BioTechnologia
vol. 95(1) • pp. 73-82 • 2014

Session 4
Biogenesis and function of small RNAs

Lecture 4.1

Insights from global analysis of miRNAs and target RNAs

P.J. Green[1-4], Dong-Hoon Jeong[1,2], S.R. Thatcher[1,5], S. Park[1,2], S.A. Schmidt[1,2]
J. Zhai[1,2,6], B.C. Meyers[1,2], K.R. Franke [1,4], M. Accerbi[1,2], E. De Paoli[1,7]

[1] Delaware Biotechnology Institute, University of Delaware, Newark, Delaware, USA
[2] Department of Plant and Soil Sciences, University of Delaware, Newark, Delaware, USA
[3] Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware, USA
[4] Department of Biology, University of Delaware, Newark, Delaware, USA
[5] DuPont Agricultural Biotechnology, Experimental Station, Wilmington, Delaware, USA
[6] Department of Molecular, Cellular and Developmental Biology, University of California, Los Angeles, California, USA
[7] Department of Agriculture and Environmental Sciences, University of Udine, Udine, Italy

MicroRNAs (miRNAs) are post-transcriptional regulators of gene expression that control development, stress responses and other processes. They most often function by guiding cleavage of mRNA targets and some inhibit translation. The deep sequencing of miRNAs and their cleaved targets continues to lead to the discovery of new miRNAs and new regulation such as regulation in response to cold, heat, drought, submergence, and metal stress and many examples of tissue or organ preferential expression (Jeong et al., Plant Cell 23: 4185; Plant Phys. 162: 1225; Genome Biol. 14: R145). Among new cases of miRNA regulation are distinct miRNA family members exhibiting differential accumulation in different organs that impact target selection and cleavage. Genome-wide target cleavage data from Parallel Analysis of RNA Ends (PARE) has proved instrumental in these studies and can be even more powerful when interpreted with the emerging diversity of miRNA variants, ARGONAUTE immunoprecipitation data, and small RNA expression data. Examples from model plants and beyond that have led to new insights about particular miRNAs will be highlighted. Some of these derive from an analysis of miRNAs in five floral organs and six panicles stages, which was augmented with PARE data from anther and pistil. Most of the previously reported rice miRNAs were identified in this study as well as tens of new miRNAs using a computational pipeline. About 20% of the miRNAs were preferentially expressed in a specific floral organ, with half being anther preferential. More than 170 miRNAs were differentially expressed during panicle development including some miRNA variants that showed differential expression patterns in different stages. In addition to enhancing our understanding of miRNAs and their targets, these studies are providing rich data resources for future work. Funded by the NSF, DOE and USDA.

Lecture 4.2

Processing of plant microRNA precursors

J. PALATNIK

Institute of Molecular and Cell Biology of Rosario (IBR), School of Biochemistry and Pharmacy, University of Rosario, Rosario, Argentina

MicroRNAs (miRNAs) are transcribed as larger transcripts that contain fold-back structures that are processed by RNAse III complexes. In both plants and animals, the precursor contains spatial clues that determine the position of the miRNA along its sequence. However, at a difference with their animal counterparts, plant precursors are very heterogeneous in size and shape. They are processed completely in the nucleus by a complex formed by DICER-LIKE1 (DCL1) and accessory proteins such as HYL1 and SERRATE. In many precursors a 15 nucleotide lower stem below the miRNA is essential for its processing. This structural determinant present in many, but not all plant miRNAs, is recognized by the processing machinery to produce a first cleavage at the base of the precursor, while a second cut below the loop releases the miRNA. In contrast, the biogenesis of miR319 and miR319 proceeds through a loop-to-base direction. DCL1 produces first a cleavage at the loop of the precursor and continues towards the base of the precursor, while the mature miRNAs are finally release between the third and fourth cleavage sites. To bring insights into the biogenesis of miRNAs from a genome-wide perspective in *Arabidopsis thaliana*, we designed a strategy to detect precursor intermediates using high-throughput sequencing. Using this strategy we were able to map the cleavage sites for most *Arabidopsis* miRNA precursors. We found a good correlation between the cuts in the precursors and the miRNA profile obtained by deep-sequencing small RNAs. The results indicate that miRNAs are released by at least four different mechanisms starting from either end of the precursors. A genome-wide view of miRNA processing in *Arabidopsis* will be presented.

Short talk 4.1

Functional analysis of SERRATE interacting proteins

S. LAUBINGER

Center for Plant Molecular Biology (ZMBP), University of Tubingen, Tubingen, Germany

Plant microRNAs (miRNAs) regulate diverse aspects of plant development including hormone responses, floral development and phyllotaxy. Mature miRNAs associate with ARGONAUTE (AGO) proteins to bind and regulate target mRNAs. Mature miRNAs are released from longer primary-miRNAs (pri-miRNA) by the RNAseIII-like enzyme DICER-LIKE 1 (DCL1). Additional RNA-binding proteins including SERRATE (SE), HYPONASTIC LEAVES1 (HYL1), TOUGH (TGH) and the CAP BINDING COMPLEX (CBC) facilitate efficient and precise processing of pri-miRNA transcripts. SE and the CBC are of particular interest for us, because they fulfill an additional function in pre-messenger RNA (pre-mRNA) splicing. In order to identify partners of SE/CBC that assist the complex in miRNA processing and/or splicing, we screened yeast cDNA libraries for potential interactors. Our results suggest that SE/CBC interact with a wide range of scaffolding proteins and regulatory proteins, including RNA-binding proteins and transcript_ion factors. Interestingly, we also identified components of the U1 snRNP, suggesting that SE/CBC regulate splicing by a direct physical interaction with the spliceosome. Further genetic and molecular analyses will be presented.

Short talk 4.2

The crosstalk between microRNA biogenesis and splicing machineries in plants

A. STEPIEN[1], D. KIERZKOWSKI[2], K.D. RACZYNSKA[1], M. TAUBE[1,3], Z. SZWEYKOWSKA-KULINSKA[1], A. JARMOLOWSKI[1]

[1] Department of Gene Expression, Institute of Molecular Biology and Biotechnology, Faculty of Biology, Adam Mickiewicz University in Poznan, Poland

[2] Department of Comparative Development and Genetics, Max Planck Institute for Plant Breeding Research, Koln, Germany

[3] Department of Macromolecular Physics, Adam Mickiewicz University in Poznan, Poland

MicroRNAs (miRNAs) are small non-coding RNAs of about 21 nt in length, which take part in a wide variety of physiological and cellular processes. They act by regulating gene expression by cleaving or inhibiting translation of target mRNAs. In plants, miRNAs are encoded mostly by independent transcription units or, less frequently, within introns of protein-coding genes. In addition, the miRNA genes that are transcribed into long non-coding transcripts, may contain more than one pre-miRNA in both, introns and/or exons. Plant miRNA biogenesis, which is still not fully understood process, occurs inside the cell nucleus, and is performed by a complex comprising at least five different proteins: DCL1 (an RNase III ribonuclease), HYL1 (a dsRNA-binding domain containing protein), SERRATE (SE) (a zinc-finger-domain protein) and CBC (a nuclear cap-binding protein complex) that is composed of two subunits, CBP20 and CBP80. DCL1 provides nucleolytic activity of the miRNA processing complex, and other factors involved in miRNA maturation are required for the efficient and correct excision of miRNAs from plant pri-miRNAs. Interestingly, SE and CBC are also involved in pre-mRNA splicing. It is unclear, however, how this dual function of SE and CBC is fulfilled. Moreover, we have already shown that splicing of pri-miRNA 163 influences biogenesis of mature miRNA 163. Thus, correlation between splicing and miRNA maturation machineries seems to be obvious. We decided to characterize interactions between SERRATE, CBC complex and spliceosome in plants. In the case of the latter machinery we chose U1snRNP, that recognizes the 5′ splice site, as a potential candidate for crosstalk studied. First, we performed experiments on the direct interactions between CBP20, CBP80 and the SERRATE protein using Bimolecular Fluorescence Complementation (BiFC) and *in vitro* pull down assays. The CBP20/SE and CBP80/SE complexes seem to be localized exclusively in the nucleus of *Arabidopsis thaliana* cells where they are mainly accumulated in specific subnuclear bodies. Moreover, *in vitro* analyses have shown stronger interactions of overexpressed in *E. coli* MBP-SERRATE with the whole CBC complex, obtained by *in vitro* translation, in comparison to the single interaction between SE and CBP20 or SE and CBP80. Furthermore, we tested interactions of SERRATE with all ten *A. thaliana* specific U1snRNP proteins by yeast two-hybrid system followed by pull down assay. We found five binding partners of SE among them, which were PRP39a, PRP39b, PRP40a, PRP40b and LUC7rl. We also performed experiments on the subcellular localization of these five proteins and SERRATE in *A.thaliana* cells. All U1snRNP binding partners co-localize with SE in the cell nucleus. Taking together, we proved the connection between proteins from miRNA biogenesis machinery and spliceosome which functionality is under further studies. This work was funded by Polish National Science Center (UMO-2012/05/N/NZ2/00880).

Lecture 4.3

Post-transcriptional regulation of auxin signaling homeostasis

D. WINDELS, F. VAZQUEZ

Botanisches Institut, Universitat Basel, Basel, Switzerland

RNA silencing encompasses a wide set of recently discovered RNA-dependent regulatory mechanisms that act as a major bandmaster to coordinate the expression, protection, stability, and inheritance of eukaryotic genomes. Our work has recently provided pioneer insights into the regulation of auxin signaling homeostasis and its impact on plant development. We showed that, during leaf development, the expression of *TIR1/AFB2 AUXIN RECEPTOR* (*TAAR*) genes and are regulated by the miRNA miR393 and by a specialized secondary siRNA network, which we termed siTAARs, to fine-tune auxin signaling homeostasis. We have also now identified an additional layer in the regulation of *TAAR* transcripts that involves RNA decay, and that appears as a major regulator of auxin signaling homeostasis and plant development. I will present these most recent data and highlight the future directions of our work that aims to understand the biological role of the simultaneous regulation of *TAARs* by RNA silencing and RNA decay.

Short talk 4.3

Regulation of pri-miRNA processing by an hnRNP-like protein

T. KOSTER[1], K. MEYER[1], C. WEINHOLDT[2], L.M. SMITH[3], M. LUMMER[1], C. SPETH[4,5], I. GROSSE[2], D. WEIGEL[3], D. STAIGER[1]

[1] Molecular Cell Physiology, Bielefeld University, Germany

[2] Institute of Computer Science, Martin-Luther-University Halle-Wittenberg, Halle/Saale, Germany

[3] Max Planck Institute for Developmental Biology, Tuebingen, Germany

[4] Center for Plant Molecular Biology, University of Tuebingen, Tuebingen, Germany

[5] Chemical Genomics Centre of the Max Planck Society, Dortmund, Germany

The hnRNP-like RNA-binding protein *At*GRP7 (*Arabidopsis thaliana* glycine-rich RNA-binding protein 7) regulates pre-mRNA splicing. Small RNA-seq was used to show that *At*GRP7 also affects the miRNome. Overexpression of *At*GRP7 caused a significant reduction of at least 2-fold for around 30 miRNAs. Among those, reduced levels of miRNA398 were accompanied by an increased level of its targets COPPER ZINC SUPEROXIDE DISMUTASE 1 (CSD1), CSD2, and the COPPER CHAPERONE CCS. Furthermore, reduced accumulation of miR390, an initiator of trans-acting small interfering RNA (ta-siRNA) formation, led to lower *TAS3* ta-siRNA levels and increased mRNA expression of the target *AUXIN RESPONSE FACTOR4*. An accumulation of several primary transcripts at the expense of the mature miRNAs suggested that *At*GRP7 affects pri-miRNA processing. Indeed, RNA immunoprecipitation revealed that *At*GRP7 interacts with several pri-miRNAs *in vivo*. Mutation of an arginine residue in the RNA recognition motif abrogated *in vivo* binding and the effect on miRNA and pri-miR levels, indicating that the RNA-binding activity of *At*GRP7 is required for these functions. Moreover, we show that *At*GRP7 affects alternative splicing of pri-miRNAs. Thus, *At*GRP7 is an hnRNP-like protein with a role in processing of specific pri-miRNAs in addition to its role in pre-mRNA splicing.

Short talk 4.4

Salt stress reveals a new role of AGO1 in the miRNA biogenesis pathway at both, transcriptional and post-transcriptional levels

J. DOLATA[1], M. BAJCZYK[1], D. BIELEWICZ[1], K. NIEDOJADLO[2], W. WALCZAK[1], Z. SZWEYKOWSKA-KULINSKA[1], A. JARMOLOWSKI[1]

[1] Department of Gene Expression, Faculty of Biology, Adam Mickiewicz University in Poznan, Poland

[2] Department of Cell Biology, Faculty of Biology and Environment Protection, Nicolaus Copernicus University, Torun, Poland

MicroRNAs (miRNAs) are short (21-24 nt) RNA molecules that control gene expression at the post-transcriptional level, by cleavage of mRNA targets or by inhibiting their translation. These small molecules are important in plant development and responses to different biotic and abiotic stresses. Recently we developed a high-throughput platform, mirEX, to simultaneously examine of all known *A. thaliana* miRNA precursors (pri-miRNAs) in different developmental stages, using Real Time PCR. We applied the mirEX panel to test the influence of high salt stress conditions on *MIR* genes expression in *A. thaliana* T87 cells. Our data clearly indicate that salt stress has a great influence on the expression pattern of *A. thaliana MIR* genes: the profile of 40% of all pri-miRNAs was significantly changed under salt stress conditions. Many of them (63pri-miRNAs) were down-regulated in comparison to control conditions. There was also a large group of up-regulated miRNA precursors (53pri-miRNAs). Data obtained from the qPCR and northern blot analyses shows that the majority of pri-miRNA and miRNA follows in the same direction (both are up or down regulated). under stress conditions. These results indicate that the mentioned above *MIR* genes are transcriptionally regulated. Selected examples were confirmed using ChIP (Chromatin Immunoprecipitation) technique and GUS system. We also observed following profiles of *MIR* gene expression regulation upon salt stress: the level of pri-miRNA was down-regulated but the level of the mature miRNA was increased (e.g. *MIR*161, *MIR*173). Such *MIR* gene expression profiles indicate post-transcriptional regulation of miRNAs biogenesis. In further experiments we have shown that miR161 and miR173 are more stable in salt stress condition and that AGO1 is responsible for this phenomenon. Additionally, we found that AGO1 is present not only in cytoplasm but also in nuclei. Using ChIP approach we have shown that AGO1 co-localizes on *MIR*161 and *MIR*173 genes and its level is increased under stress conditions. Parallely, we observed that RNA Pol II drops off during the transcription of these *MIR* genes. Our results indicate that AGO1 function is not only limited to the last step of miRNA pathway but has also important role in co-transcriptional regulation of *MIR* gene expression. This project was supported by Foundation for Polish Science (MPD/2010/7).

Short talk 4.5

Parallel evolution of two microRNAs controlling AGL17-like MADS-box genes in distantly related flowering plants

L. Gramzow, D. Lobbes, G. Theißen

Friedrich Schiller University, Jena, Germany

Even though hundreds of microRNA (miRNA) families have been found in plants so far their origin remained mostly unclear. A number of hypotheses on the origin of new plant miRNA families have been brought forward over the years. These hypotheses include the origin of miRNA families by inverted duplication of their target genes, from inverted-repeat containing transposable elements, transcription of genomic regions containing inverted repeats, and by duplication of existing miRNA families. Nevertheless, elucidating the origin for certain miRNA families proved difficult, especially for older families. Hence, our knowledge on the origin and evolution of miRNA families is still limited. Here, we exemplarily study the origin of the two miRNAs miR444 and miR824. Both miRNAs post-transcriptionally regulate AGL17-like MADS-box genes encoding transcription factors which have important functions in the nitrate-signaling pathway amongst others. However, both miRNAs do not share any significant sequence similarity and seem to be restricted to non-overlapping plant phyla. The miR444 has so far been discovered in several species belonging to the grass family Poaceae, and in *Musa accuminata*, a species of wild banana (from the family of Musaceae, order Zingiberales) which are all monocot plants. In contrast, the miR824 has only been detected in the eudicot species from the crucifer family Brassicaceae, such as the major model plant *Arabidopsis thaliana*, so far. We use data obtained from large-scale transcriptome projects, sequencing efforts and Northern blot hybridization to elucidate when and how the two miRNAs miR444 and miR824 originated and started to regulate the same family of target genes. Our analyes indicate, that both miRNAs may have originated by an inverted duplication of their target genes. Our data thus reveal the intriguing case of the independent origin and parallel evolution of two miRNAs targeting a highly conserved class of developmental control genes in distantly related clades of flowering plants.

Short talk 4.6

AtERI has suppressor function on PTGS via changing the population of small RNAs

I. JUPIN[1], G. HONIG[2], G. REUTER[2], M. KUHLMANN[3]

[1] Laboratoire de Virologie Moleculaire, CNRS-Univ Paris Diderot, Institut Jacques Monod, Cell Biology Department, Paris, France

[2] Institute of Genetics/Developmental Genetics, Martin-Luther University Halle-Wittenberg, Halle, Germany

[3] Research group Abiotic Stress Genomic, IPK Gatersleben, Gatersleben, Stadt Seeland, Germany
Interdisziplinares Zentrum fur Nutzpflanzenforschung (IZN), Martin-Luther-Universitat Halle-Wittenberg, Halle (Saale), Germany

In all known eukaryotic organisms short interfering RNAs (siRNAs) are the effector molecules of gene silencing. The presence of siRNAs, homologous to the affected gene is a typical hallmark for post-transcriptional gene silencing (PTGS). In plants the mechanism of silencing is very well understood and the most important factors are known. Although in other organisms siRNA degrading enzymes are already described, up to now no investigation was aiming to the degradation pathway of siRNAs. The function of the *Arabidopsis thaliana* enhancer of RNAi (ERI) homolog from *C. elegans* in PTGS was analyzed *in planta*. Therefore plants with *ERI* loss-of-function mutation and plants over expressing AtERI (*Pro35S::ERI*) were studied. Plants with modulated level of AtERI show an increased accumulation of the virus load after infection with turnip yellow mosaic virus (TYMV). Utilizing a *2xGUS* reporter silencing system for analysis of PTGS frequency, an increase of silencing frequency was found in the *ERI* loss-of-function mutant. Molecular analysis of siRNAs by small RNA sequencing and Northern hybridization revealed an ERI related change of a specific class of siRNAs in the 5′region of the silenced GUS reporter gene. Ectopic overexpression of AtERI result in an increase in biomass after backcrosses to wild type plants resembling the heterosis effect.

Short talk 4.7

Small RNAs in chloroplasts and mitochondria represent footprints of RNA-binding proteins

H. RUWE[1], YUJIAO QU[1], K. HOWELL[2], C. SCHMITZ-LINNEWEBER[1], I. SMALL[2]

[1] Molecular Genetics, Institute of Biology, Faculty of Life Sciences, Humboldt University of Berlin, Berlin, Germany

[2] Australian Research Council Centre of Excellence in Plant Energy Biology, The University of Western Australia, Crawley, Australia

Regulatory small RNAs like miRNAs and siRNAs are well studied in plants. Using high-throughput sequencing small RNAs are usually sequenced from size fractionated total RNA. About 20% of sequence reads have an organellar origin and did not get a lot of attention so far. We investigated small RNAs from a wide variety of plant species that map to the chloroplast and mitochondrial genome. We found about 100 of such small RNAs that show a strong bias towards localization in intergenic regions. We mapped transcript 5′- and 3′-ends and found that small RNAs and transcript ends coincide in most of the investigated cases. Whereas in chloroplasts, most 5′- and 3′-ends of mRNAs overlap with small RNAs, in mitochondria small RNAs are found to map mostly to 3′-ends of messages. Several mRNA ends have been reported to be dependent on RNA-binding proteins and we find small RNAs with sequences that have been described as binding sites for RNA-binding proteins. Investigating "knock-outs" for RNA binding proteins we show that processed mRNAs and small RNAs are missing in these mutants. This finding indicates that small RNAs might represent naturally occurring footprints of RNA-binding proteins. Many of the known RNA-binding proteins that affect RNA stability in chloroplasts belong to the class of pentatricopeptide repeat proteins (PPR-proteins). This class of proteins has strong sequence specificity and members of this class bind with high affinity (Kd in the picomolar range). Given the high specificity and affinity, it is speculated that these proteins act as protein caps preventing RNA degradation by exonucleases (Pfalz J. et al., 2009). We show that sequencing of small RNAs in mutants of PPR proteins allows the identification of their target sites. We show that some of these targets are in transcripts antisense to messenger RNAs, indicating that PPR proteins act in the metabolism of chloroplast sense and antisense RNAs.