New technologies accelerate the exploration of non-coding RNAs in horticultural plants

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Non-coding RNAs (ncRNAs), that is, RNAs not translated into proteins, are crucial regulators of a variety of biological processes in plants. While protein-encoding genes have been relatively well-annotated in sequenced genomes, accounting for a small portion of the genome space in plants, the universe of plant ncRNAs is rapidly expanding. Recent advances in experimental and computational technologies have generated a great momentum for discovery and functional characterization of ncRNAs. Here we summarize the classification and known biological functions of plant ncRNAs, review the application of next-generation sequencing (NGS) technology and ribosome profiling technology to ncRNA discovery in horticultural plants and discuss the application of new technologies, especially the new genome-editing tool clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) systems, to functional characterization of plant ncRNAs.

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INTRODUCTION

Horticultural plants (for example, such as fruits, vegetables, ornamental trees and flowers, herbs, and tea trees) have been domesticated to satisfy human’s food and aesthetic needs via various forms of hybridization breeding, mutation breeding, and transgenic breeding.1–3 Protein-coding genes related to specific target agricultural trait were chosen as major targets in the early time of transgenic breeding.2 Recently, non-coding RNAs (ncRNAs) have been shown to play key roles in the regulation of plant growth, development and response to environmental stresses at either transcriptional or post-transcriptional levels.3,4 Thus, ncRNAs are emerging as a spotlighted target materials to accelerate the domestication of horticultural crops.

Though discovery and functional characterization of ncRNAs have been carried out for more than half a century,5 their widespread occurrence and myriad functions in various organisms have not been truly appreciated until the post-genomics era. An unexpected finding from the annotation of sequenced genomes is that DNA sequences encoding proteins occupy only a small portion (2–25%) of the genomic space.6 The advent of next-generation sequencing (NGS) revolutionized the exploration of ncRNAs, and as a result, many novel ncRNAs have been recently discovered.7,8 Which were highlighted by the new discovery of circRNAs.7,9–12 One of the big challenges in ncRNAs discovery is the determination of the coding potential of RNA sequences. Recent advances in ribosome profiling have shown a great potential for distinguishing between coding and non-coding transcripts and consequently improve the accuracy of ncRNA annotations.13,14

Molecular genetics approaches have been applied to functional characterization of ncRNAs via gain-of-function analysis or loss-of-function analysis.7,15,16 Precision genome engineering is a powerful tool for functional characterization of ncRNAs. Recently, a platform using RNA-guided engineered nucleases was developed for genome editing. The type II clustered, regularly interspaced, short palindromic repeat, (CRISPR)/CRISPR-associated protein 9 (Cas9) system found naturally occurring in Streptococcus pyogenes has been used to obtain rapid and efficient editing of genomes in plant species, and could facilitate the analysis of loss-of-function, gain-of-function and gene expression.17

In this review, we describe the classification and known functions of plant ncRNAs. Then, we review the application of NGS and ribosome profiling technology to ncRNAs discovery in horticultural plants, followed by a discussion of the new technologies for functional characterization of ncRNAs.

CLASSIFICATION AND FUNCTIONS OF PLANT NCRNAS

Based on the molecular structure, plant ncRNAs can be classified as linear ncRNAs and circular ncRNAs (circRNAs; Figure 1). The catalog of ncRNAs is currently dominated by linear ncRNAs compared with circRNAs that were just recently discovered as an emerging new class of ncRNAs.7,9–12,18 On the basis of molecular function, linear ncRNAs can be divided into two categories: (1) housekeeping ncRNAs, including ribosomal RNAs (rRNAs), transfer RNAs (tRNAs) and small nucleolar RNAs (snoRNAs); and (2) regulatory ncRNAs, which can be further divided into two sub-categories: (a) small RNAs (sRNAs), including microRNAs (miRNAs) and small interfering RNAs (siRNAs); and (b) long ncRNAs (lncRNAs), including long intronic ncRNAs and long intergenic ncRNAs.7,10,20 On the basis of the genome region from which circRNAs arise, circRNAs can be divided into (1) exonic circRNAs, (2) intronic circRNAs, (3) UTR circRNAs, (4) intergenic circRNAs and (5) other circRNAs deriving from two or more genes (Figure 1).12 So far, functional characterization of ncRNAs has focused on sRNAs, IncRNAs and circRNAs. The known biological functions of these three types of ncRNAs are summarized as follows.

The function of sRNAs
sRNAs are involved in the regulation of plant growth, development and stress response via silencing endogenous gene

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expression at either transcriptional or post-transcriptional levels.\textsuperscript{21,22} miRNAs, derived from single-stranded hairpin RNAs,\textsuperscript{23} can be classified as conserved miRNAs and non-conserved miRNAs.\textsuperscript{24} Many miRNAs have been characterized from plants, which play important roles in different signaling pathways (Table 1). Usually conserved miRNAs are abundantly expressed, targeting transcription factors that directly regulate gene expression (Table 1). The relationships between conserved miRNAs and their targets have been considered to be stable during the evolution process,\textsuperscript{25} but it

Table 1. Function of miRNAs validated by experiments in plants

| miRNA   | Conservation | Targets                              | Functions                                             | References |
|---------|--------------|--------------------------------------|-------------------------------------------------------|------------|
| miR156  | Conserved    | SPL                                  | Development                                           | 102        |
| miR159  | Conserved    | MYB, SGN-US67133                     | Signaling pathway and development                     | 103,28     |
| miR160  | Conserved    | ARF                                  | Seed germination                                      | 104        |
| miR163  | Non-conserved| PXMT1, FAMT                          | Metabolite biosynthesis                               | 105        |
| miR165/166| Conserved   | HD-ZIPIII                           | Leaf and vascular development                        | 106        |
| miR167  | Conserved    | ARF                                  | Signaling pathway, flower development                 | 107        |
| miR172  | Conserved    | AP2                                  | Signaling pathway, flower development, stress response| 108        |
| miR173  | Non-conserved| TAS1, TAS2                           | Uncharacterized                                       | 109        |
| miR319  | Conserved    | TCP                                  | Flower development                                    | 110        |
| miR390  | Conserved    | TAS                                  | Development                                           | 111        |
| miR395  | Conserved    | Sulfate transporter                  | Sulfate transport                                     | 112        |
| miR396  | Conserved    | GRF, bHLH74, HaWRKY16                | Leaf development, heat tolerance                      | 27,113,26  |
| miR400  | Non-conserved| PPR                                  | Heat tolerance                                        | 114        |
| miR408  | Conserved    | Gene coding Copper ion binding protein| Copper homeostasis                                   | 115        |
| miR444  | Non-conserved| MADS57                               | Tilling development, nutrition accumulation          | 115        |
| miR472  | Non-conserved| CNL5                                 | Pathogen resistance                                   | 116        |
| miR482  | Non-conserved| NBS-LRR                             | Pathogen resistance                                   | 117        |
| miR820  | Non-conserved| DRM2                                 | Epigenetic silencing                                  | 118        |
| miR824  | Non-conserved| AGL16                                | Stomata development, plant flowering                  | 119        |
| miR828  | Non-conserved| MYB2                                 | Fiber development                                     | 120        |
| miR842/846| Non-conserved| Jacalin lectin                      | Vegetative storage                                    | 121        |
| miR858  | Non-conserved| MYB2                                 | Fiber development                                     | 120        |
| miR1512 | Non-conserved| Gene coding copine-like calmodulin-binding protein| Nodule development                                   | 122        |
| miR1863 | Non-conserved| OsOg38480                           | DNA methylation                                       | 122        |
| miR4376 | Non-conserved| Ca\textsuperscript{2+}-ATPase        | Flower and fruit development                          | 123        |
| miR5200 | Non-conserved| FTL1/2                               | Flowering initiation                                  | 124        |
| miR6019 | Non-conserved| NB-LRR/LRR                          | Pathogen resistance                                   | 125        |
| miR6020 | Non-conserved| NB-LRR/LRR                          | Pathogen resistance                                   | 125        |
| miR7695 | Non-conserved| Nramp6                               | Pathogen resistance                                   | 126        |

Abbreviations: AGL16, agamous-like 16; AP2, apetala2-like transcription factor; ARF, auxin response factor; bHLH74, basic Helix-Loop-Helix 74; CNLs, coiled-coil nucleotide-binding leucine-rich repeat; DRMs, domains rearranged methyltransferase 2; FTL1/2, flowering locus T-like1/2; FAMT, farnesoic acid carboxyl methyltransferase; GRF, growth regulating factor; HD-ZIPIII, class III homeodomain-leucine zipper transcriptional factor; Nramp6, natural resistance-associated macrophage protein 6; NBS-LRR, nucleotide-binding site-leucine-rich repeat; PPR, pentatricopeptide repeat; SGN-US67133, encoding a protein with unknown function; SPL, squamosa-promoter binding protein-like; TAS, tasiRNA-generating; TCP, teosinte branched1/cycloidea/proliferating cell factor.
The function of IncRNAs

IncRNAs are linear ncRNAs of greater than 200 nt in length,\(^\text{37}\) which have been demonstrated to involve in multiple biological processes such as phosphate homeostasis, flowering, photomorphogenesis and fertility (Table 2). The molecular mechanisms underlying the biological function of plant IncRNAs include: (1) processing into shorter ncRNAs for functioning,\(^\text{38}\) (2) acting as the target mimics of miRNAs,\(^\text{39,40}\) (3) repressing histone-modifying activities and direct epigenetic silencing via interaction with specific chromatin domains,\(^\text{41–44}\) (4) acting as molecular cargo for protein re-localization\(^\text{45,46}\) and (5) post-translational regulation through protein modification and protein–protein interactions.\(^\text{47}\)

The function of circRNAs

Discovery of thousands of circRNAs across a range of plant species have been summarized in other review paper,\(^\text{47}\) and recently demonstrated in horticultural plants, for example, Solanum lycopersicum\(^\text{48}\) and Actinidia chinensis.\(^\text{18}\) However, little is known about the function of circRNAs in plants. In Arabidopsis, Conn et al.\(^\text{49}\) reported that the circRNAs derived from exon 6 of the SEPALLATA3 (SEP3) gene can bind strongly to its cognate DNA locus, forming an RNA:DNA hybrid, or R-loop, whereas the linear RNA equivalent bound significantly more weakly to DNA. R-loop formation results in transcriptional pausing, in turn driving floral homeotic phenotypes. The function of circRNAs reported in mammalian may serve as an initial guidance for future studies on the function of plant circRNAs. For example, Hansen et al.\(^\text{50}\) reported that circular transcript ciRS-7 from human and Sny9 from mouse acts as a ‘molecular sponge’ of miR7 and miR138, respectively. The human circRNA ITCH was reported to act as a sponge for miR7, miR17 and miR214, respectively.\(^\text{51}\) Another circRNA ZNF91 containing 24 miR23 sites, as well as 39 additional sites for miR296, was discovered in mammals.\(^\text{18}\) Zhang et al.\(^\text{48}\) showed that an intronic circRNA, ci-ankrd52, positively involves in the regulation of RNA polymerase II transcription. Also, exon-intron circRNAs have been shown to enhance the expression of their parental genes in a cis configuration.\(^\text{7}\)

Table 2. Function of the IncRNAs reported in plants

| IncRNAs         | Species                              | Biological function                     | Regulation mechanism            | Refs         |
|-----------------|--------------------------------------|-----------------------------------------|---------------------------------|--------------|
| APOLO           | Arabidopsis thaliana                 | Auxin-controlled development            | Chromatin topology              | 127          |
| ASCO-IncRNA     | Arabidopsis thaliana                 | Lateral root development                 | Alternative splicing regulators  | 127          |
| asHSF2a         | Arabidopsis thaliana                 | Gametophytic development                | Antisense transcription          | 128          |
| cis-NAT_TEPL12  | Oryza sativa                         | Phosphate homeostasis                   | Translational enhancer           | 129          |
| COOLAIR         | Arabidopsis thaliana                 | Flowering                               | Histone modification             | 42           |
| ENOD40          | Medicago truncatula, Glycine max     | Nodule development                      | Protein re-localization           | 130,131      |
| HvCesA6 Inc-NAT | Hordeum vulgare                      | Photomorphogenesis                      | Association with chromatin       | 132          |
| LDMAR           | Oryza sativa                         | Cell wall biosynthesis                  | siRNA precursor                   | 133          |
| XLOC_057324     | Oryza sativa                         | Photoperiod-sensitive male sterility    | Promoter methylation             | 134          |

Abbreviations: APOLO, auxin-regulated promoter loop; ASCO, alternative splicing competitor; asHSF2a, natural long non-coding antisense RNA of heat stress transcription factor B; PHO1;2, PHOSPHATE1;2; COOLAIR, cold-assisted intronic non-coding RNA; ENOD40, early nodulin 40; HID1, hidden treasure 1; CesA6 lnc-NAT, natural antisense of CesA6 cellulose synthase gene; IPS1, induced by phosphate transcription factor B; PHO1;2, PHOSPHATE1;2; COLDAIR, cold-assisted intronic non-coding RNA; COOLAIR, cold induced long antisense intragenic RNAs; ENOD40, early nodulin 40; HID1, hidden treasure 1; CesA6 lnc-NAT, natural antisense of CesA6 cellulose synthase gene; IPS1, induced by phosphate starvation 1; LDMAR, long day-specific male-fertility-associated RNA.

Application of new technologies to discovery of ncRNAs

A variety of experimental approaches have been used for discovering ncRNAs in plants, such as molecular cloning, microarray, next-generation sequencing (NGS), third-generation sequencing,\(^\text{52}\) epitone tagging, mass-spectrometry and ribosome profiling.\(^\text{53}\) These approaches heavily rely on bioinformatics tools, such as TopHat,\(^\text{54}\) Cufflinks,\(^\text{55}\) CIRCexplorer,\(^\text{56}\) CIRI,\(^\text{57}\) CPC\(^\text{58}\) and
HMMER\textsuperscript{60} for the discovery of ncRNAs. Recently, some new computational tools, for example, miRDeep-P\textsuperscript{61} miRDeepFinder\textsuperscript{62} and miR-PREFeR\textsuperscript{63} were developed for the identification of plant miRNAs, which are often belong to large families with high-sequence similarity among the paralogous members. Moreover, these tools do not necessarily rely on a reference genome and are useful for species-specific ncRNA detection. A pipeline for discovery of ncRNAs in plants is illustrated in Figure 2. Most of above approaches for ncRNA discovery have been discussed in some recent review articles.\textsuperscript{7,15} Currently, more and more horticultural plant genomes and transcriptomes were decoded by third-generation sequencing such as Pacific Biosciences (PacBio, Menlo Park, CA, USA), Illumina Tru-seq Synthetic Long-Read technology (San Diego, CA, USA) and the Oxford Nanopore Technologies sequencing platform (Oxford, UK).\textsuperscript{53,64–66} These platforms offer longer read sequencing to facilitate the accurate \textit{de novo} assembly of full-length RNAs without needs for mapping of the transcriptome sequencing reads to the reference genomes. Thus, while still under active development, the third-generation sequencing platforms will definitely accelerate the discovery of ncRNAs and their targets. In combination with appropriate bioinformatics tools such as PLEK\textsuperscript{67} the ongoing and future efforts for transcriptome sequencing using third-generation sequencing technologies are expected to shed new light on the ncRNA landscape of horticultural plants without reference genomes. Here we focus on two frequently used technologies that offer potential for the discovery and characterization of ncRNAs in horticultural plants: that is, NGS and ribosome profiling.

NGS as a new powerful tool for the prediction of ncRNAs

The ncRNAs can be identified through the direct detection of the transcribed RNAs.\textsuperscript{58} Initially, direct cloning approach has been used to discover ncRNAs in plants.\textsuperscript{69,70} Subsequently, the hybridization-based microarray technology has been used to discover a large number of ncRNAs in the intergenic regions of \textit{A. thaliana}\textsuperscript{71,72} and rice.\textsuperscript{73} However, the ability of these hybridization-based technologies suffer several limitations such as reduced dynamic range, high false positives\textsuperscript{6} and difficulty defining splice junctions and connecting transcribed regions into transcript models.\textsuperscript{74,75}

NGS overcomes the challenges related to microarray technology,\textsuperscript{76} providing a powerful tool for defining the ncRNA domain. For example, miRNAs were previously thought to be dominant members in the sRNAs landscape; however, recent global analysis of plant transcriptomes revealed millions of sRNAs, making them the most abundant class of sRNAs in plants.\textsuperscript{77} More recently, circRNAs were recognized as a large new category of RNAs with thousands of members in animals and plants through high-throughput transcriptome sequencing (RNA-Seq) data using new computational algorithms customized for ncRNAs (Figure 2).\textsuperscript{7,11,12,57,58} With advancement of NGS technology, many ncRNAs are being discovered in an expanding list of horticultural plant species (Table 3).

Ribosome profiling as a new tool for the validation of ncRNA predictions

A key aspect of ncRNA validation is to determine the coding potential of predicted ncRNAs. The length of 18 to 30 nucleotides is the threshold commonly used for the prediction of miRNA\textsuperscript{78,79} whereas the length of greater than 200 nucleotides is often used as the threshold for IncRNA prediction.\textsuperscript{80} Presence of an open-reading frame (ORF) of at least 100 amino acids (aa) is the threshold commonly used for defining a protein-coding transcript and as such, many important small proteins (< 100 aa) were not annotated in plants.\textsuperscript{78,79} More recently, a large number of protein sequences have been predicted by translation of the longest ORFs without any further evidence.\textsuperscript{74} It is possible that some of the predicted protein-coding genes, based on an arbitrary ORF length, might be mis-annotated. For example, some well characterized human IncRNAs, such as H19, Hotair, Kcnq1ot1, Meg3 and Xist, contain ORFs of 100 aa or longer.\textsuperscript{81} Most of predicted IncRNAs contain putative ORFs, which may be translated into non-functional proteins or may be unable to be translated at all.\textsuperscript{74}

Recently, ribosome profiling, which uses deep sequencing to monitor \textit{in vivo} translation, has shown high potential for the genome-wide examination of protein-coding potential (Figure 2). Ribosome profiling has been used to segregate several hundred small proteins (< 100 aa) from predicted IncRNAs in zebrafish and humans.\textsuperscript{13,14} Also, Pamudurti et al.\textsuperscript{85} demonstrated that a group of circRNAs was associated with translating ribosomes by performing ribosome profiling from fly heads and found a circRNA generated from the \textit{muscleblind} locus encodes a protein. In \textit{Arabidopsis}, 237 protein-encoding transcripts from the existing compendia of ncRNAs were found based on the ribosome profiling technology.\textsuperscript{86,87} Thus, the ribosome profiling technology can be used as a high-throughput tool for removing false positives in the ncRNAs predictions of horticultural plants.

APPLICATION OF NEW TECHNOLOGIES TO FUNCTIONAL CHARACTERIZATION OF NCRRNAS

Thanks to the advance in the aforementioned new technologies, the universe of ncRNAs is currently expanding at an increasing rate. However, the biological function of these ncRNAs remains largely unknown.\textsuperscript{16} Various approaches have been developed for functional studies of ncRNAs (Figure 3). The primary goal of functional studies on ncRNAs is to understand the biological processes in which the ncRNAs are involved. To achieve this goal, many researchers have used gain-of-function and loss-of-function mutants for functional characterization of ncRNA genes.\textsuperscript{7} CRISPR/ Cas9, a new genome-editing technology, holds great potential for generating knockout and knock-in mutants in plants, as demonstrated in a range of plant species,\textsuperscript{17} and recently demonstrated in horticultural plant species, for example, \textit{Citrus sinensis},\textsuperscript{88} \textit{Malus pumila},\textsuperscript{89} \textit{Solanum lycopersicum}\textsuperscript{90} and \textit{Solanum tuberosum}.\textsuperscript{91} Compared with RNA interference (RNAi) that has several limitations such as incomplete gene knock-down and extensive off-target activities, CRISPR/Cas9 technology has the advantage of complete gene knockout with relatively
low off-target activities.92 In addition, the action of RNAi is restricted in cytoplasm where RNA-induced silencing complexes are located.93 However, many ncRNAs have been shown to be localized in the nucleus, which cannot be manipulated in similar manner using RNAi.94,95 Thus, CRISPR/Cas9 provides an efficient and effective alternative to RNAi for characterizing the function of ncRNAs. In fact, this new genome-editing technology has been used to knock out several ncRNAs in animals such as humans, mouse, zebrafish,94–97 as well as in plants such as soybean.98 Once the CRISPR/Cas9-mediated knockout and knock-in mutation is created, the NGS technology, mentioned above, can be used to profile the expression of target transcripts and other downstream genes in the biological pathways (Figure 3).

After identification of the biological roles of ncRNAs, it is important to understand the molecular mechanism underlying these biological roles (Figure 3). Examination of the secondary structure of ncRNAs is informative in studying the function of ncRNAs at the molecular level. Several experimental approaches, such as selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE), parallel analysis of RNA structure (PARS) or dimethyl sulfate-modified RNA for sequencing (DMS-seq), can be used for deciphering of the secondary structure of ncRNAs.7,15 To understand where and how the ncRNAs function, chromatin isolation by RNA purification (CHIRP), capture hybridization analysis of RNA targets, CLASH, crosslinking, ligation, sequencing of hybrids; CLIP, crosslinking immunoprecipitation.

CONCLUDING REMARKS

The discovery and functional characterization of ncRNAs could facilitate the domestication of horticultural plants, resulting in more nutritious, colorful, tasty, and esthetic fruits, vegetables, and ornamental flowers and trees. While the number of protein-encoding genes is relatively less variable among plants, the ncRNA domain in plants is very dynamic, with increasingly more ncRNA members being discovered and characterized annually. In particular, recent advances in NGS and ribosome profiling technology have offered great potential for expediting the discovery of ncRNAs in horticultural plants. Also, the simplicity, robustness and versatility of the CRISPR/Cas9 systems make such systems attractive for functional characterization of ncRNAs in general and specifically to the process of accelerated domestication in horticultural crops. It is expected that these new technologies will be widely applied in ncRNA research while they become more cost-efficient and more technically mature in the near future.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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