when addressing dynamic RNA–protein interactions in developing tissues and organs. These limitations of both RIP-chip/seq and UV crosslinking have been addressed in cultured cells by integrating RIP-chip/seq data with PAR-CLIP (Photoactivatable Ribonucleoside Crosslinking and Immunoprecipitation) UV crosslinking data obtained under the same conditions in the same cell system. Ideally, however, entirely new methods will be developed that avoid the limitations of the classical RIP and CLIP approaches, while taking advantage of their respective advantages.

In sum, it is still early in this rapidly expanding field of global protein–RNA interactions, but the implications are exciting given the enormous numbers of small regulatory RNAs and RBPs that are being discovered that affect posttranscriptional regulation and epigenetic changes. It is a bold proposition to suggest, but investigations of global RNA dynamics may provide researchers access to entirely novel solutions for understanding and manipulating human diseases.

ACKNOWLEDGEMENTS
I thank Matt Friedersdorf and Jeff Blackinton for critically reading the manuscript, and I apologize to many colleagues for not being able to cite their valuable contributions to this field.

FUNDING
This work was supported by grants from the National Cancer Institute (R01CA157268 to J.D.K.) and the National Science Foundation (0842621 to J.D.K.).

Jack D. Keene
Department of Molecular Genetics & Microbiology, Duke University Medical Center, USA
E-mail: keene001@mc.duke.edu

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doi: 10.1093/nsr/wnu004
Advance access publication 14 May 2014

PLANT & ANIMAL SCIENCE

Special Topic: Frontiers in RNA Research

Non-coding RNAs as potent tools for crop improvement

Renyi Liu1,* and Jian-Kang Zhu1,2

Since the seminal discovery of small interfering RNAs (siRNAs) [1] more than 10 years ago, significant progress has been made in understanding plant non-coding RNAs (ncRNAs). The first discovery was the different types of small ncRNAs, including microRNAs (miRNAs) and various types of siRNAs [2]. These small RNAs differ in their origins and biogenesis, but their final products function in a similar way, i.e. to silence genes of complimentary sequences. The second breakthrough was the identification and functional characterization of major components of the biogenesis pathway of small RNAs. An interesting discovery in this area is the coupling of miRNA biogenesis and mRNA maturation through proteins such as SE, ABH1/CPB80, CPB20, STA1, and SIC [3–5]. Lastly, functional roles of small RNAs in RNA-directed DNA methylation, viral defense, transposon suppression, abiotic and biotic stress responses, DNA double-strand break repair, and plant development have been demonstrated [6]. These advancements in basic research have greatly increased our knowledge of plant ncRNAs and facilitated the effective design of ncRNA-based strategies for crop improvement.

ncRNAs function by repressing the expression of endogenous or exogenous genes at the transcriptional, post-transcriptional, or translational levels in a sequence-specific manner. Therefore, ncRNAs can be used to specifically control the expression of target genes. Before the biogenesis of siRNAs and their mechanisms of expression regulation had been fully understood, RNA silencing was already being exploited in the early 1990s to produce desired crops by introducing antisense or sense transcripts of target genes to transgenic plants. The first commercial crop that was genetically modified based on RNA silencing was the Flavr Savr tomato [7]. The antisense transcript of polygalacturonase (PG) was introduced in tomato to suppress the...
expression of PG, which is responsible for the degradation of the cell wall during tomato ripening. The suppression of PG delayed the natural softening of tomatoes and allowed tomatoes to ripen on the vine longer resulting in a more flavorful fruit. The engineered antisense transcript, once transcribed, could hybridize with the sense PG RNA to form double-stranded RNAs (dsRNAs). It could also serve as a template to produce complementary RNA through RNA-dependent RNA polymerase (RDR). The dsRNAs were then cleaved by Dicer-like proteins (DCL) into siRNAs that led to PG mRNA degradation. It was later observed that engineering an inverted repeat (IR) construct, which contains both sense and antisense transcripts of the target gene, would result in higher efficiency of gene silencing than engineering the sense or antisense transcript alone. This is presumably because the IR transcript can form dsRNA directly, eliminating the requirement for RDR. An IR sequence, often separated by a spacer sequence that is unrelated to the target gene, has become the most commonly used transgene design for RNA silencing and has been applied to a variety of crops for crop improvement (Fig. 1).

RNA silencing-based genetic engineering has contributed significantly to two of the major goals of crop improvement: resistance to pests and pathogens and improved nutritional value. RNA interference (RNAi) evolved as a defense mechanism against invading nucleic acid molecules such as those from viruses. One of the earliest applications of the plant transformation technology was to ectopically express genes encoding viral coat proteins in transgenic plants. Such transgenic plants were found to be resistant to viruses containing the coat proteins [8]. For a number of years it was thought that the viral resistance was due to recognition of the coat proteins by the transgenic plant cells and consequent plant immune responses against the coat proteins [9]. However, it became subsequently clear that the viral resistance required the expression of the coat protein gene transcript but not the protein itself [9]. Now we know that the viral resistance of the transgenic plants was caused by sense transgene triggered RNAi against the viral RNAs. It is now common practice to use RNA silencing-based biotechnology to engineer crops that are resistant to viral infection. Viral sequences that match coat proteins, replication-associated proteins, ATPases, or promoter regions in the viral genomes have been expressed in transgenic crops allowing derived transgene siRNAs to target viral RNAs for degradation upon infection [10–12]. This strategy has led to the development of a series of commercially successful viral resistant biotech crops such as papaya, plum, cassava, bean, sugar beet, tobacco, rice, tomato, and corn [13]. To account for the high diversity and rapid evolution of viral sequences, sequences from several viral strains can be engineered into one IR construct so that the transgenic plant can obtain resistance to several viruses through a single transformation.

Although the expression of Bacillus thuringiensis (Bt) insecticidal protein remains the dominant biotechnology for controlling insect pests, RNA silencing has the potential to become a worthy alternative, particularly when insect resistance against the Bt protein has developed or the expression of Bt protein starts to cause consumer concern. Recent studies showed that RNAi-based technology could be a potent tool for crop protection against insect pests [14]. Although the injection or feeding of dsRNAs has been an extremely effective gene-knockdown
tool for studying gene function in insects and nematodes, such methods are not suitable for field applications. Rather, RNA silencing can be achieved by insect ingestion of dsRNAs expressed from target sequence-containing constructs in transgenic host plants, bacteria, or non-disease-causing plant viruses (Fig. 1). This strategy has been shown to effectively induce RNAi and protect plants from the western corn rootworm Diabrotica virgifera, the cotton bollworm Helicoverpa armigera, and the tobacco hornworm Manduca sexta, among others [14].

Similarly, transgenic plants expressing dsRNAs that are complementary to key nematode target genes were resistant to parasitic nematodes (Fig. 1). For example, transgenic tobacco, soybean, tomato, and Arabidopsis producing siRNAs targeting house-keeping, parasitism effector, or reproduction genes of nematodes exhibited significant reductions in parasite infection or reproduction [15].

RNA silencing-based technology has also enjoyed success in improving the nutritional value of crops. By down-regulating key genes in plant metabolic pathways using RNAi constructs, transgenic crops may accumulate more favorable metabolites or produce less unwanted ingredients (Fig. 1). Examples include corn with increased essential amino acids, soybean, canola and cotton with improved fatty acid composition, wheat with increased fiber content, alfalfa and poplar with reduced lignin, and soybean, rice, peanut, and apple with reduced allergens [13].

A limiting factor for the success of RNAi-based biotechnology for crop improvement is the identification of appropriate target genes. Thus, far target genes have been limited to genes with a known function. For pests and pathogens with limited genomic resources, choosing appropriate target genes can be quite difficult. As demonstrated in a recent study [16], this can be addressed by the systematic profiling of gene expression in various key developmental stages of pests or pathogens using next-generation RNA-Seq technology. By profiling gene transcripts of embryonic, larval, pupal, and adult stages of the Asian corn borer Ostrinia furnacalis, the authors were able to identify 14,690 stage-specific genes that could serve as potential targets. Among ten highly expressed larval stage-specific genes tested for dsRNA induced RNAi response, nine out of ten corresponding dsRNAs caused significant developmental stunting and high mortality. This is an effective way to identify potential target genes and eliminates bias towards genes with a known function.

A major concern regarding the application of RNAi-based technology for crop improvement is off-target effects. If sequence homology exists, transgene-derived dsRNAs may interfere with the expression of some host plant genes or genes in beneficial insects. Transgene-derived dsRNAs or siRNAs may even interfere with the expression of some human genes. This concern was heightened after a report claiming that plant miRNAs were found in human tissues and the plant miRNAs affected the expression of some human genes [17]. These findings have far-reaching implications on transgenic plant-based biotechnologies and on ncRNAs as plant ingredients of potential nutritional and medicinal value. However, more recent studies from independent groups failed to reproduce or support the findings [18–20]. Regardless, the careful design of a siRNA-producing construct, including an exhaustive search for sequence homology using software tools such as NEXT-RNAi [21], is necessary to avoid any potential off-target effects. Using artificial microRNAs (amiRNAs), rather than IR constructs, may be effective in reducing off-target effects. An amiRNA contains a known miRNA gene with the seed region replaced with a sequence complementary to target genes [22]. While a long IR transcript may be cleaved to produce many small RNAs, an amiRNA would produce a single small RNA.

Another major class of ncRNAs is long non-coding RNAs (lncRNAs) that are longer than 200 nt. Recent studies show that lncRNAs are widely present in animal and plant genomes. For example, by mining transcriptomic sequencing data in different cell lines, two groups identified 6736 and >8000 lncRNAs in the human genome [23,24]. The ENCODE project also detected 5609 out of 9277 annotated lncRNAs in the human genome [25]. A genome-wide investigation of lncRNAs has not been widely performed in plants. However, 6480 lncRNAs were recently identified in the intergenic regions of the model plant Arabidopsis using data from 200 tiling arrays [26]; 439 lncRNAs were identified in maize using full-length cDNA data [27]. Although many lncRNAs exhibit tissue or growth condition-specific expression, suggesting their functional roles in development and stress responses, the functions of most lncRNAs are largely unknown. Only a handful of lncRNAs have been functionally characterized in plants. The best studied lncRNAs are two lncRNAs that function in plant vernalization, a process where long periods of cold treatments make plants competent for flowering. On the Flowering Locus C (FLC) in Arabidopsis, an antisense lncRNA, COOLAIR, and a sense lncRNA, COLDAIR, are produced during vernalization to form an epigenetic switch for silencing the expression of the FLC gene to promote flowering [28]. While COOLAIR enhances the cold-induced down-regulation of FLC, COLDAIR interacts with the histone methyltransferase subunit of the Polycomb Repressive Complex 2 (PRC2) and targets PRC2 to the FLC locus [29,30]. Because lncRNAs may function in cis or in trans by co-transcriptional targeting of chromatin factors or acting as an enhancer, decoy, scaffold, co-activator, or co-repressor [31,32], identification of novel lncRNAs in crop plants and analysis of their function have the potential to open new avenues for crop improvement through genetic engineering.

A recent and exciting ncRNA-based technology is the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) technology for gene editing in plants [33]. CRISPR/Cas is a bacterial defense strategy against invading DNA such as phages or plasmids [34,35]. In this strategy, the CRISPR ncRNA guides a Cas endonuclease to cleave invading homologous DNA. Engineered CRISPR single guide RNAs (sgRNAs) and the Cas9 endonuclease can be expressed in transgenic plants, so that the Cas9
generates double-stranded DNA breaks at target genes that are complementary to the sgRNA sequences. Through cellular DNA repair, mutations or corrections of the target genes can be achieved [36,37]. This CRISPR/Cas-based gene editing or genome engineering technology is a very powerful tool for plant functional genomics and crop improvement.

RNAi-based technologies have proven to be potent tools for crop improvement. As genomic resources for major crops and pests and pathogens are accumulated at a fast pace, additional target genes will be exploited for crop improvement. RNAi-based technologies also have the potential to play a major role in achieving other goals for crop improvement such as increased yield and enhanced resistance to abiotic stresses once the relevant pathways are better understood. New ncRNA-based strategies may be designed when we have an improved understanding of the functions and regulatory networks of other ncRNAs, especially miRNAs and lncRNAs.

ACKNOWLEDGEMENTS

The authors thank Mrs. Qi Jie Zheng for her assistance in preparing Fig. 1.

FUNDING

This work was supported by the Chinese Academy of Sciences.

Renyi Liu1,* and Jian-Kang Zhu1,2
1 Shanghai Center for Plant Stress Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, China
2 Department of Horticulture and Landscape Architecture, Purdue University, USA
*Corresponding author.
E-mail: ryliu@sibs.ac.cn

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doi: 10.1093/nsr/nwu006
Advance access publication 14 May 2014.