J Plant Growth Regul (2000) 19:371–384
DOI: 10.1007/s003440000043

J P G R
Journal of Plant Growth Regulation

The Efficacy of RNAi in the Study of the Plant Cytoskeleton

Vincent P. Klink and Stephen M. Wolniak*

Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, Maryland 20742, USA

Abstract

Recent studies on a variety of organisms point to the ubiquity of RNA interference (RNAi) as a means to induce a gene-specific block to translation. RNAi has gained popularity in the last few years in the study of a number of problems in development. In this review, we highlight recent findings with RNAi using several different kinds of animals and fungi, and we show how these responses parallel cosuppression effects described in plants nearly a decade earlier. We then point to the efficacy of RNAi in studying minor and regulatory components of the plant cytoskeleton, and we highlight some recent studies using this approach with the water fern, *Marsilea vestita*.

Key words: Cytoskeleton; RNAi; Tubulin; Actin; *Marsilea*

Introduction

The cytoskeleton of plant cells is composed of dynamic arrays of microtubules and microfilaments that serve in a variety of diverse functions [reviewed in Marc 1997; Pierson and Cresti 1992; Volkmann and Baluska 1999]. The cytoskeleton has been implicated in the distribution and reorganization of cytoplasm in a variety of processes through its involvement in the transport of organelles and other cytoplasmic complexes to specific domains within the cell. The tubulin and actin proteins that are present in the microtubules and microfilaments of plant cells are present in relatively small gene families. These conserved proteins interact with a variety of regulatory and motor proteins that affect patterns of cytoskeletal organization and directions of particle movement. Although, the general organization and composition of the plant cytoskeleton has been documented during the last two decades, largely through time course image reconstructions from fixed and labeled cells (*reviews,* Hoffman and Vaughn 1994, 1995; Marc 1997; Pickett-Heaps and others 1999; Renzaglia and Maden 2000; Vaughn and Harper 1998), the dynamic nature of the plant cytoskeleton has been revealed in living cells through the microinjection of fluorescently tagged cytoskeletal proteins (Hepler and others 1993; Zhang and others 1990, 1993) or drugs that bind to cytoskeletal elements (Cleary 1995; Schmit and Lambert 1988).

It is now possible to probe the function of specific proteins that interact with microtubules or microfilaments. This review focuses on the efficacy and usefulness of RNA interference (RNAi) (Fire and others 1998) in studying the plant cytoskeleton. RNAi is a process in which a sequence-specific pool of mRNA can be eliminated from the cytoplasm of cells by an endogenous surveillance mechanism triggered by the introduction of exogenously supplied dsRNA. This technique has proven to be a powerful method to investigate gene function when genetics or biochemical methods are difficult. Although the identification of new proteins that interact with plant actin and tubulin has lagged behind comparable characterizations in other eukaryotic organ-

Online publication 2 May 2001
**Corresponding author; e-mail:* sw36@umail.umd.edu

isms, it is evident from ever-expanding databases of gene sequences and expressed sequence tags (EST) that many of the cytoskeletal proteins found in animals and fungi have plant homologs. With the advent of RNAi, it is now possible to expand screens for the role(s) of known and unknown genes with relative ease to study the organization and function of plant cytoskeletal gene products. This is especially important because RNAi can be used to study plant cytoskeletal regulation in a functional context, using living cells, tissues, or organs. RNAi strategies allow for the inducible, heritable, reversible, gene-specific inhibition of translation in a variety of organisms.

Discovery of the Ubiquity of RNAi

Direct inhibition of gene function at the RNA level without knockout genetics can be readily achieved by use of RNAi. This phenomenon, first described in *C. elegans* (Fire and others 1998), has been demonstrated in organisms such as the frog (Epstein and others 1997), chicken (Buchberger and others 1998), fruit fly (Kennerdell and Carthew 1998), trypanosome (Ngo and others 1998), protozoans (Ruiz and others 1998), planarian (Alvarado and Newmark 1999), zebrafish (Wargelius and Ellingsen 1999), *Neurospora* (Cogoni and Macino 1999a, 1999b), and mouse (Wianny and Zernicka-Goetz 2000). The unexpected complexities of gene expression in cells containing introduced RNAs were initially uncovered in plants about a decade earlier, and these patterns in gene silencing became known as cosuppression (Cameron and Jennings 1991; English and others 1997; Jones and others 1987; Jorgensen 1995; Matzke and Matzke 1995; Napoli and others 1990; Ratcliff and others 1997).

It has long been known that introducing an antisense RNA provides an efficient way to eliminate endogenous mRNA (Fire and others 1991; Holt and others 1988; Kemphues and others 1988). In the simplest case, antisense RNA added to cells blocks translation of specific messages through the formation of stable RNA dimers that would then be targeted for degradation. Also in the simplest case, sense RNA added to cells should have no effect on the translation of that specific message. By the use of antisense methods, genes of interest that were subcloned into bacterial plasmids containing T7, T3, or SP6 transcriptional promoters were used to generate 100–1,000 nt RNA transcripts in vitro. The single-stranded RNA was then delivered to the tissue by injection, culture, feeding, perfusion, or electroporation (Buchberger and others 1998; Holt and others 1988; Kelly and others 1997; Ngo and others 1998; Tabara and others 1998; Timmons and Fire 1998). Initially, antisense effects were compared with known gene knockouts (Guo and Kemphues 1995; Kelly and others 1997). When the antisense effects mimicked those of the knockout, it was said to have *phenocopied* the knockout and served as confirmation of eliminated gene function. In this context, phenocopy describes a change induced after the introduction of RNA-based inhibitors in the absence of mutations. In *C. elegans,* the injection of antisense RNA into a germline syncytium can eliminate maternal and zygotic gene activity (Kelly and others 1997). Surprisingly, sense RNA was found to achieve the same effect (Kelly and others 1997). Occasionally, no effects were observed after injection of antisense RNA into nematode zygotes until late into embryogenesis (Fire and others 1998) or even until adulthood (Kuwabara 1998). In these experiments, it was clear that by the time the antisense inhibitory effects were observed, the original high concentration of antisense RNA delivered to the zygote had been reduced to just a few copies per cell. At this low level, the antisense RNA would not be sufficiently abundant to deplete endogenous mRNA levels through stoichiometric binding (Fire and others 1998). Therefore, the antisense paradigm of complementary dimer formation could not apply in all cases, and thus led to the conclusion that *the dissipation of endogenous mRNA may result from the formation of dsRNA complexes and the consequent activation of surveillance mechanisms that are involved in regulating mRNA abundance in a wide variety of cells* (Montgomery and Fire 1998; Montgomery and others 1998; Ngo and others 1998).

Thus, antisense experiments have revealed unforeseen tiers of mRNA metabolism that are manifested by the disappearance of specific mRNAs and the absence of their products of translation. These events are induced by small quantities of RNA molecules in processes now known collectively as dsRNA interference (RNAi) (Fire and others 1998; Sharp 1999). Tests demonstrated that the activity of antisense and sense RNA was actually achieved through cryptic reverse strand transcription that resulted in the formation of low concentrations of double stranded RNA during standard in vitro transcription reactions (Fire and others 1998; Montgomery and Fire 1998; Montgomery and others 1998). It is this dsRNA that is responsible for catalytically inactivating endogenous RNA (Fire and others 1998), and as an RNAi effect, dsRNA is typically effective at concentrations approximately 2 orders of magnitude lower than either sense or antisense RNA (Fire and others 1998). Specificity of the dsRNA was demonstrated by a series of inhibition experiments using

intron sequences and 5′ upstream sequences. In all cases, the introns and upstream sequences elicited no RNA inhibition phenocopies. Embryos appeared unaffected, resembling wild-type controls. This set of control experiments eliminated speculation that dsRNA could induce a panic response mechanism, resulting in degradation or inactivation of all cellular mRNA (Proud 1995). Hence, RNAi serves to abolish zygotic and maternal reserves of mRNA in a gene-specific, nonstoichiometric manner (Fire and others 1998). However, the mechanism of how RNAi actually eliminates endogenous mRNA appears to be complex (Figure 1) and is still under scrutiny.

Mechanism of RNAi

Recent work has focused on the mechanism of RNAi (Figure 1). RNAi can be activated in vitro (Tuschl and others 1999) requiring ATP but is independent of mRNA translation and target mRNA cap recognition (Zamore and others 2000). The dsRNA is cleaved from large sequences that are hundreds of nucleotides (nt) in length to fragments of only 21–23 nt. This cleavage is not dependent on target mRNA, because cleavage has been shown to occur in the absence of the target mRNA (Zamore and others 2000). Target mRNA is cut in the same region having sequence identity to the cleaved dsRNA, thereby suggesting that the dsRNA oligomers may be involved in the scanning, surveillance, and recognition of specific mRNAs (Zamore and others 2000). The presence of these short oligomeric dsRNA sequences was found in plants undergoing a form of RNAi called posttranscriptional gene silencing (PTGS) (Fire 1999; Hamilton and Baulcombe 1999; Kasschau and Carrington 1998). PTGS, which is similar to "quelling" in fungi, requires the presence of an RNA-dependent RNA polymerase, an enzyme that synthesizes dsRNA in vivo and thereby alters patterns of gene expression (Dalmay and others 2000; Mourrain and others 2000). We suspect that RNAi, PTGS, and quelling are all related, if not identical, pathways for the regulation of gene expression.

Recently, a nuclease has been isolated from cultured *Drosophila* S2 cells that were undergoing RNAi (Hammond and others 2000). This nuclease degraded mRNA in a fashion that resembles RNAi effects. In addition, short 21–25-mer dsRNA fragments copurified with the nuclease, consistent with the prediction that dsRNA fragments were acting directly in the RNAi process. To substantiate that these 21–25-mers are directly involved in RNAi, incubation of the dsRNA-nuclease mix with micrococcal nuclease, a nuclease that will degrade both DNA and dsRNA, eliminated the RNAi effect (Hammond and others 2000). Incubation with either DNAse or a tRNA carrier used as a competitor for mRNA was without effect on RNAi (Hammond and others 2000). From these and other observations, it appears that the cleavage of dsRNA involves an RNAse III-like enzyme (Hammond and others 2000); RNAse III exhibits appropriate specificity and activity for the cleavage of dsRNA at specific intervals into fragments that resemble those found in the RNAi systems (Abou Elela and Ares 1998; Rotondo and others 1997). For dsRNA to bind to RNAse III, the dsRNA must be at least approximately 22 nt in length, because the dsRNA must wrap around the enzyme twice. RNAse III cleavage produces populations of 23- and 25-nt dsRNAs with staggered ends (Hamilton and Baulcombe 1999; Zamore and others 2000) that are subsequently cleaved by ATP-dependent ssRNA-directed ribonucleases so that each fragment of the dsRNA is 21 nt in length. In the absence of ATP, only the 23-nt dsRNA is present (Hammond and others 2000).

With the observation that only very few dsRNA molecules are required to induce RNAi effects in developing organisms (Clemens and others 2000), it appears that very low concentrations of dsRNA deplete endogenous mRNA in vivo. Thus far, only high levels of RNA between 100–1100 nt at concentrations of several milligrams per milliliter have been found to be effective in vitro. A possible explanation for this discrepancy is that adenosine deamination of long dsRNA can occur at 50–60% of adenosine sites on completely paired dsRNAs such as those used in RNAi experiments (Morse and Bass 1999). Deamination of adenosine results in inositol formation and causes base pair mismatching, which lowers RNAi activity of dsRNA molecules (Bass 2000). The antagonism seems to occur because the 21-mer dsRNA that would attach to the nuclease would no longer be able to recognize its endogenous mRNA substrate, thereby losing its scanning and surveillance properties. However, even though deamination is occurring in the in vitro RNAi extracts, only 0.4–0.7% of the adenosines are observed to be deaminated in short 21-mer dsRNAs (Zamore and others 2000). Although high doses of dsRNA used in RNAi experiments both in vivo and in vitro can overcome problems posed by deamination of adenosine residues, it appears that heterologous probes may be of little use for RNAi in the analysis of biologic problems.

These emerging perspectives make it clear that RNAi effects are multitiered, because the genes cloned thus far from mutants of RNAi are not RNAse

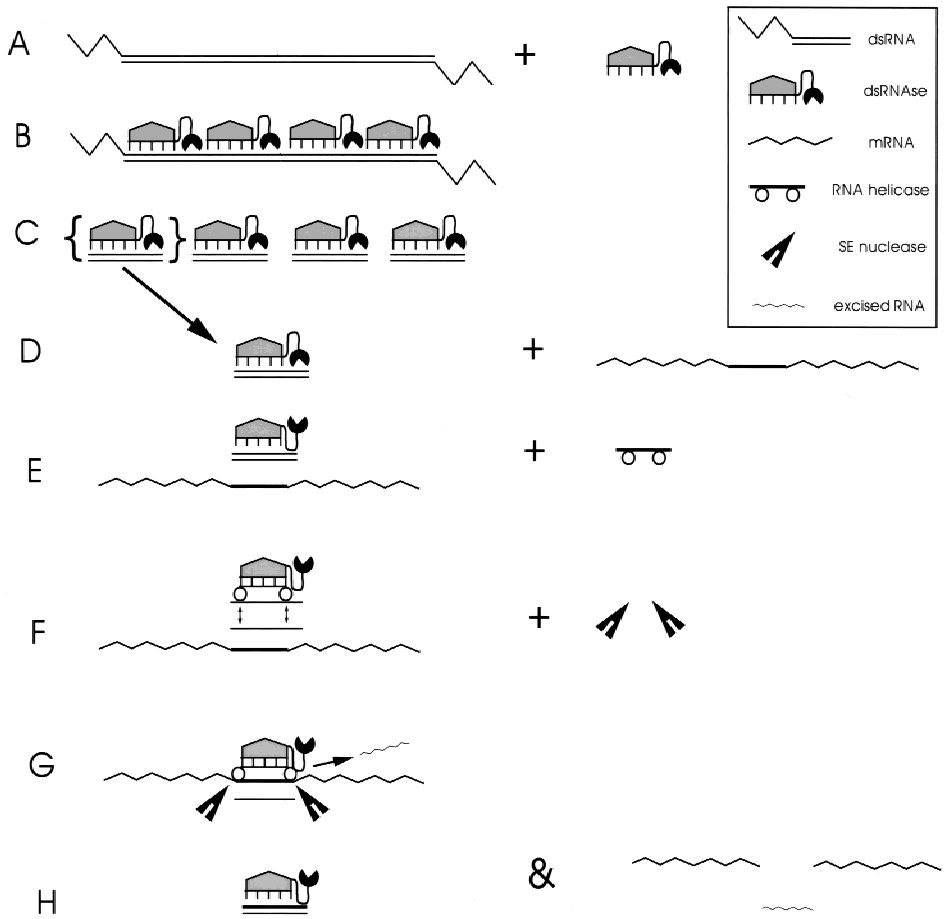

Figure 1. Model of the RNAi-mediated degradation of cognate endogenous mRNA. RNAi involves a multistep process of recognition, sequence-specific cleavage, unwinding, and sequence-specific replacement of the dsRNA oligomer sense strand by its cognate mRNA through successive interactions with a multimeric protein complex. **(A)** Double-stranded RNA is recognized by a dsRNAse-like protein. **(B)** The dsRNAse proteins bind to the dsRNA by a dsRNA-binding motif (gray pentagon), coating the dsRNA in a sequence independent, but RNA-dependent manner. **(C)** The dsRNA is then cleaved by the dsRNAse (black semicircle facing down) into small 23-mer fragments. **(D)** After cleavage of the dsRNA (black semicircle facing up), the dsRNAs in the RNAse-dsRNA complex interact with their cognate mRNA pool in a sequence-specific manner. **(E)** A dsRNA helicase becomes associated with the complex. **(F)** The dsRNA helicase then unwinds the dsRNA. **(G)** The mRNA interacts with a strand exchange (SE) nuclease-helicase complex, resulting in the ATP-dependent excision and replacement of the sense strand RNA of the original oligomeric RNA duplex. **(H)** The result of the reaction is a new dsRNA oligomer composed of the original antisense oligomer and the new sense oligomer excised from the cognate mRNA. The remaining mRNA fragments are available for further degradation **(A)** after entering the RNAi pathway.

III-like. The gene products involved in RNAi responses (PTGS) may serve a role in protecting organisms from viruses and transposons in a fashion similar to a primitive immune system (Boscher and Labousse 2000; Fire 1999; Kasschau and Carrington 1998). Some of the components in this response cascade show homology to RNAseD and the Werner syndrome helicase (Ketting and others 1999), DNA helicases (Cogoni and Macino 1999a), and RNA-dependent RNA polymerase (Boscher and Labousse 2000; Cogoni and Macino 1999b; Dalmay and others 2000; Mourrain and others 2000; Smardon and others 2000). Homologs have been found in organisms that span the eukaryotic realm (Hunter 2000; Tabara and others 1999). There may be several discrete modes of action (Figure 1) that allow different RNAi pathways to function in diverse cell types and tissues (Fire 1998). Analyses have shown that some tissue types are refractory to RNAi, and some mutants have functional RNAi in some tissues that is absent in others (Bass 2000).

Use of RNAi in Model Systems to Study the Cytoskeleton

Although *C. elegans* has been used extensively as a model genetic system, there are many genes for which no known mutants exist. Shortly after the discovery of RNAi in *C. elegans* (Fire and others 1998), investigators in a number of laboratories (Gonczy and others 1999; Jansch-Plunger and Glotzer 1999; Schumacher and others 1998; Shelton and others 1999; Skop and White 1998) used this new approach to facilitate rapid identification of regulatory and structural components of the cytoskeleton (Table 1). In many cases, no mutants were available.

The use of RNAi on a whole genome scale has already begun on *C. elegans* and will likely begin in *Drosophila* and other organisms in which their respective genomes are being sequenced. In *C. elegans,* Shelton and others (1999) used an RNAi-based screen to isolate genes involved in establishing po-

Table 1. RNAi and the Cytoskeleton

Gene	Product	Role	Reference
AIR-1	Aurora-like	Centrosome	Schumacher and others 1998
DNC-1	$p150^{glued}$	Dynactin complex	Skop and White 1998
DNC-2	p50/dynamitin	Dynactin complex	Skop and White 1998
Dhc-1	Dynein heavy chain	Centrosome migration	Gonczy and others 1999
MLKP1	Myosin light chain kinase	Cell division	Powers and others 1998
Syn-4	Syntaxin	Cell division	Jansch-Plotzer and Glotzer 1999
mlc-1	Myosin light chain	Cell division	Shelton and others 1999

larity of the embryo. Unlike standard reverse genetic assays, they took random inserts from a phage library and used polymerase chain reaction (PCR) to amplify them in 96-well plates. Double-stranded RNAs were pooled (8 inserts per pool), injected into zygotes and scored for embryonic lethalities exhibiting polarity defects (Shelton and others 1999). Those pooled dsRNAs eliciting defects were then split and reinjected individually. By this method, they were able to observe the effects of a large number of genes within a few rounds of screening. Tony Hyman and coworkers (cited in Fisler 2000) are studying mitotic spindle dynamics by injecting worms with RNAi from random open-reading frames. Defects in mitotic spindle function are assessed with standard video microscopy in developing embryos. After potential candidate genes are identified, appropriate antibodies are generated for immunolocalizations. Restoration microscopy is used for comparisons of antibody localization patterns between control and RNAi-treated samples and for quantitative evaluations of fluorescent intensities that exist between the two groups. The fact that these studies could be accomplished without mutational analysis underscores the significance of an RNAi approach. In addition, because RNAi eliminates the requirement of mutants or the maintenance of mutants, RNAi can be also be used on tissues or cell cultures. This can be especially important in studying organisms that exhibit long life cycles or are difficult to sustain in the laboratory.

Use of RNAi in Other Systems

Success with RNAi has now reached the stage at which important biologic questions can be directly explored in a wide array of organisms, cells, or tissues. RNAi has been used to study the process of tissue regeneration in planarians (Alvarado and Newmark 1999). In this study, planarian myosin dsRNA (myoRNAi) was injected into worms as a means to inhibit regeneration dynamics that would normally involve the myosin heavy chain. After injection of myoRNAi, the planarian fragments were allowed to heal for 3 days and then were processed for immunocytochemistry (Alvarado and Newmark 1999). Three-day-old fragments showed little regeneration of the body wall musculature (Alvarado and Newmark 1999). Very few preexisting myosin fibers were noted in dsRNAi-treated fragments, and the fibers that were present appeared to be remnants that existed before injection (Alvarado and Newmark 1999). The observed defects were specific to the abrogation of myosin translation, because no other gross abnormalities were observed. RNAi affected both the newly regenerated cells and those that were preexisting and terminally differentiated (Alvarado and Newmark 1999). Finally, the dsRNA injection was gene specific; the injections only affected body wall myosin, whereas the abundance and distribution of a different myosin isoform responsible for pharynx formation, was totally normal (Alvarado and Newmark 1999). After treatments with myosin dsRNA, immunolocalizations with antiacetylated tubulin antibodies revealed that the planarians initiated abnormal ciliogenesis on their basal epithelia (Alvarado and Newmark 1999). A negative control with α-tubulin RNAi injections resulted in reduced ciliogenesis but normal myosin accumulation. Ciliary anomalies resulted from lowered abundance of the α-tubulin transcript and the α-tubulin protein (Alvarado and Newmark 1999). Similar studies in trypanosomes (Ngo and others 1998) and *Paramecium* (Ruiz and others 1998) have also shown that RNAi can be used to study specific cytoskeletal components.

Spermiogenesis in Ferns as a Model for Investigating Plant Cytoskeletal Reorganization

Spermiogenesis in lower land plants is accomplished through the differentiation of a typical nonmotile plant cell into a multiciliated gamete (Hoffman and Vaughn 1995; Renzaglia and Maden 2000; Wolniak

and others 2000). At the onset of spermiogenesis, neither the spermatocyte, nor the cell from which it was derived, possess centrioles or basal bodies. An important facet of this developmental pathway is that it contradicts a basic tenet of animal cell biology, in which new centrioles are built from a parent centriolar template. In these plant cells, basal bodies are assembled de novo and then serve as templates for ciliogenesis (Hepler 1976).

During the last century, an impressive record of the structural aspects of development during lower land plant spermiogenesis has been characterized. These studies established that in bryophytes, hornworts, and lycopods, a spermatocyte synthesizes a coaxially oriented pair of basal bodies within a centrosome-like amorphous mass (Kreitner and Carothers 1976; Moser and Kreitner 1969; Vaughn and Renzaglia 1998). In *Isoetes, Equisetum, Psilotum,* a number of pteridophytes, *Ginkgo,* and a number of cycads, the undifferentiated cell (generative cell in *Ginkgo*) condenses a nonmembrane-bound amorphous structure known as the blepharoplast (that is, eyelash), (Webber 1897). The blepharoplast is a precursor structure for the de novo genesis of basal bodies (Hepler 1976). In *Isoetes* (a lycopod), *M. vestita,* (a fern) and *Ceratozamia* (a cycad), this developmental event results in the synthesis of 12, 140, and more than 40,000 centrioles in each spermatid, respectively. Basal bodies are made from no apparent preexisting centriolar template, but instead, are synthesized during one synchronous event (Hepler 1976; Norstog , 1974,1975, 1978, 1986). Subsequently, a massive transformation of these cells begins when newly made basal bodies are deployed for the developing spermatid ciliary array.

Concomitant with basal body formation in the plant spermatids, the nucleus and anterior mitochondrion become tightly associated with a forming cytoskeletal array known as a multilayered structure (MLS), which serves as a signature structure in these gametes (Figure 2). The uppermost stratum of the MLS is a ribbon of cross-linked microtubules situated on the dorsal side of the coiled cell body. On the dorsal face of the microtubule ribbon (known as a spline) are the basal bodies of the motile apparatus. The basal bodies are attached to the cross-linked microtubules of the spline. An elongated nucleus and mitochondrion can be found on the ventral side of the spline (for reviews see Carothers 1975; Duckett 1975). A MLS exists in the spermatozoids of Charophycean algae (that is, *Chara* (Mottier 1904), *Nitella* (Turner 1968), and *Coleochaetae,* (Graham and Repavich 1989)), and comparable MLS arrays can be found in liverworts, mosses, lycopods, horsetails, and ferns, and in certain gynmosperms, namely the

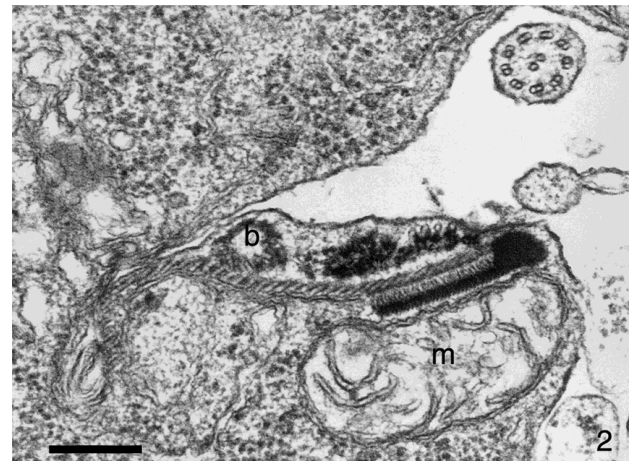

Figure 2. The multilayered structure (MLS) in a developing spermatid of *Marsilea vestita.* The MLS consists of a series of vanes and fins that subtend a planar ribbon of microtubules (the spline). On the ventral side of the MLS, there is an osmophilic zone and a mitochondrion (m) that appears to be attached to the lowermost stratum of the MLS. On the dorsal face of the spline, there is an obliquely sectioned basal body (b) and osmiophilic material that has been observed between the basal bodies (Myles and Hepler 1977). Bar = 0.2 µm.

cycads and *Ginkgo biloba.* There can be variation in the numbers of strata that underlie the spline microtubules in different organisms, and the width of the spline is also dependent on the organism and (Carothers and Kreitner 1968; Carothers 1975; Hoffman and Vaughn 1995). In addition to the MLS, there is an osmophilic crest associated with the basal bodies (Myles and Hepler 1977) in some spermatozoids that has been purported to play a function in ciliary beat coordination (Wolniak and others 2000). The basal bodies can be quite long in some organisms, with elaborate transition zones (Renzaglia and Maden 2000). The entire complex, including the ciliary array, is visible at the light microscopic level and was described early in the 20th century by plant cytologists in a variety of organisms (Table 2).

M. vestita has been used in the past to study spermiogenesis at the structural (Hepler 1976; Mizukami and Gall 1966; Myles and Hepler 1977, 1982; Sharp 1914), biochemical (Hyams and others 1983; Pennell and others 1988), and molecular levels (Hart and Wolniak 1998, 1999; Klink and Wolniak 1999; Tsai and Wolniak 1999; Wolniak and others 2000; Klink and Wolniak 2001). *M. vestita* is a heterosporous fern; heterospory allows easy separation of male from female spores. *M. vestita* is endosporous (all gametophytic development occurs within the spore wall), and most of the gametophytic mass and

Table 2. Blepharoplasts from Different Groups of Plants Vary in Size

Organism	Division	BS[a]	BB#[b]	References
Anthoceros	Anthocerophyta	0.2–0.5	2	Moser and Kreitner 1969
Hepaticum	Hepatophyta	0.2–0.5	2	Carothers 1975
Lycopodium	Lycophyta	0.2–0.5	2–3	Carothers and others 1975
Isoetes	Lycophyta	0.2–0.5	12	Duckett 1973a
Equisetum	Sphenophyta	0.5–0.75	40–60;120	Sharp 1912; Duckett 1973b
Pteridium	Pterophyta	0.5–1.0	40	Bell 1979; Bell and Duckett 1976
Platyzoma	Pterophyta	0.2–0.5	50–60	Doonan and others 1986
Ceratopteris	Pterophyta	0.5–0.6	70–80	Hoffman and Vaughn 1994
Marsilea	Pterophyta	0.5–0.6	130–140	Hepler 1976; Mizukami and Gall 1966
Ginkgo	Ginkgophyta	3.5–5.0	1,000–1,200	Gifford and Lin 1975
Microcycas	Cycadopyta	9.0	2,000–5,000	Norstog 1990; Caldwell 1907
Cycas	Cycadopyta	12.0	2,000	Norstog 1990
Dioon	Cycadopyta	16.0–20.0	20–40,000	Chamberlain 1909
Zamia	Cycadopyta	22.5–26.0	45–75,000	Norstog 1986; Mizukami and Gall 1966
Ceratozamia	Cycadopyta	27.0	~40,000	Norstog 1990; Chamberlain 1909

[a] *Blepharoplast size in μm.*
[b] *Basal body number.*

volume is directed toward the production of spermatozoids. The spores grow quickly and synchronously in liquid culture. Like others, we have found that inhibitors can be added directly to the culture medium, and their effects can be easily assayed at the cytologic, biochemical, and molecular level (Hart and Wolniak 1998, 1999; Hyams and others 1983; Myles and Hepler 1982; Wolniak and others 2000). Thus, the plan of the gametophyte, combined with the synchrony of development, permits the study of spermiogenesis in cell populations harvested at defined time intervals after imbibition. These analyses can be performed without a loss of signal because of the mass and volume of a large prothallus like that found in homosporous land plants. In *M. vestita,* the diploid sporophyte produces single-celled haploid spores. The smaller male spores (microspores) germinate almost immediately on immersion into water or aqueous culture medium. Over the next 2.5 h, this single cell will undergo five mitotic divisions. Four of these divisions (divisions 1, 3, 4, and 5) are asymmetric, resulting in six smaller sterile jacket cells; 2 larger central, undifferentiated antheridial initials; and one sterile prothallial remnant. Each sterile cell loses the capacity to proliferate further. Subsequently, the antheridial initials undergo four symmetric divisions in the next 2 h to produce a total of 32 undifferentiated spermatids. During the next 5.5 h, the undifferentiated spermatids undergo a massive, synchronous transformation into 32 spermatozoids, with each having approximately 140 cilia (Mizukami and Gall 1966; Myles and Hepler 1977; Sharp 1914).

In *M. vestita,* blepharoplasts form approximately 4 h after imbibition, when cultured at 20°C (Hepler 1976). Just after its initial formation, the blepharoplast acts as a centrosome for the last mitotic division in the gametophyte, and then becomes the formation/assembly site for basal bodies (Hepler 1976); the blepharoplast acts like a basal body factory (Wolniak and others 2000). Basal bodies are made in a single, simultaneous event in all spermatids, at about 6 h after imbibition (Hepler 1976; Mizukami and Gall 1966). The basal bodies assemble sequentially, by first acquiring a-subfibers and then b-subfibers and finally c-subfibers of the microtubule triplets (Hepler 1976). The dry microspores contain large quantities of stored proteins, including α-, β- and γ-tubulin, and large quantities of stored mRNA (Hart and Wolniak 1998; Pennell and others 1988). We have found that a number of proteins, including centrin, are synthesized from stored mRNA during the formation of the blepharoplast and during basal body assembly (Figure 3) (Wolniak and Hart 1998, 1999; Wolniak and others 2000). Our results make it reasonable to suspect that the translation of regulatory proteins at specific times during spermiogenesis control the rate, extent, and pattern of assembly of basal bodies, the cytoskeleton, and ciliary axonemes (Wolniak and Hart 1998, 1999; Wolniak and others 2000). We are interested in understanding how these lower land plant sperm synthesize basal bodies and the complex cytoskeletal array.

M. vestita provides a unique opportunity to study the de novo formation of basal bodies at a discrete cytoplasmic site in populations of cells lacking pre-

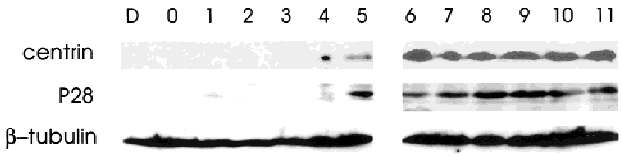

Figure 3. Some of the proteins present in the gametophytes of *M. vestita* are made at specific stages of spermiogenesis. Protein isolates were obtained from gametophytes at successive intervals (numbers at the top of the figure denote the time after imbibition, in hours; D = dry spores). Blots of these isolates were probed with anticentrin antibody (top panel), anti-P28 antibody (middle panel), and an anti-β-tubulin antibody (bottom panel). The immunoblots reveal that translation of centrin and P28 (an axonemal protein, see [LeDizet and Piperno 1995]) occurs midway through gametophyte development, whereas β-tubulin translation only occurs late in spermiogenesis. Centrin protein levels rise dramatically approximately 4 h after imbibition, whereas β-tubulin levels are high in the dry spores and only increase slightly during the last 2 h of spermiogenesis. P28, an axonemal component, accumulates to significant levels approximately 5–6 h after imbibition and remains abundant for the remainder of the developmental process.

existing centrioles. In addition, these spermatids engage in the synchronous formation of a large, complex cytoskeleton and a motile apparatus, composed of approximately 140 cilia. By focusing on blepharoplast formation and composition, we may be able to discern the identity of heretofore unknown components that play key roles in the assembly of basal bodies. The high level of structural and functional conservation that exists in basal bodies and ciliary axonemes of motile cells throughout the eukaryotic realm makes the identification of new components widely applicable in divergent organisms.

Spermiogenesis in *M. vestita* is Dependent on Stored mRNA

Spermiogenesis in *M. vestita* occurs in microspores immediately on immersion into a liquid culture medium. Even microspores that have been quiescent for decades can initiate development with high efficiency. We and others have found that the addition of inhibitors to the liquid culture medium before imbibition results in rapid uptake of these agents into the newly activated male gametophytes. When dry spores are treated at the point of imbibition with the transcriptional inhibitor, α-amanitin, or with the translational inhibitor, cycloheximide, mature sperm are not released into the culture medium (Hart and Wolniak 1998, 1999; Hyams and others 1983). Hyams and others (1983) have shown a decreased DNA content for spores cultured in the presence of both transcriptional and translational inhibitors. We (Hart and Wolniak 1999) demonstrated that protein profiles from α-amanitin–treated spores appeared similar to untreated controls, both for total protein isolates and those using labeled methionine. In addition, microspores that were fed translational inhibitors made no labeled proteins. We (Hart and Wolniak 1998) also showed that 30–50 new proteins were detectable with radiolabeled methionine during the 11-h developmental period. Although these results did not directly establish transcriptional and translational contributions to spermiogenesis, they revealed clues about its control. From these data, we conclude that most components for basal body/MLS development are present in the dry microspore at the time of imbibition, as stored proteins or "building blocks." We found that relatively few mRNA species must be translated during spermiogenesis, and, thus, suspected that these transcripts serve as rate-limiting components for the formation of the motile apparatus. The proteins that are translated from stored mRNA are made at specific times during development and appear to regulate the pattern and extent of basal body/ciliary array assembly. Therefore, it appears that transcription plays a minimal role while translation of stored messages accounts for the bulk of new protein synthesis during the rapid process of spermiogenesis in *M. vestita.* Radiolabeling experiments performed with *Ceratopteris* gametophytes by Schedbauer and coworkers (1973) and in situ hybridization experiments performed on developing embryos of *M. vestita* (Kuligowski and others 1991) reveal that rapid development relies on stored protein and the translation of stored mRNA in both of these cases, much like that in microspores of *M. vestita* during spermiogenesis (Hart and Wolniak 1998).

In a series of cytologic studies, we found that development in the presence of α-amanitin, a transcriptional inhibitor, is essentially normal up through 11 h of development when spermatozoids are about to be released from the antheridia (Klink and Wolniak 1999; 2001. In contrast, the imbibition of spores in cycloheximide resulted in arrested development before any cytoplasmic reorganization or cell division. These gametophytes closely resemble the single-cell gametophytes of spores fixed right after imbibition, even after culturing for 11 h (Klink and Wolniak 1999; 2001). With these observations in hand, we reasoned that if specific translational events control development, they could best be analyzed by exerting specific blocks to translation with antisense RNA or with dsRNA probes. We suspected

that if RNA probes could become internalized by dry spores at the time of imbibition, we could observe the contribution of specific gene products to spermiogenesis in this organism. Preliminary reports of our findings have been published in abstract form (Klink and Wolniak 1999; Tsai and Wolniak 1999).

Centrin Translation is Necessary for the Formation of Basal Bodies in *M. vestita*

Little is known about the molecular composition of the blepharoplast or the ciliary array of these lower land plant spermatozoids. We (Hart and Wolniak 1998) speculated that development is controlled by "keystone proteins" that act as master regulators of developmental events, and without their synthesis, development cannot proceed. Most components that assemble into these cytoskeletal and axonemal arrays appear to be present in the gametophyte at the time of imbibition in large pools of stored protein. We found that α, β, and γ-tubulin are all abundant in the gametophyte from the onset of development (Hart and Wolniak 1998, 1999). P28, an axonemal protein (LeDizet and Piperno 1995), becomes detectable only after 5 h of gametophyte growth (Figure 3, and Klink and Wolniak 2001). In contrast, we demonstrated that centrin protein synthesis begins after 4 h of development, a time that corresponds to the synthesis of the blepharoplast (Figure 3 and Hart and Wolniak 1998). It seemed reasonable to suspect that centrin could play an essential role in blepharoplast formation and a crucial regulatory role in spermiogenesis.

Centrin is a highly conserved (Bhattacharya and others 1993; Hart and Wolniak 1999; Salisbury 1998; Salisbury and others 1978, 1984; Salisbury 1995) , small acidic protein that has been found to be abundant in microtubule organizing centers (MTOCs) (Hoffman and Vaughn 1995; Hohfeld and others 1994; Renzaglia and Maden 2000; Taillon and others 1992; Vaughn and others 1993). Centrin is thought to function in a yet-to-be-characterized fashion in the microtubule nucleation process (Levy and others 1996; Moudjou and others 1991; Salisbury 1995). Some evidence implicates centrin in a scaffolding function that links microtubules with other cytoskeletal proteins, facilitating mechanochemical interactions (Vaughn and others 1993). In *Ceratopteris,* centrin has been localized to the lower strata of the MLS (Vaughn and others 1993), as well as the osmiophilic material that resides in and among the basal bodies just on the distal side of the microtubule ribbon (Hoffman and Vaughn 1995). Centrin has been shown to be involved in centrosome separation (Middendorp and others 2000), in spindle pole initiation (Lechtreck and Grunow 1999), and in basal body duplication or localization (Merten and others 1995) in a variety of cell types. In a series of immunolocalizations, we have found anticentrin antibody (Figure 4) and anti-α-, anti-β- and anti-γ-tubulin antibodies all staining the blepharoplast in developing gametophytes of *M. vestita,* fixed 4 h after imbibition (Klink and Wolniak 2001). By 10 h after imbibition, anti-β-tubulin antibodies are apparent in the anterior portion of spermatids, presumably binding to the spline and its associated basal bodies (Figure 5a). Labeling with anticentrin antibody matches the helical distribution of anti-β-tubulin antibody, but is far less intense (Figure 5b) and is presumably located in the fibrous material that overlies the spline microtubules between the basal bodies. Treatment of gametophytes with

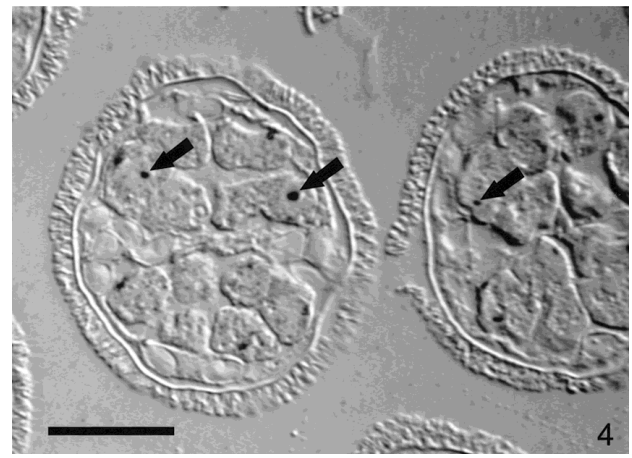

Figure 4. Anticentrin antibody labels the blepharoplast in gametophytes of *M. vestita*. Dry microspores were imbibed with Laetsch's medium and placed on a shaker at 20°C. After 4 h, the spores were fixed with 4% paraformaldehyde, buffered with phosphate at pH 7.4. At the time of fixation, the spore walls were cracked mechanically in a stainless steel mortar and pestle as described by Hepler (1976). The fixed gametophytes were embedded in methacrylate, which was polymerized by UV illumination at 4°C for 8 h. The blocks were sectioned at a thickness of approximately 4 μm and etched with acetone. We labeled the cells with anticentrin antibody, kindly provided by J.L. Salisbury (Mayo Clinic), rinsed, blocked and labeled with an gold-conjugated anti-mouse IgG. We used standard silver-enhancement protocols and observed the cells with DIC microscopy. The blepharoplasts are depicted in the spermatogenous cells as large, dense aggregates of silver (arrows point to three of the blepharoplasts in the gametophytes), and at this stage of development, the blepharoplasts also label with anti-β-tubulin antibody (data not presented). Bar = 20 μm.

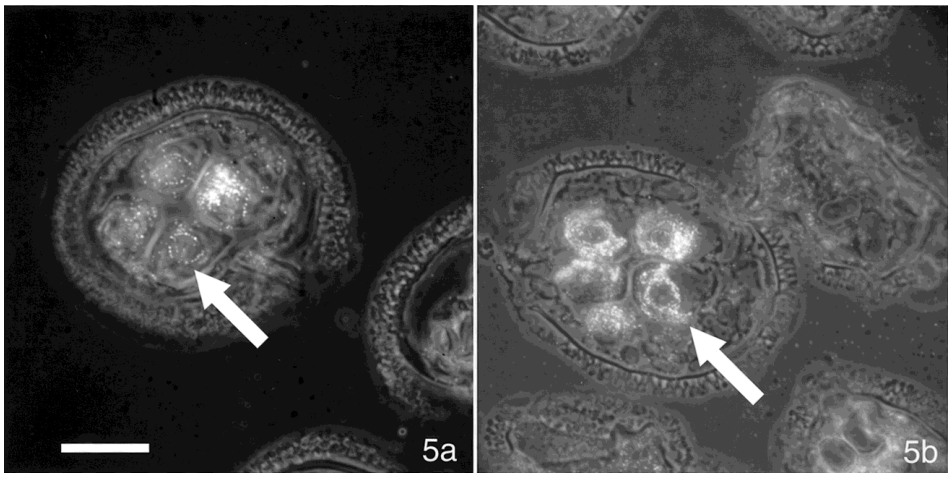

Figure 5. Anticentrin and anti-β-tubulin antibodies overlie the motile apparatus of developing spermatids of *M. vestita* 10 h after imbibition. Cells were processed as described in the legend for Figure 4, except that they were allowed to develop for 10 h after imbibition before fixation. The cells were photographed with reflection interference contrast imaging and transmitted–light phase contrast imaging. Figure **(a)** Anticentrin antibody was used as the primary antibody to label in this gametophyte. The label is distributed in the anterior portion of the spermatids, which is the coiled portion of the cell body where the motile apparatus (including the basal bodies) can be found (Myles and Hepler 1977). The arrow points to one of the spermatids oriented with its longitudinal axis perpendicular to the plane of the section. Figure **(b):** Anti-β-tubulin antibody labeling is intense in the anterior, coiled portion of the spermatids, presumably labeling the spline. We have observed no enhancement of β-tubulin labeling on the basal bodies. The arrow points to one of the spermatids oriented with its longitudinal axis perpendicular to the plane of the section. Bar = 20 μm.

α-amanitin does not perturb the localization pattern of α, β, and γ-tubulin or centrin to the blepharoplast or to the MLS. We (Hart and Wolniak 1999) showed that centrin fails to be translated in the presence of translational inhibitors. We also found that centrin mRNA cannot be translated in vitro from mRNA isolates of dry spores or mRNA isolates taken between 0–30 minutes after imbibition in untreated cultures, a result suggesting that centrin and other stored mRNAs are in an untranslatable form for some time after imbibition.

RNAi and Basal Body Formation During Spermiogenesis In *M. vestita*

If centrin translation from stored mRNA is necessary for blepharoplast formation, then its specific inhibition should block the appearance of basal bodies and the assembly of a motile apparatus. We reasoned that the best way to induce a specific block to centrin translation was through the use of an antisense approach (Klink and Wolniak 1999; 2001). We found that single- and double-stranded pieces of RNA as large as 450 nt are readily taken up into the cytoplasm of the gametophytes at the time of imbibition. Our RNAi effects appear to be gene-specific and concentration-dependent. We observed that centrin translation is blocked by the presence of centrin-derived RNA and that the dsRNA version of this probe is 10–100 times more effective than either antisense or sense. We have found that basal body synthesis and formation of the MLS is dependent on the translation of stored centrin mRNA, whereas the presence of RNA probes derived from *Marsilea* β-tubulin have no effect on spermiogenesis until late in development, when new tubulin synthesis accompanies the formation of ciliary axonemes (Klink and Wolniak 1999; 2001).

Future Prospects of RNAi in Understanding the Plant Cytoskeleton

Most experiments that focus on cytoskeletal dynamics in plants have relied heavily on drugs and inhibitors to elicit a variety of effects on cellular organization and on numerous cellular functions. Early experiments demonstrated that the movement of organelles was reversibly inhibited by culturing cells in cytochalasin B (Franke and others 1972). Disruption of microfilaments with cytochalasin or phalloidin revealed that they are responsible for organization of the cytoplasm, exocytosis, endocytosis, cell morphogenesis, growth, cell division, cell wall deposition, movement of ER and Golgi, and wound response (*reviews,* Pierson and Cresti 1992; Volkmann and Baluska 1999). Experiments using inhibitors of microtubule stability have similarly shown that patterns of order in the cytosol, long-distance organelle transport, and cell division require microtubules. In the course of these and other investigations, it has become clear that the microfilament and

microtubule networks are also linked together, structurally and functionally, both in plants and in animals (Pickett-Heaps and others 1999; Schmit and Lambert 1988; Silverman-Gavrila and Forer 2000; Staiger and Cande 1991; Waterman-Storer and Salmon 1997).

Genetic and biochemical analyses have expanded our knowledge of components associated with the cytoskeleton (Sutherland and Witke 2000), providing insight into the roles of signaling cascades in recruiting cytoskeletal elements for different functions in cells over time. Although genetic and biochemical analyses have contributed significantly to our understanding of cytoskeletal composition, large gaps remain in our understanding of regulation and signaling. The use of heterologous proteins and antibodies to perturb the cytoskeleton have been successful (Miller and others 1995; Staiger and others 1994; Tominaga and others 2000; Valster and others 1997), because the architecture of the cytoskeleton is reliant on conserved gene products (Marc and Gunning 1988). Competition studies of villin, a MF-associated protein, showed loss of villin-dependent actin bundling in *Hydrocharis* root hairs (Tominaga and others 2000), whereas microinjection of plant profilin delayed, blocked, or inhibited cell plate formation (Valster and others 1997). As we begin to study rare or nonconserved regulators of cytoskeletal function, limitations with heterologous probes will become increasingly apparent. The roles of nonconserved proteins and signal transduction pathways involved in cytoskeletal regulation can be studied by specific disruption with RNAi (Clemens and others 2000). It seems clear to us that in systems in which mutational analyses are impractical or specific processes, like the de novo formation of basal bodies in *M. vestita* can be studied in synchronous populations of rapidly developing cells, RNAi strategies stand as remarkably powerful tools.

The key limitation with RNAi protocols is the efficient introduction of the RNA probes into cells. A variety of methods of RNAi incorporation have been used with different organisms (Buchberger 1998; Holt and others 1988; Kelly and others 1997; Ngo and others 1998; Tabara and others 1998; Timmons and Fire 1998), and for plant cells, initial recommendations would include microinjection, electroporation, or even transformation with plasmids containing RNAi constructs. The dry microspores from *M. vestita* rapidly incorporate both soluble (Hart and Wolniak 1998; Hyams and others 1983; Pennell and others 1988) and insoluble materials (Pettitt 1979) from the external medium into the cytosolic compartment at the time of imbibition. We (Klink and Wolniak 1999; Tsai and Wolniak 1999) have taken advantage of this unexpected uptake capability to perform our RNAi experiments with *M. vestita* microspores (Klink and Wolniak 1999; 2001). We suspect that other dry spores exhibit equal efficiency in the uptake of macromolecules into the cytosolic compartment at the time of imbibition. An additional potential drawback of RNAi is that its effects on cells are minimal if there is a substantial pool of stable protein already present when mRNA degradation is initiated. As Alvarado and Newmark (1999) observed, remnant myosin fibers remained in cells for extended periods after RNAi(myosin) treatments.

The mechanisms that allow functional plasticity from reorganization of the same cytoskeletal elements in diverse types of cells are only now becoming clear in fungal and animal systems, in which signaling and regulatory pathways have been linked with changes in cytoskeletal activity. It may be possible to use sequence comparisons and heterologous library screens of likely plant cytoskeletal gene families as a means to identify important functional homologs. Once isolated from the experimental organism as one or more cDNA clones, these homologs can then be used to generate dsRNA in vitro or in vivo for specific translational disruption by RNAi to investigate their role(s) in development. The advantage of RNAi is the introduction of various nucleic acid sequences in a variety of ways to induce the specific inhibition of translational activity. Therefore, it is not surprising that RNAi has gained so much attention since its discovery.

ACKNOWLEDGMENTS

We gratefully acknowledge support for this work from National Science Foundation Grants (MCB-9809950, MCB-9904435). We are grateful to Chia-Wei Tsai and Jeff Molk for comments on the manuscript and to Kevin Vaughn, Peter Hepler, Zane Carothers, Zac Cande, Jeremy Hyams, Sue Wick, and Diana Myles for input and ideas over the years that contributed importantly to the initiation of our new studies on spermiogenesis in *Marsilea*.

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