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Artificial miRNAs for Specific Gene Silencing and Engineering Virus Resistance in Plants

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Introduction

The sequence-specific gene silencing has gained widespread acceptance for its ease and direct use for functional analysis of genes. Of all the methods used for gene silencing, RNA interference (RNAi) has emerged as the potential tool which involves the targeting of gene at mRNA level. This works on the phenomenon of generating siRNAs from the transcribed double stranded RNA (dsRNA) precursors. The siRNAs hence formed target the mRNA and leads to its cleavage resulting into decreased transcript level [1,2]. This technique can be used to silence the genes to get the desired traits and hence it has proved to be a promising tool for crop improvement [2,3].

Apart from siRNAs, RNAi pathway also comprises another class of small RNAs known as micro RNAs (miRNAs). miRNAs are 21-24 nt long single-stranded RNA which negatively regulate the gene expression [1]. Victor Ambros and colleagues discovered the first miRNA lin-4 in *Caenorhabditis elegans* in the year 1993 [4]. In plants, miRNAs were first discovered in *Arabidopsis thaliana* in 2002 [5]. miRNAs are processed by RNase III enzyme, Dicer like-1 (DCL1) from the precursor miRNA (pre-miRNA) derived from the primary miRNA (pri-miRNA) transcripts [6]. The processed miRNA duplex is then loaded into RNA-induced Silencing Complex (RISC) where one of the miRNA strand, usually guide strand (antisense strand) binds to the target mRNA [7]. The binding of miRNA to the target mRNA in plants is through perfect and near perfect complementarity and leads to mRNA cleavage and translational blockage respectively [8]. miRNAs have been studied extensively and manipulated for crop improvement [9-11]. Keeping in view the conserved miRNA biogenesis pathway, plant scientists have come up with a novel strategy to manipulate the miRNA sequences, popularly known as artificial miRNA (amiRNA) technology, and it is emerging as a potential tool for gene silencing studies in plants. It involves the use of amiRNA/amiRNA* sequence targeting the desired gene sequence by replacing the native miRNA/miRNA* sequence of the endogenous pre-miRNA. The amiRNA may or may not be perfectly complementary to the target gene and hence can also be used to down-regulate single or more than one gene with sequence conservation [11]. This article highlights the design of amiRNA and their use in gene silencing and virus resistance in plants.

Biogenesis and mode of action of miRNAs

miRNAs are transcribed majorly from the intergenic and intragenic (intron) regions. The plant miRNA genes are mainly transcribed by RNA pol-II and forms capped polyadenylated long pri-miRNAs. These pri-miRNAs are then processed into 80-500 nt long pre-miRNAs by DCL1. DCLs generate 21-24 nt long small RNAs with 2 nt overhang at 3' end of the duplexes. miRNAs are processed from DCL1 while the other isoforms DCL2 and DCL4 are involved in siRNA formation [12]. Pri-miRNA to pre-miRNA conversion also requires proteins like Hyponastic Leaves 1 (HYL1) and Serrate (SE) which are present in the nuclear processing centers called D-bodies or SmD3-/SmB-bodies along with other factors like CBL, DDL and TOUGH [6,13-17]. The pre-miRNAs are unstable and hence are processed into miRNA duplexes by DCL1 and are modified with 2'-methylation by a methyl transferase Hua Enhancer 1 (HEN1), before they leave the nucleus. This prevents the sRNA duplexes from degradation by sRNA degrading nucleases (SDN) [18]. The miRNA/miRNA* is exported to cytoplasm by Hasty (an ortholog of animal transporter Exportin 5) having Ran-GTPase activity which helps the RNA to move through the nuclear pore [19,20]. The miRNA strand with 5' instability known as guide or antisense strand, is loaded into RISC where it regulates mRNA expression [21,22] while the other strand called passenger or sense strand is degraded in the cytosol. An important component of the silencing complex is Argonaute (AGO) protein having conserved PAZ and PIWI domains [23]. Multiple members of AGO family are present in the plants. RISC along with AGO proteins leads to either mRNA cleavage or translational inhibition depending on the miRNA-target binding [24,25]. Compared to animal miRNAs, plant miRNAs bind to their target with high sequence complementarity [26].

The biogenesis pathway is highly conserved and is very essential for normal functioning of plants. The pathway is exploited for the expression of amiRNAs which target the desired gene sequence. The miRNA processing pathway recognizes the amiRNAs in the same way as the native miRNAs and leads to the formation of the desired mature miRNA to regulate the gene expression [27].

Artificial miRNA designing and construct preparation

Web MicroRNA Designer 3 (WMD3) developed by Warthmann and colleagues in 2008 is widely used platform for amiRNA designing, which can be used for more than 100 plants [28]. The tool has a section called "designer" which is used to generate amiRNAs from the desired gene sequence. The tool also generates amiRNAs for gene sequences whose complete genome sequence is not available in the database.

The "designer" section follows the steps for optimization of small RNAs for highest effectiveness while targeting the mRNA. The 21-mer candidates are chosen from the reverse complements of the target sequence. These 21-mers will have A at position 10 (A or U for multiple target amiRNAs) and display 5' instability, i.e., higher AU content at 5' end and higher GC content at 3' end at around 19th position. Position 1 is invariably given a U in all cases. *In silico* mutations are then introduced at positions 13-15 and 17-21. These mutated amiRNAs should hybridize to the target sequence with no more than 2 mismatches between positions 13-21 while maintaining the 5' instability [29]. In addition

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70% of free energy hybridization energy for a perfectly complementary amiRNA is essential with at least -30 k Cal/mol which is determined by M-fold [30].

Selection of candidate amiRNA

After the mutation process, all amiRNAs generated are ranked, taking into consideration all criteria required for an efficient amiRNA. amiRNAs completely complementary to the desired target are avoided or listed at last as they may lead to transitive siRNA production causing off-target effects. To avoid this, amiRNAs with one or two mismatches between positions 17 and 21 are preferred. Hence, the WMD3 output will list the best amiRNA candidates at the top in green color, followed by yellow and orange having the intermediate ones and lastly, the red having most penalty points at the bottom. amiRNAs shown in red do not imply their non-functional character but shows their increased tendency for off-targets. Hence, it is recommended that the amiRNAs should be selected from top to the bottom [29].

amiRNA construct preparation

After the selection of potential amiRNA, the sequence has to be incorporated into the amiRNA precursor. This can be done by overlapping PCR to replace the native miRNA sequence. WMD3 has 'oligo tool' which generates the oligonucleotide primers which can be used to engineer amiRNA in miR319a precursor from *A. thaliana*. The detailed procedure of overlap PCR can be viewed on WMD3 (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi). Other precursors are also used for amiRNA expression, including miR159a [31], miR164b [32], miR171 [33] and miR172a [34]. The native miRNA/miRNA* sequence is replaced by the amiRNA/amiRNA* sequence in such a way that the mismatch positions present in the endogenous pair is retained which is essential for efficient DCL-mediated processing.

Another way of replacing the native miRNA sequence is by custom synthesizing precursor miRNA carrying the desired miRNA/miRNA* sequences. The modified precursor sequence can then be cloned into the desired cloning and expression vector and used for plant transformation [35].

Testing of the efficacy of amiRNA

The amiRNA expression after the plant transformation can be tested by Northern hybridization. The antisense sequence of amiRNA can be used as a probe for the detection of desired small RNA. The expression level of amiRNA is strongly influenced by the promoters. The expression of amiRNA under a strong promoter will lead to higher gene silencing [27,33]. Target gene expression level can be monitored by RT-PCR using the primers spanning the region of amiRNA and target binding. Other details of molecular characterization for amiRNA expressing plants can be found in "Help" section of WMD3 (http://weigelworld.org).

Artificial miRNAs and virus resistance in plants

In plants, amiRNAs have been successfully used for down-regulating endogenous genes [25,29,31] and developing transgenic virus resistance against turnip yellow mosaic virus (TYMV), turnip mosaic virus (TuMV) in Arabidopsis [31]; tomato leaf curl virus [34]; cucumber mosaic virus (CMV)in tobacco [35]; CMV in tomato [36] and cassava brown streak virus (CBSV) and Cassava brown streak Uganda virus (CBSUV) in cassava [37].

Many reports suggest that amiRNA strategy is an efficient tool for establishing stable virus resistance line. Niu et al. [31] modified an *A. thaliana* miR159 precursor to express artificial miRNAs (amiRNAs) targeting viral mRNA sequences encoding two gene silencing suppressors, P69 of turnip yellow mosaic virus (TYMV) and HC-Pro of turnip mosaic virus (TuMV). Targeting the TuMV coat protein sequences also conferred TuMV resistance. Transgenic plants expressed both amiR-P69 (159) and amiR-HC-Pro (159) from a dimeric pre-amiR-P69 (159)/amiR-HC-Pro (159) transgene were resistant to both the viruses. The virus resistance trait was displayed at the cell level and transferred to subsequent generations. The resistance trait was maintained at 15°C, a temperature that compromises siRNA-mediated gene silencing [28]. Qu et al. [35] targeted the silencing suppressor 2b of CMV, by amiRNA strategy. amiRNA efficiently inhibited 2b gene expression and protein suppressor function in transient expression assays and conferred an effective resistance to CMV infection on transgenic tobacco. The anti-CMV effect of amiRNA when compared with that of a short hairpin RNA- derived small RNA targeting the same site, the amiRNA proved better method, for both transient assays and in transgenic plants. The wheat streak virus (WSMV) has always been a persistent treat to the wheat production; therefore new strategies need to be developed to curb the issue. Fahim et al. [38] came up with a similar artificial miRNA strategy against WSMV. They made use of the polycistronic amiRNA strategy, i.e., incorporated five amiRNAs within one polycistronic amiRNA precursor. These replaced the natural miRNA in each of five arms of the polycistronic rice miR395, producing amiRNA precursor, FanGuard (FGmiR395), which was transformed into wheat behind a constitutive promoter. The resistance was assessed over two generations. Three types of responses such as completely immune; initially resistant with resistance breaking down over time; and initially susceptible followed by plant recovery has been noted in T_1 plants of different transgenic families. The deep sequencing of small RNAs from inoculated leaves showed that the amiRNA targets are fully conserved in all three isolates namely, immune transgenic, susceptible transgenic and susceptible non-transgenic plants. This indicated that virus replication of some transgenics was not a result of mutational escape by the virus. Moreover, the resistance segregated with the transgene. Analysis of T_2 generation confirmed the inheritance of immunity as no symptom developed and no virus detection was observed by ELISA [38]. Another interesting study by Martinez et al. [39] reported the fate of amiRNA-mediated resistance in mixed infection of plant viruses. Transgenic line of *A. thaliana* 12-4 was challenged with a second virus along with TuMV showed resistance to TuMV when this virus was co-inoculated with tobacco mosaic virus (TMV), tobacco rattle virus (TRV), cucumber mosaic virus (CMV), turnip yellow mosaic virus (TYMV), cauliflower mosaic virus (CaMV), lettuce mosaic virus (LMV), or plum pox virus (PPV). When the plants were pre-infected with the different viruses, TuMV was able to co-infect the transgenic line [39]. Vu et al. [34] designed two amiRNAs, which targeted the pre-coat and coat protein of tomato leaf curl New Delhi virus (ToLCNDV) in transgenic tomato plants. The amiRs targeted the middle region of the AV1 (coat protein) transcript (amiR-AV1-3) and the overlapping region of the AV1 andAV2 (pre-coat protein) transcripts (amiR-AV1-1). According to their study, the transgenic tomato plants expressing amiR-AV1-1 advanced to T_2 generation showed tolerance to ToLCNDV, as compared to the amiR-AV1-3, which showed only moderate tolerance. Another virus namely wheat dwarf virus (WDV) possess a major treat to barley production by causing dwarf disease. The natural resistance resources against this virus are limited, and the use of amiRNA strategy proved beneficial. A similar approach to target this particular virus was used [34]. A barley miRNA precursor was used as backbone which targeted different conserved sequence elements of the WDV strains [40].The polycistronic amiRNA precursor construct (VirusBuster171),

expressing three amiRNAs simultaneously was checked and confirmed to work at low temperatures. A stable barley transgenic line resistant to insect-mediated WDV infection was established. Studies suggested that amiRNA technology is indeed an efficient method to curb the devastation caused by viruses.

Conclusions and Future Directions

Gene silencing mediated by amiRNAs is an effective technique and has become an important tool for gene function analysis. The gene silencing mediated by amiRNA is highly specific and is stably inherited. amiRNA- mediated silencing approach can be used for studying the function of genes whose mutational studies are not possible as they may result into deleterious effect. amiRNAs can also be used to study the functions of multiple genes which share the same sequences making the functional analyses easy. Apart from this, amiRNA technology has proved to be a potential approach for engineering virus resistance in different plants, besides the improvement of several agronomically important traits by silencing of the appropriate gene(s) in plants by amiRNAs [41]. Viruses release RNAi suppressors in defense and hence the resistance is lost, and amiRNA has been used as an alternative for this defense mechanism. The precise designing of amiRNAs by using the tools also increases the specificity of the technique and reduces the off-target effects. The application of amiRNAs in crop improvement is increasing at a fast pace. In fact, amiRNA technology has not yet been exploited for crop improvement, and therefore it can be explored for engineering crop plants for resistance against bacterial and fungal pathogens as well as insects and nematode pests through the silencing of host genes that are associated with biotic stresses and vital genes of the target pathogens and pests by amiRNAs.

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