSP – polyketide synthase (melanin biosynthesis pathway) and FKS1 β-(1,3) glucan synthase (cell wall polysaccharide)	500 bp inverted repeats of ALB1, FKS, and FKS/ALB1	29% of pALB1 transformants showed 5% (white colonies) or 24% (light-green colonies) reduction in ALB1 expression, 1% of pFKS1 transformants showed complete RNAi phenotype	Mouyna et al. (2004)
Aspergillus niger	xlnR encodes xylanases and cellulases	Inverted repeat of 834 bp of xlnR	12% transformants showed decreased activities	Oliveira et al. (2008)
Aspergillus parasiticus and A. flavus	AflR – transcription factor for expression of aflatoxin biosynthetic genes	Inverted repeat of 670 bp of aflR	aflR IRT transformants expressed verA at levels below the detection limits by N.Blot	McDonald et al. (2005)
	VerA – gene which is a part of aflatoxin biosynthetic pathway			
Fusarium graminearum	Tri6 – transcription factor regulating expression of trichothecene (mycotoxin) biosynthetic genes	Inverted repeat constructs of tri6 ORF with 602 nt in sense and 588 nt in antisense direction	Wheat head blight did not spread to neighboring spikelets	McDonald et al. (2005)

Fusarium verticilloides (or *F. moniliforme*)	*gus* gene-encoding beta-glucuronidase	Inverted repeat of 627 bp of *gus* gene	Two transformed colonies showed a reduction of 62% and 96% in the *gus* gene expression	Tinoco et al. (2010)
Neurospora crassa	Albino gene *al-1* involved in carotenoid biosynthesis	Full length *al-1* gene (1971 bp)	24% of transformants showed albino phenotype	Cogoni and Macino (1997)
Magnaporthe oryzae (*M. grisea*)	Enhanced GFP (eGFP)	Constructs with sense-sense, antisense-antisense, and sense-antisense orientation of ~700 nt of eGFP	40 out of 80 IRT transformants emitted 20% fluorescence relative to original GFP	Kadotani et al. (2003)
Schizosaccharomyces pombe	Enhanced GFP (eGFP)	Inverted repeat of 760 bp of eGFP	Gfp mRNA reduced more than twofold	Sigova et al. (2004)
Histoplasma capsulatum	AGS1 or α-(1,3)-glucan synthase, cell wall polysaccharide	Inverted repeat of 678 bp of coding sequence of AGS1	Loss of α-(1,3)-glucan synthase from cell walls	Rappleye et al. (2004)
Cryptococcus neoformans (JEC21 strain)	CAP59 – capsule synthesis ADE2 – adenine biosynthesis	520 nt inverted repeats of CAP59 and ADE2	~25% reduction giving dull (CAP59suppression) and pink (ADE2 suppression) colonies	Liu et al. (2002)
Bipolaris oryzae	PKS1 – polyketide synthase gene, involved in melanin biosynthesis pathway	Inverted repeats of PKS1 gene fragment (756 bp)	70% of the transformants showed a melanin-deficient (white color) phenotype	Moriwaki et al. (2007)
Sclerotinia sclerotiorum	*rgb1* encoding PP2A (type 2A phosphoprotein phosphatase) B regulatory subunit	Inverted repeat of 1.1 kbp of *rgb1* under the control of *A. nidulans* promoter and terminator	*rgb1* suppression inhibited sclerotial maturation and caused reduced pathogenesis	Erental et al. (2007)
Colletotrichum lagenarium	Enhanced GFP (eGFP)	Construct consisted of 0.72 kb eGFP under the control of *A. nidulans* promoter and terminator	GFP fluorescence reduced to less than 20% of the parent strain	Nakayashiki et al. (2005)
Basidiomycetes				
Coprinus cinereus	recA-like recombinase in meiosis – Lim15/Dmc1	Vector construct with 750 nt antisense and 650 nt sense strand	23% having meiotic defects, i.e., fruiting body having white cap	Namekawa et al. (2005)

(continued)

Table 12.3 (continued)

Presence of RNAi machinery in fungi	Gene targeted	Strategy	Inhibition	Refs.
Schizophyllum commune	Structural gene – SC15	334 nt hairpin construct of SC15	80% reduction in aerial hyphae formation and attachment	Jong et al. (2006)
Zygomycetes				
Mortierella alpina	Δ12-desaturase desaturates oleic acid to linoleic acid	467 nt inverted repeat of Δ12-desaturase	25–88% decrease in fatty acid production	Takeno et al. (2005)
Mucor circinelloides	*carB* – encodes phytoene dehydrogenase enzyme, involved in carotenoid biosynthesis pathway	pMAT647 with 2483 nt of *carB* pMAT754 with complete cDNA sequence (2334 nt) of *carB*	pMAT647 and pMAT754 gave a silencing frequency of 3.16% and 3.12% with a complete albino phenotype	Nicolás et al. (2003)

filamentous fungus, *Aspergillus nidulans*, 10 nM, 15 nM, 20 nM, and 25 nM siRNA were used, and a growth inhibition of 10%, 26%, 33%, 33% was observed, respectively, along with a 50% reduction in germ tube length following treatment with 10 nM siRNA-treated samples (Khatri and Rajam 2007; Amarzguioui et al. 2004). The soaking method results in transient expression making it less potent than direct microinjection (Schepers 2005), and therefore its efficiency in targeting the fungal thick cell walls consistently is not much significant.

12.4.2 Transgenic RNAi

The soaking method, transient RNAi, has limitations in terms of duration and experimental variability of the RNAi effect. The expression of inverted repeat (IR) transgenes, transgenic RNAi, has been shown to induce stable RNAi in fungi (Liu et al. 2002). However, variability, often, has been observed in the gene silencing effect by dsRNA-producing transgenes depending on the target gene, the type of construct, site of integration, and transgene copy number. An IRT is constructed using a target gene sequence, which is incorporated in an organism-specific plasmid. The plasmid containing IRT is transformed into the organism that upon transcription forms a hairpin loop which is cleaved by endogenous Dicer to generate siRNA(s). The generated small interfering RNAs (siRNAs) cleave the endogenous mRNA(s) with the help of RISC. Considering that there are several hairpin-expressing RNA systems available for robust RNA silencing using a tissue-specific RNA pol II promoter for tissue-specific expression of dsRNA and that hairpin RNA assures efficient formation of dsRNA, it is the frequently used method for induction of RNA silencing (Paddison and Vogt 2008). One type of IRTs is a long-hairpin RNA (lhRNA) which generally consists of more than 300 bp open reading frame, a spacer of approximately 250–500 oligonucleotides and an inverted repeat of the gene sequence. The IRT construct is inserted into the plasmid specific for a particular fungus and is transformed into the respective fungi either by protoplast formation or electroporation. But the transformation/cloning efficiency for long inverted repeats of a cDNA is very low, and the selection process is very time-consuming. The construction of lhRNA(s) is difficult as it involves several rounds of PCR and cloning steps, and moreover, it's necessary that vectors/plasmids should be available for each species to carry out siRNA delivery. Generally the longer size of spacer helps in the easy cloning of the inverted repeat and improves the efficiency of RNAi. Here, the use of IR constructs containing a short spacer (20–50 bp) in the middle of an inverted repeat improves the cloning efficiency (Yu et al. 2004). Moreover, the presence of introns as a spacer in the constructs also improves their effectiveness and enhances cloning efficiency [48, 49] (Wesley et al. 2001, Kalidas and Smith 2002). Nevertheless, these strategies have been successfully used to trigger silencing in various fungal systems like *Bipolaris oryzae* (Moriwaki et al. 2007) in which an IRT was constructed consisting of sense and antisense polyketide synthase gene of 756 bp encoding for melanin production separated by a spacer of cutinase gene intron obtained from a study on
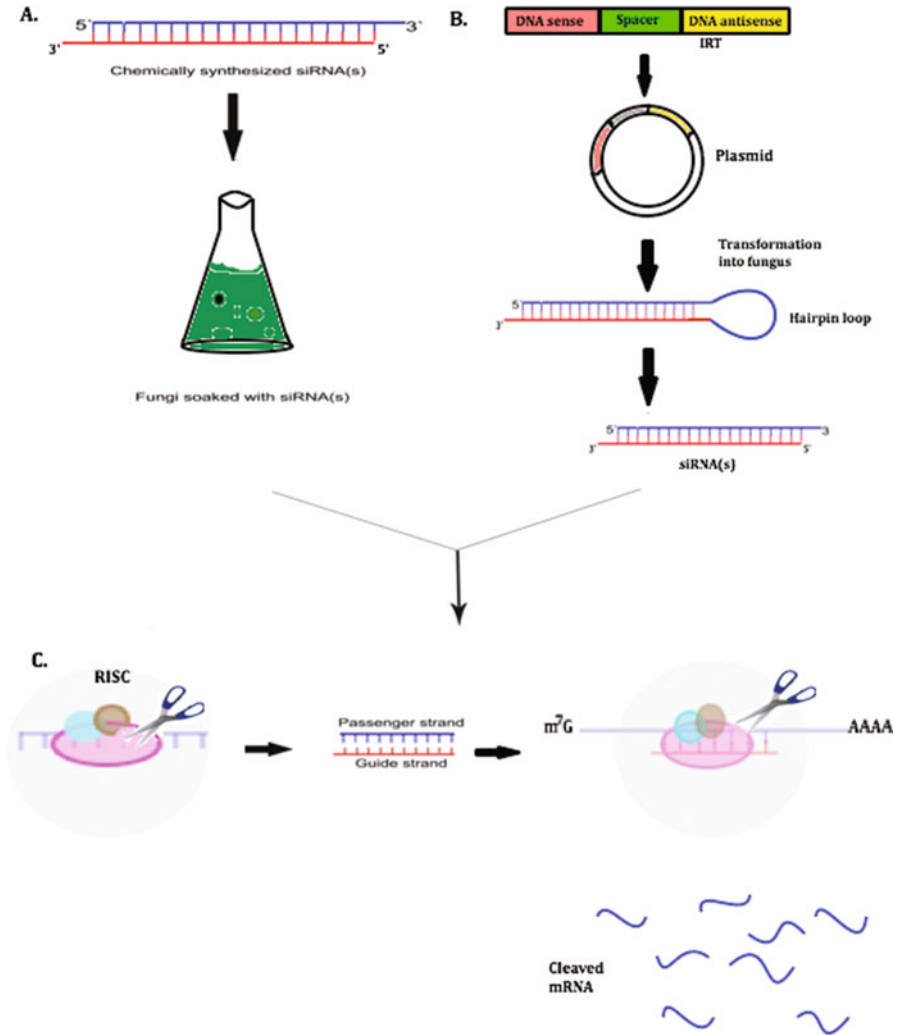
Fig. 12.2 A schematic representation of the two delivery methods of siRNA is shown. (**a**) Soaking method which involves soaking of fungi with siRNA. (**b**) IRT consists of the sense and antisense orientation of the gene separated by a spacer. It is incorporated into plasmid and transformed into fungi. Endogenous Dicer cleaves the hairpin loop which is formed upon transcription of IRT and siRNA(s) is generated. (**c**) The passenger strand is cleaved, and the guide strand of siRNA enters the RISC where it cleaves the target mRNA

Magnaporthe oryzae (Kadotani et al. 2003), and this yielded melanin-deficient (white) phenotype in 70% transformants. Another study reported only 12% transformants of *xlnR* encoding xylanases and cellulases of *A. niger* (Oliveira et al. 2008) showing decreased activities compared to the control strain. The reason

was attributed to either rearrangement in silencing constructs or apparent decrease in xylanolytic activities or may be absence of "true" co-transformants.

Another prominent IRT successfully used in mammalian systems is the short-hairpin RNA (shRNA) which consists of a 19 bp siRNA sense and antisense sequence separated by a spacer of 9 nucleotides where the siRNA sequence should be 100% homologous to the target mRNA. The shRNA expression vectors available for mammalian systems require RNA polymerase III system as shown in Fig. 12.2.

12.5   Validation of Gene Silencing

A preliminary way to investigate the knockdown of a gene and percentage of silencing in fungus is to measure the mycelial growth of fungus by either colony diameter method or mycelial dry weight method (radial growth assays). Blotting techniques, fluorescence imaging, and biochemical assays are the further confirmatory tests which are used to analyze silencing at the RNA and protein levels. A study was carried out by Kadotani et al. (2003) in the blast fungus *M. oryzae* (Holen 2006) in which they investigated RNA silencing using enhanced green fluorescent protein (eGFP). Sense-sense, antisense-antisense, and sense-antisense IRT constructs of eGFP separated by a partial sequence of β-glucuronidase gene as internal spacer were employed. Significant silencing was induced only by sense-antisense IRT construct as detected by the loss of GFP fluorescence using an image analyzer. Several studies have employed Northern blot analysis for studying gene silencing in various fungi (Yamada et al. 2007; Kadotani et al. 2003; Hammond et al. 2007, 2008; Hammond and Keller 2005; Segers et al. 2006; Janus et al. 2007). The captured fluorescence images of plasmids (IRTs) containing fluorescence tags like GFP (Segers et al. 2006; Janus et al. 2007) assist in better understanding of protein localization, confirm the presence of plasmid in the cell, and also facilitate in differentiating the silenced from the non-silenced transformants. Real-time PCR and the reverse transcription PCR (Khatri and Rajam 2007; Oliveira et al. 2008; Janus et al. 2007; Liu et al. 2002) setup are also employed using appropriate gene-specific primers to detect and quantify the gene transcript levels. Liu et al. conducted a study (Liu et al. 2002) on RNA silencing in the pathogenic fungus *Cryptococcus neoformans* where they constructed IRTs on genes related to capsule synthesis and adenine biosynthesis pathway. They observed 25% reduction in the gene expression imparting the transformants with dull and pink colonies, respectively, and silencing analyzed by employing reverse transcription PCR with gene-specific primers and PCR product stained with SYBR Green I nucleic acid gel stain for analysis of DNA-associated fluorescence. Janus et al. investigated a reporter system *DsRed* to identify silencing transformants by cosilencing *DsRed* with the protein isopenicillin N synthase (*pcbC*) involved in cephalosporin C biosynthesis in the filamentous fungus *Acremonium chrysogenum* and employed immunoblotting using a polyclonal antibody against the isopenicillin N synthase to assess the downregulation of the *pcbC* gene (Janus et al. 2007). Biochemical assays can be

performed as well which are specific for the enzyme/protein of interest to evaluate silencing of the target protein by specific siRNA(s). Hammond and Keller in 2005 executed the norsolorinic acid (NOR) analysis by thin-layer chromatography and observed whether the *A. nidulans aflR* IRTs can suppress NOR production (Hammond and Keller 2005). Research discoveries in vitro need to be accompanied by establishment of the potentiality of siRNA as a therapeutic. The antifungal drug dosages within the therapeutic window have been tested and determined on animal models, mostly murine models (Ibrahim et al. 2008a, b) for fungal diseases like zygomycosis, aspergillosis, candidiasis, cryptococcosis; but no pharmacological studies have yet been conducted on animal models employing siRNA therapy for fungal infections. Thus, dedicated efforts are needed to assess siRNA therapy for mycoses in various animal models and further investigate in humans through clinical trials.

12.6   Conclusion

RNAi has been explored in fungi in the post-genomics era, not only to understand the RNA silencing machinery but also as a tool to treat fungal diseases which cannot be effectively managed by conventional drugs. For instance, the past few decades have seen a rise in the opportunistic invasive fungal infections (mycoses) due to significant increase in the population of immunocompromised patients. The limitations of existing antifungal therapies have provided impetus toward exploring RNAi as a therapeutic option. *N. crassa* was the organism in which the first RNAi gene was discovered which was succeeded by numerous in vitro studies to achieve improved silencing in fungi and promising RNAi as a therapeutic tool. Identification of a potential target gene is necessitated against which siRNA is designed in silico, and recent whole-genome studies in fungi have greatly expedited the novel drug discovery process. Even though RNAi-related research discoveries have made rapid progress from in vitro to in vivo and clinical trials for neurodegenerative diseases and cancer, proper pharmacokinetic parameters of safety and efficacy for RNAi still remain to be answered for mycoses. RNAi as a therapeutic approach is relatively at a nascent stage, and uninterrupted probing should prove useful to drive it as a preferred option for the treatment of mycoses in the long run.

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