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Review

Advances in highly specific plant gene silencing by artificial miRNAs

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Endogenous microRNAs (miRNAs) are potent negative regulators of gene expression in plants and animals. Through artificially transformed miRNA (amiRNAs) to target one or several genes of interest is becoming a powerful tool for silencing genes. The characteristics and application prospect of artificial microRNA (amiRNA) technology were reviewed.

Key words: Gene-silence, RNA interference, MiRNA, Artificial-microRNA.

INTRODUCTION

Transgene-mediated gene silence through RNA interference (RNAi) offers a direct way of inactivating one or several specific genes (Small, 2007; Tang et al., 2007). RNAi transgenes are dominant and can be applied in many different genetic backgrounds for any known gene in the genome. The RNA interference effects were all acted through the small silencing RNAs (sRNAs) derived from the transcribed double-stranded RNA precursors (Watson et al., 2005; Sen and Blau, 2006). Some studies have systematically compared different silencing strategies and found that hairpin RNA interference (hpRNAi) produced more efficient silencing triggers than separately transcribed sense and antisense RNAs (Wesley et al., 2001; Chuang and Meyerowitz, 2000).

MicroRNAs (miRNA), which negatively regulate gene expression, are endogenous single-stranded small RNA molecules 21 to 23 nucleotides long. They were first discovered in the Victor Ambros Laboratory (Lee et al., 1993), but the term microRNA was first introduced in 2001 (Ruvkun, 2001). The miRNAs are processed by RNaseIII-like enzyme Dicer from short hairpin-loop structures known as miRNA precursors (premiRNA) that are derived from longer primary miRNA transcripts (Brodersen and Voinnet, 2006). In plants, miRNAs trigger target mRNA cleavage and destruction through perfect or near perfect base pairing (Moxon et al., 2008). Many reports the numerous protein–protein and protein–RNA interactions can Influence the regulation of miRNA metabolism and function. The studies have shown that both siRNAs and microRNAs can move out of their domain of expression, which also means alteration of several nucleotides that does not affect miRNA

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Abbreviations: amiRNA, Artificial microRNA; **RNAi**, RNA interference; **sRNAs**, small silencing RNAs; **hpRNAi**, hairpin RNA interference; **premiRNA**, miRNA precursors; **RdRP**, RNA-dependent RNA polymerase.

biogenesis (Chuck and O'Connor, 2010; Krol et al., 2010). For the plant breeder, modifying the endogenous miRNA precursors can target genes of interest with the generate artificial miRNA (amiRNA). So, gene discovery will certainly be accelerate by gene silence (Niu et al., 2006; Warthmann et al., 2013; Schwab et al., 2010). Artificial microRNA (amiRNA) is becoming a powerful tool for silencing genes in plants, and several amiRNA vectors have recently been developed based on the natural precursor structures. The amiRNA sequence does not have to be perfectly complementary to the target sites; it can be optimized to target only one or, alternatively, several sequence-related genes. In plants nucleus and cytoplasm some RNA cleavage can be guided by the small RNA (sRNA). If there are not much non-autonomous effects, a few of the stronger promoters may cause higher degrees of gene silence (Schwab et al., 2006; Eamens et al., 2011).

miRNA BIOSYNTHESIS

Most miRNA genes are far from protein genes in genetic distance and may have their own promoter and can be transcribed independently. Research found that when the host genes are expressing, large number of intronic miRNAs may not express. Whether it is expressed or not is dependent on the host gene promoter. The host genes contain significant fraction of miRNA genes in their introns with the same orientation and are thought to be co-processed from the host gene (Isik et al., 2010). miRNA first digest in nuclear within RNaseIII-Drosha, releasing a 60 to 80 nt intermediates with hairpin structure named miRNA precursor (Lee et al., 2002). miRNA precursor need to be transferred to the nucleus, then through the cytoplasm Dicer enzyme further processing, can become mature molecules. This process depends on the transfer mechanism---RanGTP/exportin-5 (Lund et al., 2004).

miRNA maturation process: First Dicer recognizes hairpin structural parts of miRNA precursor and digests two chains, then the rest of the precursor will be cut off, generating an incomplete pairs of small molecules of double-stranded RNA that has been phosphorylated at the 5'end and has a 2nt outstanding at the 3'end. Because of double-stranded RNA molecular thermodynamics instability, the chain miRNA * would degraded immediately. Almost all of the eukaryotic biological processes can be regulated by the miRNAs (microRNAs). The cell development and function sustaining can be dependent on the levels of miRNAs in their organism (Tran and Hutvagner, 2013).

ARTIFICIAL MICRORNA

AmiRNA technology refers to the use of miRNA expression characteristic, using endogenous miRNA precursor as expression framework, to produce small molecule RNA mediated gene silencing. The research showed that miRNAs biogenesis cannot be affected by the alteration of several nucleotides, which makes it possible to modify the endogenous miRNA precursors of target genes of interest by artificial miRNA (amiRNA). The amiRNAs have the high specificity to facilitate efficient gene silencing of the target gene(s) (Niu et al., 2006; Warthmann et al., 2013; Schwab et al., 2010). Recently, the natural precursor structures of ath-miR159a, ath-miR164b, ath-miR169d, ath-miR172a, ath-miR319a and osa-miR528 were frequently used. For example, Liu et al (2010) generated a simple amiRNA vector (pAmiR169d) based on the structure of Arabidopsis miR169d precursor (premiR169d), and Wang et al. (2010) established a highly efficient method for construction of rice artificial microRNA vectors based on the structure of precursor Osa-miR528.

ARTIFICIAL miRNA CONSTRUCTION METHOD

Warthmann et al (2008) established a miRNA design platform, WMD3 (Web MicroRNA Designer，http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) system, which can design artificial miRNA for more than 100 plants. By using "designer" of WMD3 tool, selecting the plant to interfere with the genome database and inputting target gene sequences as well as online submission, this system compares through the plant genome database (or EST databases) to prevent "miss", and according to the related amiRNA parameters (for example, Tm.) lists candidate amiRNA. WMD3 provides construction based on ath-miR319a, osa-miR528, pChlamiRNA2/3 as amiRNA template, PCR method to replace the carrier with miRNA clips (detailed method of constructs artificial miRNA can consult on the site).

Choosing appropriate candidate precursor

After a two-step selection process based on empirically established criteria for efficiency and specificity, suitable amiRNA candidates are improved as the knowledge on the biology of miRNAs grows (Schwab et al., 2006; Huntzinger and Izaurralde, 2011). Then, the microRNA sequences would add a 3' modification of nucleotides (de Alba et al., 2013). And the recent reports showed that the stability and efficiency of target repression of miRNA can be influenced by the 3' modifications (Wyman et al., 2011). Then, the design of amiRNA should meet the requirements that 5'end instability, the position 10 usually with an ''A'' base, and the considered amiRNA should have appropriate annealing temperature and free will.

Currently, 21mers from the reverse complement of the target transcripts are considered effective amiRNA candidates, if they have an ''A'' (sometimes also ''U'') at position 10 and display 5'end instability (higher AU con-

tent at the 5'end and higher GC content at the 3'end). The position 1 will be replaced by an ''U'' and all candidates then undergo a series of mutations at positions 13 to 15 and 17 to 21 followed by mappings against all currently known cDNA sequences or gene models for the particular species. Allowing two mismatch within two target genes at position 13 to 21 bases, Ossowski et al. (2008) think that in amiRNA 17 to 21 and target RNA existence 1 to 2 mismatch can prevent RdRP mediated sub-siRNA generated. When the target mRNA exist senior structure, amiRNA may be unable to achieve to this point. This can be prevented either by avoiding the use of mRNA advanced structural zone, or designing two artificial miRNAs to different target gene areas because of difficulty in predicting the senior mRNA structure in cells.

POLYCISTRONIC ARTIFICIAL MICRORNA

The polycistronic pri-miRNA can be generated by these clustered miRNAs which are found in close proximity to each other. A polycistronic pri-miRNA usually contains three miRNA, which can be processed by Drosha in the nucleus. Then, they would be transported to the cytoplasm where it is further processed into mature miRNA by Dicer (Farazi et al., 2013; Jain et al., 2012; Ouda and Fujita, 2013). Polycistronic amiRNA can target with many different genes, but whether the relative position of an amiRNA in the polycistronic pri-amiRNA transcript would affect its maturation and RNAi efficiency is the mainly concerned problem. The RNAi effects of these amiRNAs had been verified previously (Hu et al., 2009). Recently, Chen et al. (2010) inserted three amiRNA cassettes, which are against Fluc, EGFP, and lacZ reporter gene respectively, into the pDsRed vector in different orders. The results directly demonstrate that the maturation and function of an amiRNA is not apparently affected by its relative position in the multi-amiRNA expression vector. Chen et al. (2010) had also inserted a series of amiRNA cassettes into three major expression vectors in tandem, found that the number of concatenated amiRNA cassettes has an impact on the RNAi efficiency of single-copy amiRNA. At the same time, the researchers also found that, the three major expression vectors showed an apparently decreased inhibitory effect with an increasing number of more than four amiRNA cassettes.

PROSPECTS

AmiRNA with high efficiency and precision in gene function research area is an effective tool to replace hpRNAi. Almost all situations that use hpRNAi can use amiRNA alternatively. The amiRNA can also be used to study gene functions of multi-copy genes with existing complementary effect. AmiRNA can also foster antiviral plants, because the amiRNA mediated digestion of target genes allows partial bases mismatch and this can avoid gene silencing interference failure caused by virus gene variants. Much microrna can interact with some microbe in plants and multiple artificial microRNAs conferred robust resistance to the great mass of plant virus (Lafforgue et al., 2013; Kung et al., 2012; Balmer and Mauch-Mani, 2013). The mutant library is an important tool in the genome research. Arabidopsis and rice plants has been built by model T-DNA insertion mutant library, because of the T-DNA insertion preference and some genes are not inserted, many gene inserted mutants could never get. Development of amiRNA mutant library by the gene silencing can compensate for this shortcoming.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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