Micro-review

Host-induced gene silencing: a tool for understanding fungal host interaction and for developing novel disease control strategies

CRISTIANO C. NUNES AND RALPH A. DEAN*

Department of Plant Pathology, Fungal Genomics Laboratory, Center for Integrated Fungal Research, North Carolina State University, Raleigh, NC 27606, USA

SUMMARY

Recent discoveries regarding small RNAs and the mechanisms of gene silencing are providing new opportunities to explore fungal pathogen–host interactions and potential strategies for novel disease control. Plant pathogenic fungi are a constant and major threat to global food security; they represent the largest group of disease-causing agents on crop plants on the planet. An initial understanding of RNA silencing mechanisms and small RNAs was derived from model fungi. Now, new knowledge with practical implications for RNA silencing is beginning to emerge from the study of plant–fungus interactions. Recent studies have shown that the expression of silencing constructs in plants designed on fungal genes can specifically silence their targets in invading pathogenic fungi, such as *Fusarium verticillioides*, *Blumeria graminis* and *Puccinia striiformis* f.sp. *tritici*. Here, we highlight the important general aspects of RNA silencing mechanisms and emphasize recent findings from plant pathogenic fungi. Strategies to employ RNA silencing to investigate the basis of fungal pathogenesis are discussed. Finally, we address important aspects for the development of fungal-derived resistance through the expression of silencing constructs in host plants as a powerful strategy to control fungal disease.

INTRODUCTION

More than 25 years ago, Sanford and Johnston (1985) proposed the concept of parasite-derived resistance (PDR) as a radical strategy to control plant disease. In PDR, the expression of a parasite gene in the plant host interferes with the parasite's ability to grow and develop, resulting in disease resistance. Perhaps the most successful application of PDR is the use of transgenic papaya plants expressing *Papaya ringspot virus* (PSRV) coat protein to protect against virus infection (Gonsalves, 1998). With the exception of viruses, it was generally believed that the lack of ready macromolecule exchange between host and parasites would be a major impediment to more widespread development of PDR.

Over the past decade or so, the discovery of small RNAs and the elucidation of RNA silencing mechanisms have rekindled an interest in PDR. Today, it is strongly suggested, on the basis of reporter gene silencing, that RNA molecules traffic between host and parasites (Tinoco *et al.*, 2010; Tomilov *et al.*, 2008; Westwood *et al.*, 2009). Growing evidence suggests that RNA silencing constitutes a crucial component of many plant–parasite interactions in addition to viruses, including responses to insects, bacteria, nematodes and fungal infections (Huang *et al.*, 2006; Katiyar-Agarwal and Jin, 2010; Mao *et al.*, 2007; Ruiz-Ferrer and Voinnet, 2009; Turner *et al.*, 2006). Several very recent published reports on the use of host-induced gene silencing (HIGS) to control invading fungi are probable forerunners of many more applications to come.

In this article, we describe: (i) the central components of RNA silencing, emphasizing recent discoveries in plant pathogenic fungi (PPF); (ii) the fundamental principles of the use of RNA silencing to study PPF; (iii) applications in HIGS using fungus-derived genes to confer disease resistance; and (iv) future perspectives of HIGS for the control of fungal disease.

GENERAL MECHANISM OF EUKARYOTIC RNA SILENCING

Approximately 20 years ago, anomalies observed during efforts to functionally characterize genes led to the discovery of post-transcriptional gene silencing (PTGS). This phenomenon is known as co-suppression in plants, quelling in *Neurospora crassa* and RNA interference (RNAi) in *Caenorhabditis elegans* and other animals (Lee *et al.*, 1993; Napoli *et al.*, 1990; Romano and Macino, 1992). Subsequent further independent investigations revealed that much of the underlying machinery and mechanisms is conserved across organisms.

PTGS can be generalized as a sequence-specific regulatory mechanism for the silencing of gene expression triggered by long double-stranded (dsRNA) or hairpin-structured RNAs. These molecules are processed into 21–35-nucleotide-long single-stranded RNAs that associate with and direct the RNA-induced silencing complex (RISC) to the target transcript. This results in mRNA degradation or translational repression (Fig. 1) (Esquela-Kerscher and Slack, 2006). There are distinct classes of small RNA molecules, such as short interfering RNAs (siRNAs), microRNAs (miRNAs), tRNA-derived RNA fragments (tRFs) and Piwi-interacting

*Correspondence: Email: ralph_dean@ncsu.edu; radean2@ncsu.edu

Fig. 1 General mechanism and potential application of RNA silencing for the development of plant disease control strategies. In plant cells (A), endogenous RNA silencing is triggered by transcription of double-stranded RNA (dsRNA) (or hairpin) molecules in the nucleus (A1). These precursor molecules are processed by ribonuclease III (RNase III) enzymes, such as Dicer (or Drosha in animals), producing RNA molecules, which are loaded and transported from the nucleus by Exportin-5. In the cytoplasm, 21-nucleotide dsRNAs are produced by Dicer activity (A3). On elimination of the passage strand, the guide single-stranded RNA (ssRNA) associates with Argonaute (A4) and directs the RNA-induced silencing complex (RISC) to bind to target endogenous transcripts (A5), resulting in the degradation of transcripts (A6). Unbound siRNA binds to mRNAs (A7) through the action of RNA-dependent RNA polymerase (RdRp) and resynthesizes dsRNA which is cut into 21-nt dsRNA molecules by Dicer (A8), leading to RNA silencing amplification. In pathogen-infected plant cells (B), integration (B1) and transcription (B2) of the host-induced gene silencing (HIGS) construct results in HIGS dsRNA molecules (or hairpins) that are processed into 21-nt dsRNA molecules (B3 and B4; as explained in A). Alternatively, virus-induced gene silencing (VIGS) (B5) is used to produce VIGS dsRNA precursors that are processed into 21-nt dsRNA molecules by Dicer (B6). VIGS dsRNA molecules lead directly to the silencing of viral target transcripts in the plant cytoplasm (B7). The action of plant RdRp also leads to viral RNA silencing amplification (B8). HIGS dsRNA molecules are exported from plant cells and lead to the silencing of nematode targets on nematode feeding (B9), or the silencing of fungal transcripts on fungal infection (B10).

small RNAs (piRNAs). Currently, siRNAs and miRNAs are the best elucidated classes of small RNAs in plants and animals, and are discussed in more detail. Although it is clear that siRNA and miRNA processing pathways are distinct, in plants they typically begin in the nucleus, where Dicer and members of the ribonuclease III (RNase III) family bind to long dsRNA or hairpin RNAs and process them into siRNAs or miRNAs, respectively. In animals, Drosha is responsible for the slicing off of the long stem-loop dsRNA structure, which is then processed into siRNAs or miRNAs by Dicer (for

more details, see elsewhere: Baulcombe, 2004; Carthew and Sontheimer, 2009; Miyoshi *et al.*, 2010; Sato *et al.*, 2011). To our knowledge, it is unclear how siRNA is exported from the nucleus. In contrast, endogenously derived short hairpin RNA molecules (miRNA) are transported to the cytoplasm by a nuclear pore protein named Exportin-5 (Katahira and Yoneda, 2011; Kim, 2004; Lund *et al.*, 2004; Yi *et al.*, 2003). In other instances, short dsRNA molecules may be derived from exogenous sources, such as viruses replicating in plant cells. Once in the cytoplasm, endogenously or

exogenously derived short dsRNA molecules are processed into small dsRNA, and loaded into RISC. Here the 'passage' strand is eliminated, and the 'guide' RNA strand directs Argonaute to specifically degrade the target RNA transcript through the slicing ribonuclease activity of Argonaute or to interfere with translation (Esquele-Kerscher and Slack, 2006). These small RNAs can also be used as primers by RNA dependent-RNA polymerase (RdRp) to resynthesize dsRNAs (Melnik *et al.*, 2011). This amplification step ensures that several rounds of RNA silencing are triggered. Several members of the Argonaute family have been described. For example, AGO associates with 21–25-nucleotide-long small RNAs (siRNAs and miRNAs), whereas PIWI binds to 23–30-nucleotide-long RNA (piRNAs) molecules to silence repetitive element transcripts (Brennecke *et al.*, 2007; Hartig *et al.*, 2007). Here, we refer to the principle RNA silencing proteins as Dicer, Argonaute and RdRp.

In fungi, a diverse number of small RNA classes have been described over the last few years. Recently, microRNA-like RNAs (miRNAs) have been discovered in *N. crassa* (Lee *et al.*, 2010). The molecular machinery involved in the biogenesis of miRNAs appears to be significantly different from that reported for miRNAs in plants and animals. Thus, it is still a matter of debate whether genuine miRNAs are present in filamentous fungi. Other small RNAs in fungi include quelling-deficient 2 (QDE-2)-interacting small RNAs (qiRNAs) and Dicer-independent small interfering RNAs (disiRNAs) in *N. crassa* (Lee *et al.*, 2009, 2010). Endogenous short RNAs (esRNAs) have been discovered in *Mucor circinelloides*, and LTR retrotransposon-siRNAs (LTR-siRNAs) and tRFs in *Magnaporthe oryzae* and *Aspergillus fumigatus* (Jöchl *et al.*, 2008; Nicolas *et al.*, 2010; Nunes *et al.*, 2011).

FUNDAMENTAL PRINCIPLES OF RNA SILENCING IN PPF

A knowledge of RNA silencing protein machinery in fungi was initially derived from studies of *N. crassa* and referred to as 'quelling' (Catalanotto *et al.*, 2002, 2004; Cogoni and Macino, 1997; Fulci and Macino, 2007; Lee *et al.*, 2009; Li *et al.*, 2010b; Maiti *et al.*, 2007; Nakayashiki, 2005; Nolan *et al.*, 2008; Romano and Macino, 1992; Schumann *et al.*, 2010; Xiao *et al.*, 2010). Although quelling was first described in 1992, it was several years later that Cogoni and Macino discovered three distinct loci responsible for PTGS through the characterization of quelling-deficient (*qde*) mutants (Cogoni and Macino, 1997). The *qde-1*, *qde-2* and *qde-3* loci encode proteins that correspond to RdRp, Argonaute and RecQ DNA helicase, respectively. Small RNAs produced by Dicer associate with *qde-2* and require *qde-1* and *qde-3* for biogenesis (Catalanotto *et al.*, 2002, 2004). Over the past few years, there have been several examples of RNA silencing in PPF, including members of the Ascomycota, Basidiomycota and Zygomycota, as well as in *Phytophthora* spp. (fungus-like organisms; Oomycetes within the Kingdom Stramenopila) (Table 1).

As shown in Table 1, a variety of target genes have been used to test whether silencing is functional in any given PPF. Transgenes, such as β -glucuronidase (GUS) and green fluorescent protein (GFP) (reporter genes), or endogenous fungal genes with an easily screened phenotype have proven to be useful as markers for PTGS (Caribé dos Santos *et al.*, 2009; Fitzgerald *et al.*, 2004; Kadotani *et al.*, 2003; Laurie *et al.*, 2008; Miyamoto *et al.*, 2008; Nguyen *et al.*, 2008; Whisson *et al.*, 2005). Further, reporters, such as a GFP, can be fused to a target gene as an endogenous positive control to provide visible evidence that silencing is functional in a particular GFP-expressing fungal transformant. This approach is particularly useful for high-throughput screens. Exploring this strategy, Nguyen *et al.* (2008) developed a pSilent-Dual1 (pSD1) vector to study 37 genes involved in calcium signalling in *M. oryzae*. Although this is a powerful approach, it requires the ability to create a GFP-tagged strain. Alternatively, endogenous genes leading to easily scorable traits, such as melanin biosynthesis genes, are ideal and have been used in *Bipolaris oryzae*, *Cochliobolus sativus*, *M. oryzae* and *Venturia inaequalis* (Fitzgerald *et al.*, 2004; Leng *et al.*, 2011; Moriwaki *et al.*, 2007; Nakayashiki *et al.*, 2005). Other suitable target genes include those coding for hydrophobins as a result of the easily wettable phenotype of silenced transformants (Nakayashiki *et al.*, 2005). Screens for silenced hydrophobin transcripts have been used in *Cladosporium fulvum*, *M. oryzae* and *Moniliophthora perniciosa* (Caribé dos Santos *et al.*, 2009; Hamada and Spanu, 1998; Lacroix and Spanu, 2009; Nakayashiki *et al.*, 2005). Other endogenous genes for screens include enzymes whose activities can be measured directly, such as polygalacturonase in *Ophiostoma novo-ulmi* (Carneiro *et al.*, 2010).

RNA silencing has been explored in a number of PPF and *Phytophthora* spp. (Table 1) amenable to genetic transformation. In general, constitutive promoters are effective for the expression of silencing constructs. The *Aspergillus nidulans* *trpC* and *gpd* are the promoters of choice in PPF within the Ascomycota. In addition, the *oliC* (*A. nidulans*) and *gpdA* (*Glomerella cingulata*) promoters have been used effectively in *Botrytis cinerea* and *V. inaequalis*, respectively (Fitzgerald *et al.*, 2004; Patel *et al.*, 2008). For PPF within the Basidiomycota, the *Ustilago maydis* HSP70 promoter has been used successfully in *Moniliophthora perniciosa* (Caribé dos Santos *et al.*, 2009). Native and species-specific promoters have also been employed, such as *AvrL567* in *Melampsora lini* and an *Ustilago*-specific HSP70 in *Ustilago hordei* (Laurie *et al.*, 2008; Lawrence *et al.*, 2010). Alternatively, *in vitro*-synthesized double-stranded RNA (dsRNA) molecules have been used successfully for RNA silencing. In this approach, sense and antisense strands of the target gene are synthesized by *in vitro* transcription and then mixed to form dsRNA molecules. This eliminates many cloning steps to construct the silencing vector prior to fungal transformation (Grenville-Briggs *et al.*, 2008; Whisson *et al.*, 2005). In the Basidiomycota, *Moniliophthora perniciosa*, hydrophobin and

Table 1 Summary of RNA silencing in plant pathogenic fungi.

Phylum	Species	Targeted gene	Promoter used	RNA precursor	Comments	Reference
Ascomycota	<i>Alternaria alternata</i>	Putative hydrolase (<i>ACTT2</i>), a host-selective ACT-toxin	<i>trpC*</i>	Hairpin†	Silenced transformants were nonpathogenic	Miyamoto <i>et al.</i> (2008)
		Green fluorescent protein (GFP)	<i>trpC*</i>	Hairpin	Silencing of GFP suggests RNA silencing functionality	Miyamoto <i>et al.</i> (2008)
		Enoyl-reductase (<i>ACTTS2</i>), a host-selective ACT-toxin	<i>trpC*</i>	Hairpin	Silencing of <i>ACTTS2</i> transcripts resulted in lack of ACT-toxin production and complete loss of pathogenicity	Ajiro <i>et al.</i> (2010)
	<i>Aspergillus flavus</i> and <i>A. parasiticus</i>	Transcription factor (<i>afIR</i>)‡	<i>gpd*</i>	Hairpin	The atoxigenic silenced strain was stable in corn and wheat	McDonald <i>et al.</i> (2005)
	<i>Bipolaris oryzae</i>	Polyketide synthase gene (<i>PKS1</i>)	<i>trpC*</i>	Hairpin	The silencing construct was stable during asexual propagation	Moriwaki <i>et al.</i> (2007)
	<i>Botrytis cinerea</i>	Superoxide dismutase (<i>BCSOD1</i>)	<i>oliC*</i>	Sense and antisense constructs	Both constructs reduced transcript levels but had no effect on virulence	Patel <i>et al.</i> (2008)
	<i>Cladosporium fulvum</i>	Hydrophobin gene (<i>HCF-1</i>)	n/a	Antisense RNA	Silenced transformants reverted to the wild-type	Hamada and Spanu (1998)
		First exons of six hydrophobin coding genes	<i>gpd*</i>	Chimeric hairpin	This work highlights that changes in gene expression of nontargeted genes pose a challenge for the silencing of multiple genes	Lacroix and Spanu (2009)
	<i>Cochliobolus sativus</i>	GFP, a host-selective toxin (<i>ToxA</i>) and a polyketide synthase (<i>CsPKS1</i>)		Hairpin	Development of pSGate1 silencing vector <i>CsPKS1</i> -silenced strains resulted in albino phenotypes	Leng <i>et al.</i> (2011)
	<i>Colletotrichum gloeosporioides</i>	Transcription factor (<i>PAC1</i>)	<i>trpC*</i>	Hairpin	pTroya, a Gateway RNA silencing vector, was developed from pSilent-1 backbone for high-throughput application in fungi	Shafran <i>et al.</i> (2008)
	<i>Colletotrichum lagenarium</i>	eGFP	<i>trpC*</i>	Hairpin	Development and application of pSilent-1 to silence fungal transcripts	Nakayashiki <i>et al.</i> (2005)
	<i>Fusarium graminearum</i>	Transcription factor (<i>Tri6</i>)	<i>gpd*</i>	Hairpin	Silencing transformants were less virulent on wheat	McDonald <i>et al.</i> (2005)
	<i>Fusarium solani</i> f.sp. <i>pisi</i>	β(1,3)-D-glucan synthase (<i>FsFKS1</i>)	<i>alcA</i>	Hairpin	Imperfect cell wall formation led to reduced spore viability and lysis of spore and mycelia in silenced transformants	Ha <i>et al.</i> (2006)
	<i>Fusarium solani</i>	Chitinase (CSN1)	<i>gpd*</i>	Hairpin	Silenced transformants were more virulent than wild-type	Liu <i>et al.</i> (2010)
	<i>Magnaporthe oryzae</i>	eGFP	<i>trpC*</i>	Sense, antisense and hairpin RNA constructs	Hairpin construct more efficiently triggered eGFP silencing	Kadotani <i>et al.</i> (2003)
		MPG1	<i>trpC*</i>	Hairpin	Development of pSilent-1 vector as tool for gene silencing for Ascomycete fungi	Nakayashiki <i>et al.</i> (2005)
		PKS-like gene	<i>trpC*</i>	Hairpin	Extends application of the pSilent-1 vector	Nakayashiki <i>et al.</i> (2005)
		eGFP	<i>trpC*</i> <i>gpd*</i>	dsRNA	Development of a vector with convergent opposing promoters vector [pSilent-Dual1 (pSD1)]; pSD1 triggers RNA silencing slightly less efficiently than pSilent-1	Nguyen <i>et al.</i> (2008)
		37 genes involved in calcium signalling	<i>trpC*</i> <i>gpd*</i>	Chimeric dsRNA	This co-silencing approach facilitates screening of transformants based on GFP fluorescence Work identifies many calcium signalling proteins involved in hyphal growth, sporulation and pathogenicity	Nguyen <i>et al.</i> (2008)
	<i>Ophiostoma novo-ulmi</i>	Endopolygalacturonase (<i>Epg1</i>)	<i>gdp</i>	Hairpin	A longer stem-loop RNA fragment (409 bp) resulted in greater reduction of mRNA transcripts compared with a 200-bp fragment	Carneiro <i>et al.</i> (2010)

Table 1 Continued.

Phylum	Species	Targeted gene	Promoter used	RNA precursor	Comments	Reference
Basidiomycota	<i>Sclerotinia sclerotiorum</i>	B regulatory subunit (<i>rgb1</i>) of 2A phosphoprotein phosphatase (<i>PP2A</i>)	<i>trpC*</i>	Hairpin	pSilent-1 vector triggered high levels of silencing in two transformants which exhibited reduced hyphal radial growth, impaired sclerotial maturation and were unable to cause disease on tomato and <i>Arabidopsis thaliana</i>	Erental <i>et al.</i> (2007)
	<i>Verticillium longisporum</i>	Chorismate synthase (<i>Vlaro2</i>)	<i>trpC*</i>	Hairpin	pSilent-1 vector was used and silenced 80% of <i>Vlaro2</i> transcripts Silenced transformants were able to infect <i>Arabidopsis thaliana</i> and <i>Brassica napus</i>	Singh <i>et al.</i> (2010)
	<i>Venturia inaequalis</i>	Trihydroxynaphthalene reductase (<i>THN</i>) and GFP	<i>gpdA</i> §	Chimeric hairpin	Silenced transformants had a light brown phenotype and were able to infect apple (Royal Gala)	Fitzgerald <i>et al.</i> (2004)
	<i>Melampsora lini</i>	Effector protein (<i>AvrL567</i>)	<i>AvrL567</i> native promoter	Hairpin	Silenced rust lines were virulent on flax plants carrying L6 immune receptor	Lawrence <i>et al.</i> (2010)
	<i>Moniliophthora perniciosa</i>	GFP, hydrophobin (<i>MpHYD3</i>) and 1-cys peroxiredoxin (<i>MpPRX1</i>)	n/a	<i>In vitro</i> synthesized dsRNA	dsRNA shown to be feasible for transforming this fungus	Caribé dos Santos <i>et al.</i> (2009)
Zygomycota	<i>Ustilago hordei</i>	GUS and mating-type gene (<i>bW</i>)	Hsp70 native promoter	Hairpin	<i>U. hordei</i> transformants (<i>MAT1</i>) mated with <i>MAT-2</i> exhibited reduced production of mating hyphae	Laurie <i>et al.</i> (2008)
	<i>Mucor circinelloides</i> ¶	Carotenogenic gene (<i>carB</i>)	Native promoter		3% of silenced transformants showed albino phenotype	Nicolas <i>et al.</i> (2003)
Fungi-like organism	<i>Phytophthora infestans</i>	G-protein β -subunit encoding gene (<i>Pigpb1</i>)	<i>Pigpb1</i> native promoter	<i>Pigpb1</i> sense strand	Silenced transformants failed to sporulate	Latijnhouwers and Govers (2003)
		Cdc 14 coding gene (<i>PiCdc14</i>)	HAM34	Sense orientation	Transformants showed reduced sporulation	Ah Fong and Judelson (2003)
		G-protein α -subunit gene (<i>Pigpa1</i>)	<i>Pigpa1</i> native promoter	<i>Pigpa1</i> sense orientation	Transformants exhibited reduced zoospore production and infection on potato leaves	Latijnhouwers <i>et al.</i> (2004)
		GFP <i>inf1</i> and <i>cdc14</i>	n/a	<i>In vitro</i> synthesized dsRNA	GFP, <i>inf1</i> and <i>cdc14</i> expression levels were reduced after exposure to dsRNA	Whisson <i>et al.</i> (2005)
		bZIP transcription factor (<i>Pibzp1</i>)	HAM34	Sense and antisense orientations	Silenced transformants showed abnormal zoospore movement, failed to develop appressoria and were incapable of infecting tomato leaflets	Blanco and Judelson (2005)
		Nuclear LIM interactor-interacting factors (<i>NIFC1</i> and <i>NIFC2</i>)	HAM34	Hairpin	Zoospore cyst germination was impaired by 60% in silenced <i>NIFC</i> transformants	Judelson and Tani (2007)
		<i>Inf1</i>		Sense, antisense and hairpin RNA constructs	Silencing occurred at the transcription level Hairpin most efficient method of silencing	Ah-Fong <i>et al.</i> (2008)
		Putative glycosylated protein (<i>Pihmp1</i>)	n/a	<i>In vitro</i> synthesized dsRNA	Silenced lines showed loss of pathogenicity	Avrova <i>et al.</i> (2008)
		Putative ATP-dependent DEAD-box RNA-helicase gene (<i>Pi-RNH1</i>)	n/a	<i>In vitro</i> synthesized dsRNA	<i>Pi-RNH1</i> -silenced lines formed large aberrant zoospores that had multiple flagella and underwent partial cleavage	Walker <i>et al.</i> (2008)
		Four members of the <i>CesA</i> encoding for cellulose synthase genes	n/a	<i>In vitro</i> synthesized <i>CesA1</i> -, <i>CesA2</i> -, <i>CesA3</i> - and <i>CesA4</i> -dsRNA	Silenced strains contained disrupted cell wall surrounding appressoria Cellulose content of the silenced strains was >50% lower than that of nonsilenced strains	Grenville-Briggs <i>et al.</i> (2008)

Table 1 Continued.

Phylum	Species	Targeted gene	Promoter used	RNA precursor	Comments	Reference
		Effector protein (<i>PiAVR3a</i>)	HAM34	Hairpin	A <i>PiAVR3a</i> -silenced line (CS12) was significantly reduced in pathogenicity on <i>Solanum tuberosum</i> cv Bintje (susceptible) and on <i>Nicotiana benthamiana</i>	Bos <i>et al.</i> (2010)
		Dicer-like (<i>Pidd1</i>), Argonaute (<i>Piago1/2</i>) and Histone deacetylase (<i>Pihda1</i>)	n/a	<i>In vitro</i> synthesized dsRNA	Stable Ns (nonsporulating, <i>Picdc14</i> -silenced) transformant protoplasts were treated with dsRNA homologous to <i>Pidd1</i> , <i>Piago1/2</i> and <i>Pihda1</i> Regenerated lines of <i>Pidd1</i> , <i>Piago1/2</i> and <i>Pihda1</i> showed an obvious sporulation	Vetukuri <i>et al.</i> (2011)
	<i>P. parasitica</i> var. <i>nicotianae</i>	A coding gene considered to be involved in cellulose-binding (CB), elicitor (E) of defence in plants and lectin (L)-like activities (<i>CBEL</i>)	<i>Bremica lactucae</i> HSP70	Antisense or sense constructs	Silenced transformants showed reduced attachment to cellulosic surfaces and cell wall thickening	Gaulin <i>et al.</i> (2002)
	<i>P. sojae</i>	Heterotrimeric G-protein α subunit (<i>PsGPA1</i>)	HAM34	Antisense construct	Silenced <i>PsGPA1</i> lines had abnormal zoospore chemotaxis, encystment and germination Silenced transformants were unable to infect soybean	Hua <i>et al.</i> (2008)
		C ₂ H ₂ zinc finger transcription factor (<i>PsCZF1</i>)	HAM34 and native <i>PsCZF1</i>	Antisense construct	Hyphal growth rate of silenced transformants was reduced about 50% and oospore production, zoospore and cyst germination were impaired Silenced strains lost virulence in soybean	Wang <i>et al.</i> (2009)
		MAP kinase encoding gene (<i>PsSAK1</i>)	HAM34	Antisense construct	Silenced transformants showed faster encystment, reduced germination rate and longer germ tubes compared with wild-type Transformants were unable to colonize wounded and unwounded soybean leaves	Li <i>et al.</i> (2010a)
		Putative seven-transmembrane G-protein-coupled receptor (<i>GPR11</i>)	HAM34	Antisense construct	Zoospore release from sporangia was drastically impaired as well as zoospore encystment and germination Silenced transformants lost pathogenicity to soybean	Wang <i>et al.</i> (2010)
		PsYKT6, a conserved member gene of the soluble N-ethylmaleimide-sensitive factor attachment protein receptors (<i>SNAREs</i>)	HAM34	Antisense construct	Silencing of PsYKT6 revealed involvement of this gene in asexual development, sexual reproduction and pathogenesis on soybean cultivars Antisense constructs were stable in all three transformants	Zhao <i>et al.</i> (2011)
		Crinkling- and necrosis-inducing proteins (CRN) (<i>PsCRN63</i> and <i>PsCRN115</i>)	HAM34	Sense construct	Silenced transformants were unable to suppress host cell death	Liu <i>et al.</i> (2011)

**Aspergillus nidulans* promoters.

†Hairpin = inverted repeats.

‡Delivery of synthetic siRNAs into *Aspergillus flavus* protoplasts resulted in significant reduction in *afID* transcripts (Abdel-Hadi *et al.*, 2011).

§*Glomerella cingulata*.

¶Nonpathogenic fungus, but it is a relevant organism within Zygomycota.

n/a, not applicable.

peroxiredoxin transcripts were successfully silenced using *in vitro*-synthesized dsRNA (Caribé dos Santos *et al.*, 2009). In *Phytophthora infestans*, synthesized dsRNA was used to silence exogenous and endogenous genes, such as GFP, *inf1* and *cdc14* (Whisson *et al.*, 2005). As *Phytophthora* spp. are diploid and homologous recombination has not been demonstrated conclusively, RNA silencing is currently the only option for functional gene analysis in Oomycetes. As listed in Table 1, the HAM34 promoter has been widely used in *Phytophthora* spp., as well as native promoters, such as *Pigpa1* and *Pigpb1*. Although silencing mediated by synthesized dsRNA is typically transient, this does not appear to be the case in *P. infestans*, where silencing may persist throughout the pathogen's entire life cycle. The use of synthesized dsRNA is likely to be valuable for high-throughput functional characterization of genes and/or silencing of gene families, such as those for the cellulose synthase gene family (*CesA*) in *P. infestans* (Grenville-Briggs *et al.*, 2008). Nevertheless, the main drawback of the use of synthesized dsRNA is the constant need to generate new silenced lines for long-term studies.

As listed in Table 1, the source of the dsRNA precursor molecule is also critical. This precursor molecule can be transcribed *in trans* independently in sense or antisense orientations (Kadotani *et al.*, 2003; Patel *et al.*, 2008). Alternatively, a short spacer may be used between *cis*-located sense and antisense sequences corresponding to the target to allow the self-formation of a hairpin RNA molecule (Kadotani *et al.*, 2003; Miyamoto *et al.*, 2008; Nakayashiki *et al.*, 2005). The hairpin structure has been used extensively with PPF (Table 1). Kadotani *et al.* (2003) showed that a hairpin-generating construct more effectively silenced GFP in *M. oryzae* than did sense and antisense constructs. The principle constraint to the use of a hairpin-like structure is that it requires the cloning of inverted fragments, which can be time consuming. To simplify cloning, Nguyen *et al.* (2008) developed the pSD1 vector, which contains opposing *A. nidulans trpC* and *gpd* promoters separated by a multiple cloning site. pSD1 offers the great advantage of a single-step, nonoriented cloning procedure.

Although gene silencing has a number of advantages compared with gene knockout, studies to date have produced variable results in terms of efficiency. For example, in *Moniliophthora perniciosa*, the silencing efficiency of hydrophobin and peroxiredoxin transcripts varied from 18% to 97% and 23% to 87%, respectively (Caribé dos Santos *et al.*, 2009). Results using RNA hairpins are also highly variable. For example, a RNA hairpin precursor used to transform the Ascomycota *Ophiostoma novo-ulmi* resulted in three knockdown transformants having 6%, 22% and 31% expression relative to the wild-type (Carneiro *et al.*, 2010). A number of factors may explain the inconsistent efficiency of silencing. For instance, the genomic context of the inserted RNA silencing vector and/or number of integration events may influence the transcriptional activity of the precursor RNA molecule. An important drawback regarding the application of gene silencing to

simultaneously silence multiple genes is that it may lead to the silencing of nontarget transcripts. As an illustration, the analysis of the expression levels of *C. fulvum* transformants carrying various hydrophobin silencing constructs resulted in a reduction in the expression levels of several off-target transcripts (Lacroix and Spanu, 2009).

RNA silencing has been applied successfully to discover and characterize a number of fungal pathogenicity genes. In *Alternaria alternata*, the silencing of two host-selective ACT-toxins resulted in the complete loss of pathogenicity (Ajiro *et al.*, 2010; Miyamoto *et al.*, 2008). In *M. oryzae*, high-throughput silencing of 37 genes involved in calcium signalling revealed that many genes affected hyphal growth, sporulation and pathogenicity (Nguyen *et al.*, 2008). The silencing of mycotoxin-specific genes in *Fusarium graminearum* resulted in reduced virulence on wheat (McDonald *et al.*, 2005). In *Melampsora lini*, transformants silenced for the AvrL567 avirulence effector were able to cause disease on flax plants carrying the corresponding L6 immune receptor (Lawrence *et al.*, 2010).

APPLICATIONS IN PDR USING HIGS

Plants naturally utilize the RNA silencing machinery to defend against invading viruses (Csorba *et al.*, 2009; Harvey *et al.*, 2011; Hu *et al.*, 2011). HIGS has also been shown to be effective against other plant pathogens (Huang *et al.*, 2006). In contrast with plant viruses, most of the nematode body resides outside of the plant cell; nevertheless, RNA silencing has been shown to target nematode parasitism genes (Urwin *et al.*, 2002). *Arabidopsis thaliana* expressing a dsRNA targeting the root-knot nematode parasitism gene *16D10* led to effective disease resistance against four major *Meloidogyne* spp. (Huang *et al.*, 2006). The expression of *MjTis11* (*Meloidogyne javanica* putative transcription factor) in tobacco resulted in the silencing of the target gene in feeding root-knot nematodes (Fairbairn *et al.*, 2007). Alternatively, dsRNA delivered by the viral infection of host plants has the potential to induce gene silencing in nematodes (Dubreuil *et al.*, 2009).

RNA silencing has been implicated in resistance to bacterial pathogens, including crown gall disease caused by *Agrobacterium tumefaciens* (Escobar and Dandekar, 2003; Escobar *et al.*, 2001, 2002; Viss *et al.*, 2003). To induce gall formation, *iaaM* (tryptophan monooxygenase) and *ipt* (AMP isopentenyl transferase) genes are horizontally transferred from the bacterium to the host genome. Escobar *et al.* (2001) showed that the silencing of *iaaM* and *ipt* oncogenes in *Arabidopsis thaliana* and *Lycopersicon esculentum* resulted in high levels of resistance to crown gall. The same approach was also effective in producing resistance to crown gall in walnut (*Juglans regia* L.) and apple (Escobar *et al.*, 2002; Viss *et al.*, 2003). Other work has suggested that a number of small RNAs participate in the bacterium–plant interaction. miRNAs (miR393, miR160, miR167 and miR825) were upregulated in plants

after challenge with bacterial pathogens (Katiyar-Agarwal and Jin, 2010). In insects, a reduction in damage caused by the coleopteran western corn rootworm was obtained by expression of dsRNA V-ATPase A in transgenic corn plants (Baum *et al.*, 2007). For other examples, see Mao *et al.*, 2007; Turner *et al.*, 2006.

Examples of the application of RNA silencing in fungus–plant interactions are now beginning to emerge. Recently, Tinoco *et al.* (2010) showed that tobacco transformed to express a GUS hairpin-structured dsRNA specifically silenced GUS transcripts in a GUS-expressing strain of *Fusarium verticillioides* during infection. Other recent publications have shown that the *in planta* expression of fungal dsRNA results in HIGS in the biotrophic pathogens *Blumeria graminis* and *Puccinia striiformis* f.sp. *tritici*. In the barley–*B. graminis* pathosystem, 76 fungal genes found to be expressed during the interaction were subjected to RNA silencing by HIGS; 21% (16 of 76) of plants transiently transformed with each of the fungal RNA silencing constructs exhibited a reduction in the number of *B. graminis* spores able to develop haustoria (Nowara *et al.*, 2010). Two genes coding for 1,3- β -glucanotransferase (*GTF1* and *GTF2*) were further investigated. Virus-induced gene silencing (VIGS), using *Barley stripe mosaic virus* (BSMV) to produce antisense *GTF1* and *GTF2* transcripts, led to a significant reduction in haustorium formation and the rate of secondary hyphae elongation, respectively. The analysis of independent T1 transgenic plants carrying inverted repeats (hairpin) targeting *GTF1* showed that the level of dsRNA-derived hairpin transcript decreased rapidly in noninoculated plants. However, some transgenic plants inoculated with *B. graminis* f.sp. *hordei* exhibited reduced symptoms compared with control plants. The same study also used HIGS to silence Avra10 transcripts produced by *B. graminis* f.sp. *hordei*. In susceptible barley hosts, Avra10 acts as a virulence effector, whereas, in plants containing the R gene *Mla10*, it is additionally recognized as an avirulence factor. As predicted, the expression of the silencing construct targeting the Avra10 transcript in susceptible plants resulted in a reduction in pathogen development (haustorium formation) (Nowara *et al.*, 2010). Furthermore, the abundance of the Avra10 transcript was reduced in plants co-bombarded with the cognate resistance gene *Mla10* and the Avra10 silencing construct following fungal infection. The authors concluded that these data are consistent with gene silencing occurring within young haustoria. Also using VIGS mediated by BSMV, Yin *et al.* (2010) demonstrated HIGS in wheat targeting *Puccinia striiformis* f.sp. *tritici* (PST) genes. These authors initially showed that a highly abundant haustorial PST transcript (PSTha12J12) was silenced by the expression of dsRNA in VIGS-mediated BSMV–wheat transformants. Further examination of other PST genes revealed that only genes highly expressed in haustoria were effectively silenced. Three predicted genes coding for secreted proteins and a fourth gene predicted to code for a chitinase with expression patterns similar to PSTha12J12 were silenced to a high level. Similarly two genes, one with unknown

function and a *Uromyces fabae* hexose transporter homologue having high expression levels in purified haustoria cells and relatively high levels in urediniospores, were silenced. Other genes that were expressed constitutively in a variety of fungal cell types, such as an elongation factor-1 homologue, predicted β -tubulin, glyceraldehyde 3-phosphate dehydrogenase and actin coding genes, were not silenced (Yin *et al.*, 2010). Although VIGS mediated by BSMV in wheat appears to be restricted to the silencing of only pathogen genes highly expressed in haustoria, these recent reports using different pathosystems strongly suggest that RNA molecules are able to move from plants into fungal cells and effectively silence their target genes. We expect more examples to be forthcoming in the near future.

FUTURE PERSPECTIVES OF HIGS IN FUNGUS–PLANT INTERACTIONS

The application of PDR to engineer transgenic plants has led to valuable outcomes. The development of transgenic virus-resistant plants represents the foremost example, and the deployment of such plants has been recognized as saving an entire commercial agricultural industry (Gonsalves, 1998). More recently, the coupling of PDR to HIGS has emerged as a feasible strategy to develop plants resistant to other important pathogens, including nematodes and fungi.

Although PDR was discovered more than 25 years ago, it is only now that promising applications of fungus-derived resistance mediated by HIGS are ready for take off. In this concept, the source of resistance is derived from the parasite's own genome. The public availability of more than 100 fungal genome sequences and many plant genome sequences, combined with transcriptome data, now offers the scientific community an arsenal of candidate genes for testing and for the design of silencing constructs to avoid off-target transcripts. Possible targets may include virulence determinants, genes essential for fungal infection development and known fungicide targets. The availability of host genome sequence data helps to ensure that HIGS constructs do not target and negatively affect the host.

The ability to identify fungal targets, as well as the means to develop stable and environmentally safe transgenic crop plants, holds great promise for the fulfilment of future food needs. The concept and use of transgenic pathogen-resistant crops are well established (Collinge *et al.*, 2010), and the recent growing acceptance of genetically modified plants around the world, including the European Union and China, is likely to attract the attention of government (funds) and researchers (scientific knowledge) to further develop PDR. Although the application of the concept of fungus-derived resistance mediated by HIGS appears to be very encouraging, a series of challenges needs to be carefully addressed before application of this strategy can be fully implemented. We conclude by highlighting a few fundamental questions.

How do RNA molecules traffic from the plant into fungal cells?

A knowledge of the mechanisms involved in the movement of protein effector molecules from pathogens into host cells is now beginning to emerge (Dean, 2011; Rooney *et al.*, 2005; Schornack *et al.*, 2009; Tyler, 2009; White *et al.*, 2009). In *Arabidopsis*, the intercellular and systemic movement of small RNAs is mediated through the plasmodesmata and phloem, respectively (Melnik *et al.*, 2011). Although virus particles are also capable of moving through plasmodesmata and in the phloem, the mechanism by which naked RNA moves between two organisms is unknown. It remains to be determined how RNA is delivered from the plant nucleus, across the plasma membrane and plant cell wall into fungal cells. Is it a passive or active process? If active, what host and pathogen genes are involved and how is the process regulated?

Can HIGS be effective for other types of fungal pathogen?

HIGS has been shown to be effective in silencing the transcripts of obligate biotrophic invading fungi, at least for *B. graminis* and *Puccinia striiformis* f.sp. *tritici*. Perhaps the intimate interaction of this group of pathogens allows sufficient opportunity for the trafficking of RNA molecules. Considering hemibiotrophic and necrotrophic fungi, is there adequate time for RNA movement from host into fungal cells before host cells die?

What are the most promising fungal targets?

In the gene-for-gene concept, pathogen avirulence (Avr) genes are constantly evolving to escape recognition by the corresponding host resistance (R) gene product (Stukenbrock and McDonald, 2009). To date, the number of successful HIGS candidates leading to reduced fungal growth development remains limited and includes effectors, a cell wall elongation gene, a chitinase, a hexose transporter and a gene with unknown function. Much work remains to be done to identify suitable fungal candidate genes. Fortunately, as we have described, opportunities exist to establish high-throughput screening pipelines to identify strong candidates.

What are the best strategies for applying HIGS to control fungal disease?

A number of possible strategies are feasible to exploit HIGS for durable resistance. The use of (i) a silencing construct that targets entire gene families, (ii) multiple lines deployed in rotation to minimize the selection pressure on pathogens, (iii) a 'polygenic' HIGS line conferring resistance to single or multiple fungal pathogens or (iv) a combination of both classical R genes and HIGS

cassettes in the same host, which may synergistically boost resistance, are all strategies worthy of investigation.

ACKNOWLEDGEMENTS

This work is part of the doctoral dissertation of Cristiano C. Nunes and was supported by the CAPES Foundation (Brazilian Educational Agency), Fulbright Program and the Graduate School at North Carolina State University. Thanks are due to Dr Gary Payne and members of the Center for Integrated Fungal Research for valuable discussions and critical reading of the manuscript.

REFERENCES

- Abdel-Hadi, A.M., Caley, D.P., Carter, D.R.F. and Magan, N. (2011) Control of aflatoxin production of *Aspergillus flavus* and *Aspergillus parasiticus* using RNA silencing technology by targeting *aflD* (nor-1) gene. *Toxins*, **3**, 647–659.
- Ah Fong, A. and Judelson, H.S. (2003) Cell cycle regulator *Cdc14* is expressed during sporulation but not hyphal growth in the fungus-like oomycete *Phytophthora infestans*. *Mol. Microbiol.* **50**, 487–494.
- Ah-Fong, A.M., Bormann-Chung, C.A. and Judelson, H.S. (2008) Optimization of transgene-mediated silencing in *Phytophthora infestans* and its association with small-interfering RNAs. *Fungal Genet. Biol.* **45**, 1197–1205.
- Ajiro, N., Miyamoto, Y., Masunaka, A., Tsuge, T., Yamamoto, M., Ohtani, K., Fukumoto, T., Gomi, K., Peever, T.L., Izumi, Y., Tada, Y. and Akimitsu, K. (2010) Role of the host-selective ACT-toxin synthesis gene ACTS2 encoding an enoyl-reductase in pathogenicity of the tangerine pathotype of *Alternaria alternata*. *Phytopathology*, **100**, 120–126.
- Avrova, A.O., Boevink, P.C., Young, V., Grenville-Briggs, L.J., van West, P., Birch, P.R.J. and Whisson, S.C. (2008) A novel *Phytophthora infestans* haustorium-specific membrane protein is required for infection of potato. *Cell. Microbiol.* **10**, 2271–2284.
- Baulcombe, D. (2004) RNA silencing in plants. *Nature*, **431**, 356–363.
- Baum, J.A., Bogaert, T., Clinton, W., Heck, G.R., Feldmann, P., Ilagan, O., Johnson, S., Plaetinck, G., Munyikwa, T., Pleau, M., Vaughn, T. and Roberts, J. (2007) Control of coleopter insect pests through RNA interference. *Nat. Biotechnol.* **25**, 1322–1326.
- Blanco, F.A. and Judelson, H.S. (2005) A bZIP transcription factor from *Phytophthora* interacts with a protein kinase and is required for zoospore motility and plant infection. *Mol. Microbiol.* **56**, 638–648.
- Bos, J.I., Armstrong, M.R., Gilroy, E.M., Boevink, P.C., Hein, I., Taylor, R.M., Zhendong, T., Engelhardt, S., Vetukuri, R.R., Harrower, B., Dixelius, C., Bryan, G., Sadanandom, A., Whisson, S.C., Kamoun, S. and Birch, P. (2010) *Phytophthora infestans* effector AVR3a is essential for virulence and manipulates plant immunity by stabilizing host E3 ligase CMPG1. *Proc. Natl. Acad. Sci. USA*, **107**, 9909–9914.
- Brennecke, J., Aravin, A.A., Stark, A., Dus, M., Kellis, M., Sachidanandam, R. and Hannon, G.J. (2007) Discrete small RNA-generating loci as master regulators of transposon activity in *Drosophila*. *Cell*, **128**, 1089–1103.
- Caribé dos Santos, A.C., Sena, J.A.L., Santos, S.C., Dias, C.V., Pirovani, C.P., Pungartnik, C., Valle, R.R., Cascardo, J.C.M. and Vincentz, M. (2009) dsRNA-induced gene silencing in *Moniliophthora perniciosa*, the causal agent of witches' broom disease of cacao. *Fungal Genet. Biol.* **46**, 825–836.
- Carneiro, J.S., Bastide, P.Y., Chabot, M., Lerch, L. and Hintz, W.E. (2010) Suppression of polygalacturonase gene expression in the phytopathogenic fungus *Ophiostoma novo-ulmi* by RNA interference. *Fungal Genet. Biol.* **47**, 399–405.
- Carthew, R.W. and Sontheimer, E.J. (2009) Origins and mechanisms of miRNAs and siRNAs. *Cell*, **136**, 642–655.
- Catalanotto, C., Azzalin, G., Macino, G. and Cogoni, C. (2002) Involvement of small RNAs and role of the *qde* genes in the gene silencing pathway in *Neurospora*. *Genes Dev.* **16**, 790–795.
- Catalanotto, C., Pallotta, M., ReFalo, P., Sachs, M.S., Vayssie, L., Macino, G. and Cogoni, C. (2004) Redundancy of the two dicer genes in transgene-induced post-transcriptional gene silencing in *Neurospora crassa*. *Mol. Cell. Biol.* **24**, 2536–2545.
- Cogoni, C. and Macino, G. (1997) Isolation of quelling-defective (*qde*) mutants impaired in posttranscriptional transgene-induced gene silencing in *Neurospora crassa*. *Proc. Natl. Acad. Sci. USA*, **94**, 10 233–10 238.

- Collinge, D.B., Jorgensen, H.J., Lund, O.S. and Lyngkjær, M.F. (2010) Engineering pathogen resistance in crop plants: current trends and future prospects. *Annu. Rev. Phytopathol.* **48**, 269–291.
- Csorba, T., Pantaleo, V. and Burguán, J. (2009) RNA silencing: an antiviral mechanism. In: *Advances in Virus Research*, Vol. 75 (Loebenstein, G. and Carr, J.P., eds), pp. 35–71, 230. New York, NY: Academic Press.
- Dean, P. (2011) Functional domains and motifs of bacterial type III effector proteins and their roles in infection. *FEMS Microbiol. Rev.* **35**, 1100–1125.
- Dubreuil, G., Magliano, M., Dubrana, M.P., Lozano, J., Lecomte, P., Favory, B., Abad, P. and Rosso, M.N. (2009) Tobacco rattle virus mediates gene silencing in a plant parasitic root-knot nematode. *J. Exp. Bot.* **60**, 4041–4050.
- Erental, A., Harel, A. and Yarden, O. (2007) Type 2A phosphoprotein phosphatase is required for asexual development and pathogenesis of *Sclerotinia sclerotiorum*. *Mol. Plant–Microbe Interact.* **20**, 944–954.
- Escobar, M.A. and Dandekar, A.M. (2003) *Agrobacterium tumefaciens* as an agent of disease. *Trends Plant Sci.* **8**, 380–386.
- Escobar, M.A., Civerolo, E.L., Summerfelt, K.R. and Dandekar, A.M. (2001) RNAi-mediated oncogene silencing confers resistance to crown gall tumorigenesis. *Proc. Natl. Acad. Sci. USA*, **98**, 13 437–13 442.
- Escobar, M.A., Leslie, C.A., McGranahan, G.H. and Dandekar, A.M. (2002) Silencing crown gall disease in walnut (*Juglans regia* L.). *Plant Sci.* **163**, 591–597.
- Esquela-Kerscher, A. and Slack, F.J. (2006) Oncomirs—microRNAs with a role in cancer. *Nat. Rev. Cancer*, **6**, 259–269.
- Fairbairn, D.J., Cavallaro, A.S., Bernard, M., Mahalinga-Iyer, J., Graham, M.W. and Botella, J.R. (2007) Host-delivered RNAi: an effective strategy to silence genes in plant parasitic nematodes. *Planta*, **226**, 1525–1533.
- Fitzgerald, A., Van Kan, J.A. and Plummer, K.M. (2004) Simultaneous silencing of multiple genes in the apple scab fungus, *Venturia inaequalis*, by expression of RNA with chimeric inverted repeats. *Fungal Genet. Biol.* **41**, 963–971.
- Fulci, V. and Macino, G. (2007) Quelling: post-transcriptional gene silencing guided by small RNAs in *Neurospora crassa*. *Curr. Opin. Microbiol.* **10**, 199–203.
- Gaulin, E., Jauneau, A., Villalba, F., Rickauer, M., Esquerre-Tugaye, M.T. and Bottin, A. (2002) The CBEL glycoprotein of *Phytophthora parasitica* var. *nicotianae* is involved in cell wall deposition and adhesion to cellulosic substrates. *J. Cell Sci.* **115**, 4565–4575.
- Gonsalves, D. (1998) Control of papaya ringspot virus in papaya: a case study. *Annu. Rev. Phytopathol.* **36**, 415–437.
- Grenville-Briggs, L.J., Anderson, V.L., Fugelstad, J., Avrova, A.O., Bouzenzana, J., Williams, A., Wawra, S., Whisson, S.C., Birch, P.R., Bulone, V. and van West, P. (2008) Cellulose synthesis in *Phytophthora infestans* is required for normal appressorium formation and successful infection of potato. *Plant Cell*, **20**, 720–738.
- Ha, Y., Covert, S.F. and Momany, M. (2006) FSKS1, the 1,3-B-glucan synthase from the caspofungin-resistant fungus *Fusarium solani*. *Eukaryot. Cell*, **5**, 1036–1042.
- Hamada, W. and Spanu, P.D. (1998) Co-suppression of the hydrophobin gene HCF-1 is correlated with antisense RNA biosynthesis in *Cladosporium fulvum*. *Mol. Gen. Genet.* **259**, 630–638.
- Hartig, J.V., Tomari, Y. and Forstemann, K. (2007) piRNAs—the ancient hunters of genome invaders. *Genes Dev.* **21**, 1707–1713.
- Harvey, J.J., Lewsey, M.G., Patel, K., Westwood, J., Heimstadt, S., Carr, J.P. and Baulcombe, D.C. (2011) An antiviral defense role of AGO2 in plants. *PLoS ONE*, **6**, e14639.
- Hu, Q., Niu, Y., Zhang, K., Liu, Y. and Zhou, X. (2011) Virus-derived transgenes expressing hairpin RNA give immunity to Tobacco mosaic virus and Cucumber mosaic virus. *Virology*, **41**, 1–11.
- Hua, C., Wang, Y., Zheng, X., Dou, D., Zhang, Z., Govers, F. and Wang, Y. (2008) A *Phytophthora sojae* G-protein alpha subunit is involved in chemotaxis to soybean isoflavones. *Eukaryot. Cell*, **7**, 2133–2140.
- Huang, G., Allen, R., Davis, E.L., Baum, T.J. and Hussey, R.S. (2006) Engineering broad root-knot resistance in transgenic plants by RNAi silencing of a conserved and essential root-knot nematode parasitism gene. *Proc. Natl. Acad. Sci. USA*, **103**, 14 302–14 306.
- Jöchl, C., Rederstorff, M., Hertel, J., Stadler, P.F., Hofacker, I.L., Schrettl, M., Haas, H. and Huttenhofer, A. (2008) Small ncRNA transcriptome analysis from *Aspergillus fumigatus* suggests a novel mechanism for regulation of protein synthesis. *Nucleic Acids Res.* **36**, 2677–2689.
- Judelson, H.S. and Tani, S. (2007) Transgene-induced silencing of the zoospore-specific NIFC gene cluster of *Phytophthora infestans* involves chromatin alterations. *Eukaryot. Cell*, **6**, 1200–1209.
- Kadotani, N., Nakayashiki, H., Tosa, Y. and Mayama, S. (2003) RNA silencing in the phytopathogenic fungus *Magnaporthe oryzae*. *Mol. Plant–Microbe Interact.* **16**, 769–776.
- Katahira, J. and Yoneda, Y. (2011) Nucleocytoplasmic transport of microRNAs and related small RNAs. *Traffic*, **12**, 1468–1474.
- Katiyar-Agarwal, S. and Jin, H. (2010) Role of small RNAs in host–microbe interactions. *Annu. Rev. Phytopathol.* **48**, 225–246.
- Kim, V.N. (2004) MicroRNA precursors in motion: exportin-5 mediates their nuclear export. *Trends Cell Biol.* **14**, 156–159.
- Lacroix, H. and Spanu, P.D. (2009) Silencing of six hydrophobins in *Cladosporium fulvum*: complexities of simultaneously targeting multiple genes. *Appl. Environ. Microbiol.* **75**, 542–546.
- Latijnhouwers, M. and Govers, F. (2003) A *Phytophthora infestans* G-protein beta subunit is involved in sporangium formation. *Eukaryot. Cell*, **2**, 971–977.
- Latijnhouwers, M., Ligterink, W., Vleeshouwers, V.G.A., Van West, P. and Govers, F. (2004) A G-alpha subunit controls zoospore motility and virulence in the potato late blight pathogen *Phytophthora infestans*. *Mol. Microbiol.* **51**, 925–936.
- Laurie, J.D., Linning, R. and Bakkeren, G. (2008) Hallmarks of RNA silencing are found in the smut fungus *Ustilago hordei* but not in its close relative *Ustilago maydis*. *Curr. Genet.* **53**, 49–58.
- Lawrence, G.J., Dodds, P.N. and Ellis, J.G. (2010) Transformation of the flax rust fungus, *Melampsora lini*: selection via silencing of an avirulence gene. *Plant J.* **61**, 364–369.
- Lee, H.C., Chang, S.S., Choudhary, S., Aalto, A.P., Maiti, M., Bamford, D.H. and Liu, Y. (2009) qRNA is a new type of small interfering RNA induced by DNA damage. *Nature*, **459**, 274–277.
- Lee, H.C., Li, L., Gu, W., Xue, Z., Crosthwaite, S.K., Pertsemliadis, A., Lewis, Z.A., Freitag, M., Selker, E.U., Mello, C.C. and Liu, Y. (2010) Diverse pathways generate microRNA-like RNAs and dicer-independent small interfering RNAs in fungi. *Mol. Cells*, **38**, 803–814.
- Lee, R.C., Feinbaum, R.L. and Ambros, V. (1993) The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell*, **75**, 843–854.
- Leng, Y., Wu, C., Liu, Z., Friesen, T.L., Rasmussen, J.B. and Zhong, S. (2011) RNA-mediated gene silencing in the cereal fungal pathogen *Cochliobolus sativus*. *Mol. Plant Pathol.* **12**, 289–298.
- Li, A., Wang, Y., Tao, K., Dong, S., Huang, Q., Dai, T., Zheng, X. and Wang, Y. (2010a) PsSAK1, a stress-activated MAP kinase of *Phytophthora sojae*, is required for zoospore viability and infection of soybean. *Mol. Plant–Microbe Interact.* **23**, 1022–1031.
- Li, L., Chang, S.S. and Liu, Y. (2010b) RNA interference pathways in filamentous fungi. *Cell. Mol. Life Sci.* **67**, 3849–3863.
- Liu, H., Zhang, B., Li, C. and Bao, X. (2010) Knock down of chitinase expression in phytopathogenic fungus *Fusarium solani* and its effect on pathogenicity. *Curr. Genet.* **56**, 275–281.
- Liu, T., Ye, W., Ru, Y., Yang, X., Gu, B., Tao, K., Lu, S., Dong, S., Zheng, X., Shan, W., Wang, Y. and Dou, D. (2011) Two host cytoplasmic effectors are required for pathogenesis of *Phytophthora sojae* by suppression of host defenses. *Plant Physiol.* **155**, 490–501.
- Lund, E., Gutttinger, S., Calado, A., Dahlberg, J.E. and Kutay, U. (2004) Nuclear export of microRNA precursors. *Science*, **303**, 95–98.
- Maiti, M., Lee, H. and Liu, Y. (2007) QIP, a putative exonuclease, interacts with the *Neurospora* argonaute protein and facilitates conversion of duplex siRNA into single strands. *Genes Dev.* **21**, 590–600.
- Mao, Y.B., Cai, W.J., Wang, J.W., Hong, G.J., Tao, X.Y., Wang, L.J., Huang, Y.P. and Chen, X.Y. (2007) Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. *Nat. Biotechnol.* **25**, 1307–1313.
- McDonald, T., Brown, D., Keller, N.P. and Hammond, T.M. (2005) RNA silencing of mycotoxin production in *Aspergillus* and *Fusarium* species. *Mol. Plant–Microbe Interact.* **18**, 539–545.
- Melnik, C.W., Molnar, A. and Baulcombe, D.C. (2011) Intercellular and systemic movement of RNA silencing signals. *EMBO J.* **30**, 3553–3563.
- Miyamoto, Y., Masunaka, A., Tsuge, T., Yamamoto, M., Ohtani, K., Fukumoto, T., Gomi, K., Peever, T.L. and Akimitsu, K. (2008) Functional analysis of a multicopy host-selective ACT-toxin biosynthesis gene in the tangerine pathotype of *Alternaria alternata* using RNA silencing. *Mol. Plant–Microbe Interact.* **21**, 1591–1599.
- Miyoshi, K., Miyoshi, T. and Siomi, H. (2010) Many ways to generate microRNA-like small RNAs: non-canonical pathways for microRNA production. *Mol. Genet. Genomics*, **284**, 95–103.

- Moriwaki, A., Ueno, M., Arase, S. and Kihara, J. (2007) RNA-mediated gene silencing in the phytopathogenic fungus *Bipolaris oryzae*. *FEMS Microbiol. Lett.* **269**, 85–89.
- Nakayashiki, H. (2005) RNA silencing in fungi: mechanisms and applications. *FEBS Lett.* **579**, 5950–5957.
- Nakayashiki, H., Hanada, S., Quoc, N.B., Kadotani, N., Tosa, Y. and Mayama, S. (2005) RNA silencing as a tool for exploring gene function in ascomycete fungi. *Fungal Genet. Biol.* **42**, 275–283.
- Napoli, C., Lemieux, C. and Jorgensen, R. (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *Plant Cell*, **2**, 279–289.
- Nguyen, Q.B., Kadotani, N., Kasahara, S., Tosa, Y., Mayama, S. and Nakayashiki, H. (2008) Systematic functional analysis of calcium-signalling proteins in the genome of the rice-blast fungus, *Magnaporthe oryzae*, using a high-throughput RNA-silencing system. *Mol. Microbiol.* **68**, 1348–1365.
- Nicolas, F.E., Torres-Martinez, S. and Ruiz-Vazquez, R.M. (2003) Two classes of small antisense RNAs in fungal RNA silencing triggered by non-integrative transgenes. *EMBO J.* **22**, 3983–3991.
- Nicolas, F.E., Moxon, S., de Haro, J.P., Calo, S., Grigoriev, I.V., Torres-Martinez, S., Moulton, V., Ruiz-Vazquez, R.M. and Dalmay, T. (2010) Endogenous short RNAs generated by dicer 2 and RNA-dependent RNA polymerase 1 regulate mRNAs in the basal fungus *Mucor circinelloides*. *Nucleic Acids Res.* **38**, 5535–5541.
- Nolan, T., Cecere, G., Mancone, C., Alonzi, T., Tripodi, M., Catalanotto, C. and Cogoni, C. (2008) The RNA-dependent RNA polymerase essential for post-transcriptional gene silencing in *Neurospora crassa* interacts with replication protein A. *Nucleic Acids Res.* **36**, 532–538.
- Nowara, D., Gay, A., Lacomme, C., Shaw, J., Ridout, C., Douchkov, D., Hensel, G., Kumléhn, J. and Schweizer, P. (2010) HIGS: host-induced gene silencing in the obligate biotrophic fungal pathogen *Blumeria graminis*. *Plant Cell*, **22**, 3130–3141.
- Nunes, C.C., Gowda, M., Sailsbery, J., Xue, M., Chen, F., Brown, D., Oh, Y., Mitchell, T.M. and Dean, R.A. (2011) Diverse and tissue-enriched small RNAs in the plant pathogenic fungus, *Magnaporthe oryzae*. *BMC Genomics*, **12**, 1–20.
- Patel, R.M., van Kan, J.A., Bailey, A.M. and Foster, G.D. (2008) RNA-mediated gene silencing of superoxide dismutase (bcsod1) in *Botrytis cinerea*. *Phytopathology*, **98**, 1334–1339.
- Romano, N. and Macino, G. (1992) Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences. *Mol. Microbiol.* **6**, 3343–3353.
- Rooney, H.C., Van't Klooster, J.W., van der Hoorn, R.A., Joosten, M.H., Jones, J.D. and de Wit, P.J. (2005) Cladosporium Avr2 inhibits tomato Rcr3 protease required for Cf-2-dependent disease resistance. *Science*, **308**, 1783–1786.
- Ruiz-Ferrer, V. and Voinnet, O. (2009) Roles of plant small RNAs in biotic stress responses. *Annu. Rev. Plant Biol.* **60**, 485–510.
- Sanford, J.C. and Johnston, S.A. (1985) The concept of parasite-derived resistance—deriving resistance genes from the parasite's own genome. *J. Theor. Biol.* **113**, 395–405.
- Sato, F., Tsuchiya, S., Meltzer, S.J. and Shimizu, K. (2011) MicroRNAs and epigenetics. *FEBS J.* **278**, 1598–1609.
- Schornack, S., Huitema, E., Cano, L.M., Bozkurt, T.O., Oliva, R., Van Damme, M., Schwizer, S., Raffaele, S., Chaparro-García, A., Farrer, R., Segretin, M.E., Bos, J., Haas, B.J., Zody, M.C., Nusbaum, C., Win, J., Thines, M. and Kamoun, S. (2009) Ten things to know about oomycete effectors. *Mol. Plant Pathol.* **10**, 795–803.
- Schumann, U., Ayliffe, M., Kazan, K. and Wang, M. (2010) RNA silencing in fungi. *Front. Biol.* **5**, 478–494.
- Shafran, H., Miyara, I., Eshed, R., Prusky, D. and Sherman, A. (2008) Development of new tools for studying gene function in fungi based on the gateway system. *Fungal Genet. Biol.* **45**, 1147–1154.
- Singh, S., Braus-Stromeier, S.A., Timpner, C., Tran, V.T., Lohaus, G., Reusche, M., Knufer, J., Teichmann, T., von Tiedemann, A. and Braus, G.H. (2010) Silencing of *Vlaor2* for chorismate synthase revealed that the phytopathogen *Verticillium longisporum* induces the cross-pathway control in the xylem. *Appl. Microbiol. Biotechnol.* **85**, 1961–1976.
- Stukenbrock, E.H. and McDonald, B.A. (2009) Population genetics of fungal and oomycete effectors involved in gene-for-gene interactions. *Mol. Plant-Microbe Interact.* **22**, 371–380.
- Tinoco, M.L., Dias, B.B., Dall'Asta, R.C., Pamphile, J.A. and Aragao, F.J. (2010) *In vivo* trans-specific gene silencing in fungal cells by *in planta* expression of a double-stranded RNA. *BMC Biol.* **8**, 1–11.
- Tomilov, A.A., Tomilova, N.B., Wroblewski, T., Michelmore, R. and Yoder, J.I. (2008) Trans-specific gene silencing between host and parasitic plants. *Plant J.* **56**, 389–397.
- Turner, C.T., Davy, M.W., MacDiarmid, R.M., Plummer, K.M., Birch, N.P. and Newcomb, R.D. (2006) RNA interference in the light brown apple moth, *Epiphyas postvittana* (Walker) induced by double-stranded RNA feeding. *Insect Mol. Biol.* **15**, 383–391.
- Tyler, B.M. (2009) Entering and breaking: virulence effector proteins of oomycete plant pathogens. *Cell. Microbiol.* **11**, 13–20.
- Urwin, P.E., Lilley, C.J. and Atkinson, H.J. (2002) Ingestion of double-stranded RNA by parasitic juvenile cyst nematodes leads to RNA interference. *Mol. Plant-Microbe Interact.* **15**, 747–752.
- Vetukuri, R.R., Avrova, A.O., Grenville-Briggs, L.J., van West, P., Soderbom, F., Savenkov, E.I., Whisson, S.C. and Dixelius, C. (2011) Evidence for involvement of Dicer-like, Argonaute and histone deacetylase proteins in gene silencing in *Phytophthora infestans*. *Mol. Plant Pathol.* **12**, 772–785.
- Viss, W.J., Pitrak, J., Humann, J., Cook, M., Driver, J. and Ream, W. (2003) Crown-gall-resistant transgenic apple trees that silence *Agrobacterium tumefaciens* oncogenes. *Mol. Breed.* **12**, 283–295.
- Walker, C.A., Koppe, M., Grenville-Briggs, L.J., Avrova, A.O., Horner, N.R., McKinnon, A.D., Whisson, S.C., Birch, P.R. and van West, P. (2008) A putative DEAD-box RNA-helicase is required for normal zoospore development in the late blight pathogen *Phytophthora infestans*. *Fungal Genet. Biol.* **45**, 954–962.
- Wang, Y., Dou, D., Wang, X., Li, A., Sheng, Y., Hua, C., Cheng, B., Chen, X., Zheng, X. and Wang, Y. (2009) The *PsCZF1* gene encoding a C2H2 zinc finger protein is required for growth, development and pathogenesis in *Phytophthora sojae*. *Microb. Pathog.* **47**, 78–86.
- Wang, Y., Li, A., Wang, X., Zhang, X., Zhao, W., Dou, D., Zheng, X. and Wang, Y. (2010) GPR11, a putative seven-transmembrane G protein-coupled receptor, controls zoospore development and virulence of *Phytophthora sojae*. *Eukaryot. Cell*, **9**, 242–250.
- Westwood, J.H., Roney, J.K., Khatibi, P.A. and Stromberg, V.K. (2009) RNA translocation between parasitic plants and their hosts. *Pest Manag. Sci.* **65**, 533–539.
- Whisson, S.C., Avrova, A.O., Van West, P. and Jones, J.T. (2005) A method for double-stranded RNA-mediated transient gene silencing in *Phytophthora infestans*. *Mol. Plant Pathol.* **6**, 153–163.
- White, F.F., Potnis, N., Jones, J.B. and Koebe, R. (2009) The type III effectors of *Xanthomonas*. *Mol. Plant Pathol.* **10**, 749–766.
- Xiao, H., Alexander, W.G., Hammond, T.M., Boone, E.C., Perdue, T.D., Pukkila, P.J. and Shiu, P.K. (2010) QIP, a protein that converts duplex siRNA into single strands, is required for meiotic silencing by unpaired DNA. *Genetics*, **186**, 119–126.
- Yi, R., Qin, Y., Macara, I.G. and Cullen, B.R. (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev.* **17**, 3011–3016.
- Yin, C., Jurgenson, J. and Hulbert, S. (2010) Development of a host-induced RNAi system in the wheat stripe rust fungus *Puccinia striiformis* f. sp. *tritici*. *Mol. Plant-Microbe Interact.* **24**, 554–561.
- Zhao, W., Dong, S., Ye, W., Hua, C., Meijer, H.J.G., Dou, X., Govers, F. and Wang, Y. (2011) Genome-wide identification of *Phytophthora sojae* SNARE genes and functional characterization of the conserved SNARE PsYKT6. *Fungal Genet. Biol.* **48**, 241–251.