Preface

We have assembled a set of protocols that we believe represent the state of the art for laboratory and computational analyses of plant microRNAs (miRNAs). These small, non-coding RNA molecules of about 21 nucleotides have made a grand entrance onto the scientific stage with their discovery in the 1990s and their stunning ascent to stardom in the early years of the current decade. Along the way, it has been demonstrated that miRNAs are simply one of several classes of small RNAs produced in plant cells, albeit a particularly important class given the broad phylogenetic conservation and strong regulatory effects of many miRNAs. Plant miRNAs are uniquely interesting for their ancient evolutionary origins and their strong post-transcriptional regulatory effects.

Most chapters of this volume focus on the identification, validation, and characterization of the miRNA class of RNAs. However, a topic that cannot avoid mention is the other classes of small RNAs that are biochemically similar in size and composition (although of somewhat genetically distinct origins). For example, in the cloning and characterization of plant miRNAs, the resulting data set is rich with another significant class of small RNAs, substantially more prevalent in plants than other organisms – the heterochromatic, small, interfering RNAs (heterochromatic siRNAs). As you will see, the methods contained in this volume emphasize miRNA analyses, but include ways to distinguish one class of small RNAs from another. One chapter describes how to characterize candidate members of a unique class of siRNAs (trans-acting siRNAs) that are dependent on the action of miRNAs to initiate their formation.

The biogenesis of miRNAs is dependent on an increasingly well-defined set of proteins that include enzymes for the precise excision of the mature miRNA from a longer precursor, as well as the modification of this mature miRNA, export from the nucleus, and interaction with its target. This volume starts with a chapter that clearly lays out the cellular participants in the production of miRNAs and the utility of studying these genes and gene products. Discussions of the genes that encode these proteins are found throughout the volume, as partial or full loss-of-function mutants in these genes are important components in the toolbox for studying miRNAs.

One set of chapters describes the standard approaches for purification of the working material for the study of miRNAs: the small RNA component of the transcriptome. While not always easy, depending on the composition and source of the plant tissue that is used, the protocols describe here should cover a broad swath of even the most recalcitrant plant materials, resulting in very high quality RNA that can be used for library construction. An alternative approach described in one of the chapters is the isolation of small RNAs associated with Argonaute proteins. With purified small RNA in hand, one is ready to begin the characterization of small RNAs by any one of numerous experimental approaches, the most common of which is deep sequencing by “next generation” technologies, a process that leads to datasets on the scale of millions of small RNAs per reaction. This volume includes a protocol for the generation of sequencing libraries from purified small RNA. After sequencing, the next significant challenge faced by the experimentalist will be the handling of the data – trimming, mapping, organizing, and analyzing the millions of short sequence reads.
Several chapters describe methods for analyzing miRNA-directed regulation of target RNAs. In its most basic form, the identification of the regulatory targets of plant miRNAs is based on the observation of a near-perfect complementarity between miRNAs and their mRNA targets. This makes computational-based target prediction simpler in plants than in animals. The pairing of miRNAs with an mRNA, in plants, typically results in cleavage of the mRNA target. Such approaches to target prediction in plants are addressed in this volume, as are both standard approaches to validating specific target cleavage events and the exciting development of genome-wide methods to characterize cleaved mRNAs in a single library.

This volume includes a series of chapters that discuss approaches to analyzing the functional role of plant miRNAs. This includes computational methods for the prediction of plant miRNA targets and the experimental methods that can supply validation data to support these predictions. Computational methods have also been applied to the study of gene regulatory sequences in promoters, an application that works well to identify promoter elements and potential transcription factor binding motifs in plant miRNA precursors. Regulatory elements contribute to the regulation of expression of miRNAs in response to stresses such as biotic and abiotic stress; under such conditions, miRNAs in turn regulate other transcripts creating variation in both their levels and expression patterns. In situ hybridizations have long been used for the localization of messenger RNAs and proteins, but an exciting recent application of this methodology is the ability to localize miRNAs in plant tissues using a new generation of highly sensitive probes. And when these data have been brought together to infer the function of a miRNA, plants are amenable to an assessment of this predicted function using transient assays. All of these topics are the subjects of chapters in this volume, and we believe that these will provide valuable contributions and useful material for our readers' experimental work.

Interestingly, studies of the biology of plant miRNAs have been somewhat turned on their head with the realization that miRNAs can also be used as tools themselves for the study of the biological function of other genes. A chapter in this volume describes the development of artificial miRNAs, a powerful tool for the investigation of gene function with current applications in both forward and reverse genetics experiments.

While most of the initial studies and basic approaches have been developed in *Arabidopsis*, many exciting advances are sure to come from the application of these and other methods to other plant species. Indeed, our goal in editing this book is to provide the community with a set of protocols that will help advance miRNA research for all plant species. To this end, all or nearly all of the protocols could be used for any plant species of interest. We have included several chapters that will be of particular interest to plant biologists working in non-model species, including a set of approaches for RNA purification from quite diverse species and tissues, as well as an overview of computational methods to handle data from a broad set of species.

MicroRNA activities and stability are dependent on a series of modifications and processing. One of the most well-characterized modifications is 3′ methylation, an activity carried out by the HUA ENHANCER 1 methyltransferase, which may stabilize the miRNA by preventing uridylation and by diminishing exonuclease-mediated degradation. One chapter in this volume describes the methods by which 3′ methylation can be assessed.

Surely there are many additional advances yet to come in this field, contributing new methods in parallel to making additional strides in our understanding of the biology of small RNAs. As an example, a collaborative effort of many of the labs that contributed to this volume led to an overhaul of the criteria used to define plant miRNAs (1). That manuscript
came out too late to be fully reflected in this volume, and other rapid changes in the field will keep this as one of the fastest moving fields in plant biology. Much as deep sequencing represented a tremendous advance, dare we say a revolution in the study of small RNAs, there are unquestionably greater things yet to come in the methods for the analysis of plant miRNAs. This may include further advances in understanding the cell specificity, abundance levels, trafficking, modifications, target interactions, or biological roles.

In summary, we are excited by the prospect of the experiments that this volume may facilitate or even inspire. As students (broadly speaking), practitioners, or theorists in the field of plant molecular biology, we hope that you will find many or all of these chapters to be of use in your work. The chapters may serve to introduce you to a new field of work, or extend your capabilities in a topic in which you are already quite familiar. While a mastery of the techniques in this volume is not a requisite for success in the field of small RNAs, an incredible group of contributors has contributed a set of protocols.

Finally, this book would not have come to fruition without the careful editorial and administrative assistance of Sharon Bancroft, along with additional administrative help by Charlotte McDermitt and Kathy Fleischut. Most of all, we are incredibly grateful to the contributing groups who have taken their time to describe in exquisite detail the methods, tips, and tricks that they use.

We hope that you find this unique collection of protocols helpful to your research.

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Reference

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