

**Final Report**  
**Grant Number: DE-FG02-04ER15541**

**Title: Regulated mRNA Decay in Arabidopsis: A global analysis of differential control by hormones and the circadian clock**

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**Publications:**

1. Lu, C., Tej, S.S., Luo, S., Haudenschild, C.D., Meyers, B.C., and Green, P.J. Elucidation of the small RNA component of the transcriptome. *Science*, 309:1567-1569 (2005)
2. Meyers, B.C., Souret, F.S., Lu, C., Green, P.J. Sweating the small stuff: MicroRNA discovery in plants. *Current Opinion in Biotechnology*, 17:139-146 (2006)
3. van Hoof, A. and Green, P.J. Nonsense-mediated mRNA decay in plants, in *Nonsense-Mediated mRNA Decay*, (L. Maquat, ed.) Landes Bioscience pp. 1-6 (2006)
4. Lu, C., Kulkarni, K., Souret, F., Valliappan, R., Tej, S.S., Poethig, S., Henderson, I.R., Jacobsen, S., Wang, W., Green, P.J., and Meyers, B.C. microRNAs and other small RNAs Enriched in the Arabidopsis RNA-Dependent RNA Polymerase-2 Mutant. *Genome Research*, 16:1276-1288 (2006)
5. Lu, C., Meyers, B.C., and Green, P.J. Construction of small RNA cDNA libraries for deep sequencing. *Methods* 43:110-117 (2007)
6. Rymarquis, L.A., Kastenmayer, J.P., Hüttenhofer, A.G., and Green, P.J. Diamonds in the rough: mRNA-like non-coding RNAs. *Trends in Plant Science* 13:329-334 (2008), EPub 2008 April 28.
7. German, M.A., Pillay, M., Jeong, D-H., Hetawal, A., Luo, S., Janardhanan, P., Kannan, V., Rymarquis, L., Nobuta, K., German, R., De Paoli, E., Lu, C., Schroth, G., Meyers, B.C. and Green, P.J. Global identification of microRNA-target RNA pairs by parallel analysis of RNA ends. *Nature Biotechnology* 26: 941 - 946 (2008), EPub 2008 June 9.
8. German, M.A., Luo, S., Schroth, G., Meyers, B.C., and Green, P.J. Construction of Parallel Analysis of RNA Ends (PARE) libraries for the study of cleaved miRNA targets and the RNA degradome. *Nature Protocols* 4:356-362 (2009), EPub 2009 February 26.
9. Green, P.J. and Meyers, B.C. The RNA World: Identifying miRNA -Target RNA Pairs as Possible Missing Links in Multi-Network Models. In Coruzzi, G.M. and Gutiérrez, R.A. (Eds.) *Plant Systems Biology*. Blackwell Publishing Limited, Oxford, UK (2009).
10. Jeong, D-H., German, M.A., Rymarquis, L.A., Thatcher, S.R., and Green, P.J. Abiotic Stress-Associated miRNAs: Detection and Functional Analysis. In Meyers, B.C. and Green, P.J. (Eds.) *Plant MicroRNAs, Methods in Molecular Biology*, 592:203-230 (2009). Humana Press, New York, NY. EPub 2009 Oct 3.

## Books

11. Meyers, B.C. and Green, P.J. (Eds.) Plant MicroRNAs, Methods in Molecular Biology, vol.592 (2009). Humana Press, New York, NY.

## Research accomplishments (reference numbers refer to the publications above):

The long-term goal of this research was to better understand the influence of mRNA stability on gene regulation, particularly in response to hormones and the circadian clock. The primary aim of this project was to examine this using DNA microarrays, small RNA analysis and other approaches. We accomplished these objectives, although we were only able to detect small changes in mRNA stability in response to these stimuli. However, the work also contributed to a major breakthrough allowing the identification of small RNAs on a genomic scale in eukaryotes. Moreover, the project prompted us to develop a new way to analyze mRNA decay genome-wide. The research described in the ten journal publications and one book listed above was supported, at least in part, by this project. Thus, the research was hugely successful beyond our objectives and led to very important publications, most notably in Science and Nature Biotechnology.

Our early analysis of Arabidopsis small RNAs (miRNAs and siRNAs) was funded in part from this grant and led the first application of deep sequencing to miRNA identification and characterization [1], an approach that is now routine in eukaryotic systems [e.g. 2-11]. In addition, our research contributed to the addition of about 2500 miRNA and other small RNA sequences to Agilent's 44K whole genome array. Although plans to commercialize this array are no longer in place at Agilent, the probe list has been provided to other groups for array construction. Currently our best example of regulated mRNA decay in response to the proposed stimuli is the *CCL* gene that is controlled by the circadian clock. Clock regulation of *CCL* mRNA decay is altered in the *dst1* mutant of Arabidopsis (Lidder et al., 2006, Plant Physiol. 138:2374-2385 (2005)). The semi-dominant *dst1* locus appears large and complex. Recently we identified two point mutations in this locus in collaboration with DuPont. Current efforts focus on using the wild-type and mutant genes corresponding to both mutations for reciprocal complementation studies. This should put us in an excellent position to elucidate the role of *DST1* in circadian control of *CCL* mRNA stability in response to the circadian clock.

In addition, we developed under this grant, an exciting method to identify the targets of miRNA-directed cleavage on a global scale. Plant miRNAs cause decay of their target mRNAs by directing cleavage near the center of the target sequence that base-pairs with the miRNA. The approach we developed called Parallel Analysis of miRNA Ends, or PARE, involves the cloning and sequencing of the cleaved end of miRNA targets *en masse*. PARE involves creation of libraries that contain 3' decay products of mRNAs. The first step in library construction is the addition of a 5' RNA adapter using RNA ligase (Fig. 1). Products of miRNA-directed cleavage are a result of Slicer activity, and the 3' fragment has a 5' monophosphate, rather than a 5' cap. Hence, they are ligation competent. Other mRNAs will not be incorporated into the library due to their 5' cap, or because they lack a 5' monophosphate. After the ligation of the 5' adapter, the transcripts are annealed to a DNA oligonucleotide consisting of an oligo(dT) and 3' adapter sequence, and the mRNA is reverse transcribed. This cDNA is amplified and then digested with the Type IIS restriction enzyme MmeI, which cleaves 20 nucleotides (nt) 3' of the recognition site; the MmeI site is located in the 5' adapter, immediately adjacent to the mRNA. Next, a 3' adapter with degenerate nucleotides in the overhang region is ligated to the MmeI digestion

products, and the resulting material is PCR amplified, gel purified and submitted for SBS sequencing.

When libraries were made from the inflorescence of *Arabidopsis*, the detection of known and predicted targets was robust. More than 27 million total reads were sequenced from libraries made from wild type (WT) plants and from an *xrn4* knockout mutant [8]. Because XRN4 degrades the products we were trying to clone (Souret et. al., 2004, Mol. Cell Biol. 15:173-183), the use of the *xrn4* mutant increased the sensitivity of detection for known targets, but most of the known validated targets were still found in the WT library (83%), compared to 93% in *xrn4*. Given that both these libraries represented only inflorescence tissue, this indicates that the inflorescence is a very rich source of target fragments, as it is for small RNAs [125]. For about 80% of known targets, the PARE sequence from the cleavage site was the sequence with the highest abundance for the target. This is easily visualized in Target-plots or “T-plots,” in which the abundance of each sequence in the PARE library that matches a given mRNA

(annotated cDNA) is plotted versus its location in the transcript (Fig. 2). The PARE sequence at the cleavage site is usually quite evident, and can be used to filter real miRNA targets from predicted targets. When 125 predicted but yet-to-be validated targets were examined, 32 and 43 cleavage site sequences were found in WT and *xrn4*, respectively. These were further filtered for characteristic T-plots and 12/13 selected in this way could be validated individually by modified 5' RACE (the conventional method for cleavage site validation, on which our high-throughput method is based). PARE can also be used to generate miRNA-target RNA pairs by bioinformatics approaches developed in the lab of our computational collaborator, Blake Meyers. In our *Arabidopsis* study, this resulted in the identification of new miRNA targets, as well as new miRNAs [1]. Similar to small RNA sequencing, PARE-like methods are now being applied to a variety of plant and other systems to identify and validate miRNA and siRNA targets on a genome-wide scale. However, PARE also reports on other RNA decay products that comprise the RNA degradome and the method can be modified to capture decay products with different types of end chemistries such as 5' hydroxyls. It is the use of PARE to examine the patterns of decay for each transcript in the

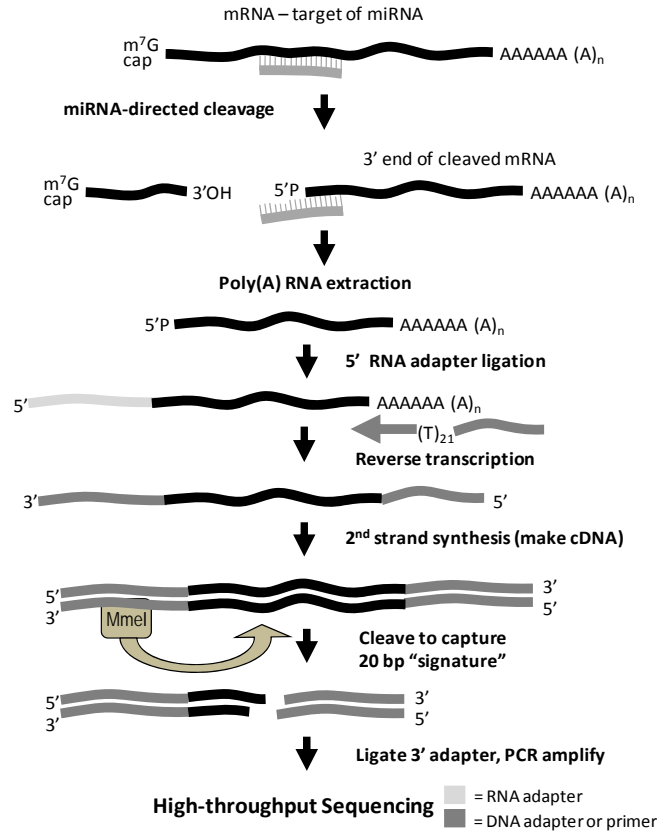


Fig.2. Schematic depiction of PARE library construction for miRNA target detection

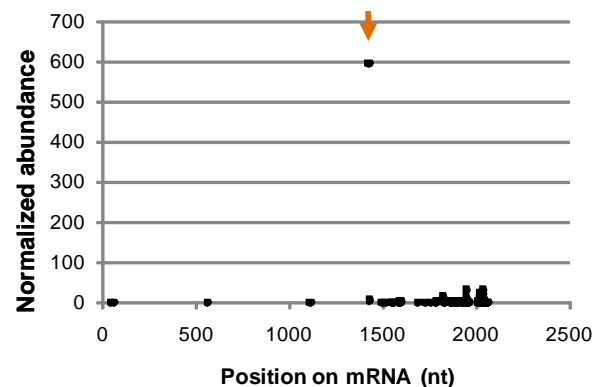


Fig. 3. Example of a T-plot used in PARE analysis. The plot compares the total abundance of PARE sequences versus their mapping position on an mRNA sequence (At1g77850). The arrow indicates the sequence (peak) corresponding to the position of miRNA-directed cleavage.

transcriptome that is expected to have the greatest impact. Not only will this reveal new mechanisms of post-transcriptional control, but PARE should also aid in the interpretation of some types of transcriptome analyses that cannot differentiate between degraded and intact mRNA.