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Micro RNA genes and their likely influence in rice (*Oryza sativa* L.) dynamic traits through evolution

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Abstract

Micro RNAs (miRNAs) are small non-coding RNAs molecules having approximately 18-25 nucleotides, they are present in both plants and animals genomes. MiRNAs have diverse spatial expression patterns and regulate various developmental metabolisms, stress responses and other physiological processes. The dynamic gene expression playing major roles in phenotypic differences in organisms are believed to be controlled by miRNAs. Mutations in regions of regulatory factors, such as miRNA genes or transcription factors (TF) necessitated by dynamic environmental factors or pathogen infections, have tremendous effects on structure and expression of genes. The resultant novel gene products presents potential explanations for constant evolving desirable traits that have long been bred using conventional means, biotechnology or genetic engineering. Rice grain quality, yield, disease tolerance, climate-resilience and palatability properties are not exceptional to miRN Asmutations effects. There are new insights courtesy of high-throughput sequencing and improved proteomic techniques that organisms' complexity and adaptations are highly contributed by miRNAs containing regulatory networks. This article aims to expound on how rice miRNAs could be driving evolution of traits and highlight the latest miRNA research progress. Moreover, the review accentuates miRNAs grey areas to be addressed and gives recommendations for further studies.

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Introduction

Rice has two main classes of small RNAs: microRNAs (miRNAs) and small interfering RNAs (siRNAs). MiRNAs are derived from endogenous single stranded transcripts that fold back to themselves, contrary to siRNAs which originate from longer exogenous double-stranded RNA taken by cell from vectors like virus (Guo *et al*., 2015; Martinez de Alba *et al*., 2013). However, both small RNAs are involved in epigenetics using RNA-induced transcriptional silencing (RITS) as well as key players in RNA interference (RNAi) process which is important for plant cell survival under various stresses (Deng *et al*., 2015;Younis *et al*., 2014). Typically miRNAs associate with mRNA via complementary base pairing to influence stability of mRNA. MiRNAs achieve these key regulatory functions by coupling with Argonaute (AGO) proteins to form a unit that degrade target messenger RNA (mRNA). Although degradation is the main mode of miRNA operations to attain regulatory functions, regulation can also achieved through mRNA translation repression or direct DNA methylation (Jones-Rhoades, 2006; Sun, 2012).

In recent years, rice miRNAs have been catalogued to be involved inpanicle branching, increased yield, improved grain quality, early flowering, immunity to diseases, among other important traits at post-transcriptional level (Baldrich and San Segundo, 2016; Chen *et al*., 2013; Miura *et al*., 2010; Wang *et al*., 2012; Zhang *et al*., 2013). Altering miRNAs activities leads to direct physiological variations in plants where they act as ubiquitous regulators in the genes expression. The latest version of important miRNA database - miRBase 21 (Released on June, 2014) stores 28645 entries representing hairpin precursor miRNAs, in which 592 precursors and 713 mature miRNAs are from rice (*Oryza Sativa* L.). Studies of miRN As has greatly advanced since it was first documented in Caenorhabditiselegans (Lee *et al*., 1993; Reinhart *et al*., 2000). The adoption of high throughput sequencing for genome discovery and analysis has identified plethora of miRNAs in plants. However, few miRNAs are fully characterized (Wang *et al*., 2004).

Moreover, there is limited information about rice miRNAs despite clear demonstrations that they play crucial roles in improving rice agronomic traits (Macovei *et al*., 2012). Present review gives an update of rice miRNAs.

Rice productivity mainly depends on genome stability, due to its sessile nature, several external factors (UV light, drought, heavy metals and pathogen infections) influences rice genome stability. Different visible traits in rice varieties are essentially caused by gene expression variation rather than gene products structure changes. MiRNA-mediated gene expression regulations have largely been employed to withstand dynamic external changes. These adaptation mechanisms are greatly achieved through constant evolution with gain or loss of miRNA binding sites caused by nucleotide mutations. Plants miRNAs have been characterized to evolve through target genes inverted duplications, random formations or via modification of existing miRNAs (Allen *et al*., 2004; Felippes *et al*., 2008; Guo *et al*., 2008). Nevertheless, plants miRNAs are generally conserved with the novel miRNAs expunged in a short evolutionary period because it's deleterious nature (Cuperus *et al*., 2011).

Biogenesis of miRNAs

miRNA biogenesis is a diverse complex process accompanied with many regulatory proteins and enzymes in a series of steps (Wen-wen *et al*., 2014). In summary, the processes begin with miRNA gene being transcribed into a primary miRNA (pri-miRNA) controlled by Polymerase II enzymes (Lee, 2004; Wang *et al*., 2013).

Thereafter, dicer-like1–serrate–hyponastic leaves 1 (DCL1-SE-HYL1) microprocessor complex (Baranauske *et al*., 2015) processes pri-miRNA to a stem loop intermediate called pre-miRNA containing miRNA/miRNA* duplex because of their self-complementary foldback structure. Consequently, HYL1, a double-stranded RNA binding protein, help in the metabolism stability (Han *et al*., 2004).

Henceforth, the miRNA/miRNA* duplex is cleaved into approximately 21nt miRNA at nucleus by DCL1 (Park *et al*., 2002) with few exceptions from miRNAs which require other DCL family members for biogenesis: DCL2 generates 22nt, DCL3 generates 24nt and DCL4 generates 21nt miRNA (Deleris *et al*., 2006; Rogers and Chen, 2013) thereafter miRNA is exported by HASTY/nuclear pore exports to cytoplasm (Wu *et al*., 2010). As showed in Fig. 1. double-stranded miRNA is loaded into RNA-induced silencing complex (RISC), subsequently the miRNA duplex unwinds facilitated by helicase-like enzyme before mature miRNA guide strand is selected, strand with lower thermodynamic relative to miRNA* and enhanced by the already associating RNA binding proteins is preferentially selected (Eamens *et al*., 2009) while the opposite (passenger) strand is removed due to conformational changes at AGO1 complex influenced by dissociation of Heat Shock Protein(HSP90) and SQUINT (SQN). Immediately after interacting with all necessary components RISC complex guided by miRNA direct mRNA activity (gene silencing) (Bartel, 2004; Iki *et al*., 2010; Schwarz, 2003).

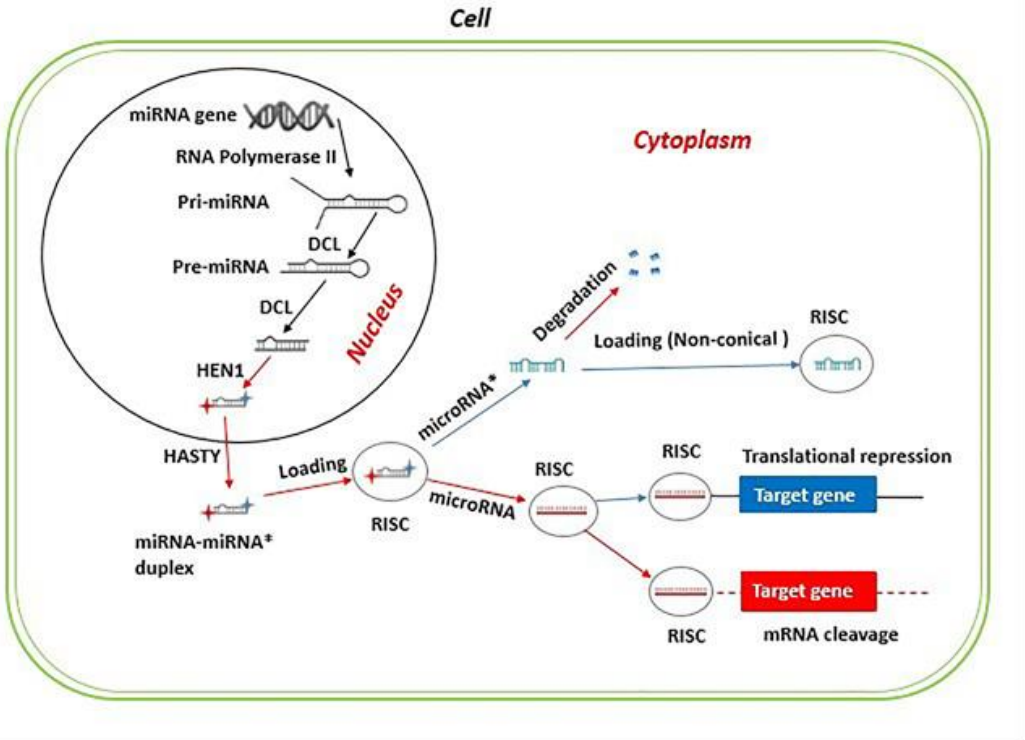

Fig. 1. Biogenesis of plants miRNA.

Plants miRNA biogenesis however have additional step compared to animal miRNA biogenesis. The miRNA/miRNA* duplexes are 2'-O-methylated on the ribose of the last nucleotide by miRN Amethyl transferase HEN1, which protect the 3' end from uridylation and degradation (Li *et al*., 2005).

It's worth noting that for rapid change of expression profiles, exoribonucleases encoded by Small RNA Degrading Nuclease (SDN1) enzyme known for degradation is necessary for mature miRNA turnover.

To recap biogenesis process, mature miRNA guide strand, often the strand with weaker 5` terminus, is retained in RISC complex where it associates with argonaute protein and other proteins complexes to mediate activity of target mRNA (Rajagopalan *et al*., 2006). The opposite miRNA strand also known as passenger strand is degraded or loaded into another RISC in non-canonical miRNA pathways (Eamens *et al*., 2012; Ramachandran and Chen, 2008; Wen-wen *et al*., 2014).

miRNAs mechanisms of action

Although the specific AGO proteins to be associated, location and action to be taken remains unclear, plants miRNA generally interact with DCL and AGO proteins to form effector complexes. The associated proteins in RISC complex guided by miRNA target mRNA or chromatin which is highly complementary in transcripts sequence thus destabilizing through slicing, translational repression or chromatin modification mostly at posttranscriptional level. (Eamens *et al*., 2008; Wu *et al*., 2009). Notwithstanding that, miRNAs can also silence at transcriptional level via DNA methylation demonstrated by rice DCL3-dependent 24nt miRNA which is loaded into AGO4 and direct methylation at the nucleus (Wu *et al*., 2010).

AGO1 deems sufficient for miRNA mediating degradation in plants (Baumberger and Baulcombe, 2005). AGO1 activity vastly depends on SQUINT (SQN) which encodes orthologue of Cyclophilin 40 (Cyp40) and Heat Shock Protein 90 (HSP90) (Smith *et al*., 2009). Rice (*Oryza Sativa* L.) plant has four AGO1 homologs (AGO1a, AGO1b, AGO1c and AGO1d) (Carbonell *et al*., 2012). Whilst, AGO2 associates with mir408 for defense actions against pathogen (Maunoury, 2011), AGO7 interacts with miR390 to regulate cellular signaling (Endo *et al*., 2013), AGO10 involves recruiting mir165/mir166 to regulate development (Zhou *et al*., 2015). Interestingly, the diverse AGO proteins family cross talk initiating alternative RNA-mediated defenses. For example, mir403 disassociated with AGO1 to activate defense pathway mediated by AGO2 thus countering viral suppression (Harvey. *et al*., 2011).

It is elucidated that miRNAs in animals, which are partially complementary to the target mRNA can also accelerate deadenylation hence rapid mRNAs degradation (Eulalio *et al*., 2009). High slicing activity and redundancy within deadenylase families in plants makes it difficult to experimentally ascertain slicing-independent target deadenylation (Wang *et al*., 2013). However, accumulation of cleavage products in most miRNA targets symbolizes some sort of slicing independent destabilization (German *et al*., 2008).

Translational inhibition in plants can also be achieved when miRNA are in perfect complementation with targets unlike in animals where it is often associated with limited miRNA/target complementation (Zeng *et al*., 2003). Overexpressed miR172 in Arabidopsis mutants demonstrated hindered proteins levels but not mRNAs, this event corroborates that translation inhibition occurred rather than cleavage (Aukerman and Sakai, 2003; Dugas and Bartel, 2008).

Roles of miRNAs

Breeding elite rice is a global objective to sustain human population which its growth has outpaced rice production (3K RGP, 2014). The proposal of designing ideal plant architecture (IPA) was bold move by rice scientists aimed at enhancing yield through point mutations of regulatory factors. Altering activity of Osmir156 which target SOUAMOSA PROMOTER BINDING PROTEIN-LIKE 14 (OsSPL14) displayed rice with increased yield, good quality and stress tolerance (Jiao *et al*., 2010; Miura *et al*., 2010).

The responses to dynamic environment prompting appropriate traits and survival strategies by cells is believed to be guided by miRNAs (Xiu-JieWang, 2004). Regulatory roles in biological processes under influence of miRNA is highly conserved in plants, this is evidenced by the fact that most miRNA families are species specific (Kamanu *et al*., 2013).

Multiple miRNA have also been manifested to play integral role in rice immunity against rice blast, a fungal disease caused by Magnapor the Oryza. The counter measures employed by rice against fungus infection is effected at Pathogen-associated molecular patterns triggered immunity (PTI) (Li *et al*., 2010) and effector triggered immunity (ETI) levels (Mentlak *et al*., 2012). Basal responses in PTI showed mir398b mediating regulation of multiple genes: Os03g22810 encoding Superoxide Dismutase (SOD), Os07g46990 encoding SOD2 and Os04g48410 encoding copper chaperone for SOD displaying reduced fungal growth and increased hydrogen peroxide at the infection site. When pathogens successfully delivered effectors to suppress responses at PTI, alternative rice defense mechanism was recorded to be activated. (Li *et al*., 2014).

Apart from basal regulations, rice miRNA also act as positive regulator. Expressions of mir160a displayed up-regulation only in resistant rice lines, the target Os04g43910 gene encoding Auxin Response Factor 16 (ARF16) showed decreased expression. This indicates positive regulation of immunity against pathogen through suppression of indole-3-acetic acid (IAA) pathways (Li *et al*., 2014).

Moreover, miRNAs have been demonstrated to be involved in stabilizing or destabilizing gene expressions depending on the mRNA effects (Xie *et al*., 2007) It is vividly clear from the first 20 experimentally discovered rice miRNAs to the novel miRNA that they play crucial role in development and abiotic stress mitigation therefore influencing growth (Jia-Fu Wang, 2004; Sunkar *et al*., 2008; Xie *et al*., 2006).

Further Research using model plant (*Arabidopsis thaliana*), verified that miRNA is controlling gene regulation systems via the target genes. For this reason, there is often positive correlation between miRNA regions and target genes sequence. (Carrington and Ambros, 2003; Takuno and Innan, 2008). When *Arabidopsis thaliana* was subjected to abiotic stresses, miRNAs among them; miR168, miR171, and miR396 responded to the high-salinity, drought, and low temperature stresses, showing great sense of cross-talk in the signaling pathways (Liu *et al*., 2008).

Most miRNA in plants mediate gene silencing of the target mRNA by base pairing in almost perfect manner hindering translation rather than slicing, miR-172 elucidated this phenomenon in Arabidopsis: base pairing complementarity with APETALA 2 (AP2) located in the coding region instead of the 3′ UTR therefore controlling cell-fate specification in flower development (Chen, 2004) By the same token, miRNA 39 was involved in root development and hormone signaling, cleaving mRNA targets which encoded Scarecrow-like (SCL) family (Llave, 2002).

Importantly, expression patterns of miRNAs support their roles not only in development but also in response to biotic/abiotic stresses.

When plants were subjected to drought stress, phytohormoneabscisic acid (ABA) worked contentiously with phytohormone auxin generally regulated by miR393 enhancing lateral root growth, transcripts encoding two auxin receptors, TIR1 and AFB2 were cleaved by the miRNA (Chen *et al*., 2012; He and Li, 2008).

To sum up, predictions of miRNA targets (BOX 1) have revealed many regulatory pathways that might be mediated by miRNA (He and Hannon, 2004) indeed miRNA are involved in almost all biological process directly or by feedback regulation of miRNA products.

Evolution of miRNAs

Advances in high throughput sequencing, have brought new insights into how the evolution of miRNA-containing regulatory networks contributed to species complexity. Although, plants miRNAs are under purifying selection, computational sequence analysis on regions outside miRNA/miRNA* duplex of ath-miR161, ath-miR163 and ath-miR822 showed some correlation with the evolved target sequences. The miRNA sequences were aligned in inverted form to target genes, asserting that inverted duplications was probably the cause of the new miRNAs genes (Allen *et al*., 2004; Ehrenreich and Purugganan, 2008).

Mutations in miRNA-related regions, basically initiated different phenotypes observed in plants, altering biological functions thus enhancing genome evolution. It is therefore likely that mutations in the inverted regions could be speed up formation of new miRNAsgenes (Cuperus *et al*., 2011; Sun *et al*., 2009).

In the quest to find out whether miRNAs have evolved during domestication of rice, it was found that some miRNA genes evolve rapidly most likely due to strong negative selection (Liu, 2013). osa-smR5864w gene in rice showed that, a single C-to-G point mutation was the cause of pollen fertility or sterility (Zhou *et al*., 2012).

During evolution, newly formed miRNAs get expressed weakly therefore face negative selective pressures to evolve rapidly compared to conserved miRNAs.

Interestingly, approximately one third of miRNAs likely increase the processing of pre-miRNAs into mature miRNAs, due to polymorphisms at miRNA stem region hence enhancing the stability of hairpin structures (Liu, 2013).

The evolution of miR395 gene families in both *Arabidopsis Thaliana* and *Oryza Sativa* plants demonstrated the homology that exist in miRNA gene members, which came as a result of gene duplications events at different time scales during evolution. The evolution of plants miRNA gene was similar to protein-coding genes, plants have smaller number of unique miRNA sequences but larger miRNA families (Li and Mao, 2007). .

To put miRNA family evolution into perspective, rice miR395 displayed 24 genes transcribed as a single transcript from the four compact clusters. Apparently, the variation of genomic organizations of miR395 gene families, and other miRNA gene families generated different regulatory profiles in plants (Li and Mao, 2007).

Lastly, transposable elements could also be speeding up miRNA genes evolution, along with rapid genetic recombination at the origin of gene structures. In the case of long terminal repeat retro transposons (LTR-RT), research elucidated that when subjected to mutation, LTR-RT formed miRNA-like hairpins that eventually became miRNA genes that led genome evolution dormancy (Zhou *et al*., 2013).

MiRNA-target evolution

In order to understand miRNA target evolution, analysis of evolution of miRNA binding sites is necessary. Plants have highly conserved miRNA binding sites and strong evolutionary selection, miR397 for example was hereditary retained in dicots to target L-ascorbate oxidase precursors (Jones-Rhoades and Bartel, 2004).

Using molecular evolution and population genetics to study miRNA target genes and binding site in rice genome, a study revealed that loss in activity of miR397 after the whole genome duplication (WGD) was due to mismatches in the likely miR397 binding site to Os01g62600 gene (Guo *et al*., 2008).

During the co-evolution of miRNAs and target genes, nucleotide polymorphism played a crucial role in determining gain or loss of miRNA binding sites.(Berezikov, 2011) in order for a miRNA to be active, there should be insignificant nucleotides variation between miRNA and target binding sites: mir161 and mir163 genes of *Arabidopsis thaliana* demonstrated that inverted duplication activities coupled with expansion of target gene families which are adopted into miRNA biogenesis pathways greatly affected its evolution(Allen *et al*., 2004).

Strategies for characterizing miRNAs

MiRNA functional characterization can be achieved using genetic mutations, metabolism changes brought by mutations of miRNA have tendency to result in pleiotropic developmental defects, increased grain productivity among other important traits in plants (Jiao *et al*., 2010; Miura *et al*., 2010; Palatnik *et al*., 2003). Defects of miR-JAW gene activity which is homologous to CINCINNATA (CIN) gene (Nath, 2003) showed crinkly leaves, the uneven leaf curvature and shape was concluded to be caused by miRNA activity on several Teosinte branched1; Cycloidea; Proliferation cell factor1 (TCP) genes which controlled leaf development in Arabidopsis (Palatnik *et al*., 2003).

Tampering with Auxin homeostasis regulated by miRNA in Arabidopsis led to down regulation of auxin signals responsible for lateral root development (Eckardt, 2005; Guo *et al*., 2005). Overexpression is another strategy employed when profiling miRNAs, Overexpressed miR444a resulted in reduced tillers in rice (Guo *et al*., 2013).

Moreover, subjecting the study plants to stresses like drought, salinity and hydrogen peroxide subsequently documenting miRNA differential expressions and physiological changes is indisputable way to characterize them; when rice was exposed to cadmium (Cd) stress various responses were observed, there was a single up-regulated gene while 18 of the genes down-regulated, miRNAs were concluded to play a major role in Cd tolerance (Ding *et al*., 2011).

Conclusions and recommendations

This review highlights the recent miRNAs research findings. Until now, hundreds of plant miRNAs have been identified by next generation sequencing (NGS) and computational approaches. However, the omics of rice miRNAs still lags behind other plant research areas. The complex networks and relationships among transcription factors, miRNAs, miRNA targets and other regulatory components remain to be fully explored.

Limited comparative data has been big hindrance to the establishment of birth and evolution of plants miRNAs. The alternative low depth cloning techniques used in profiling miRNAs is apparently biased to highly expressed regions. Vital questions raised on mechanisms of miRNA co-evolution with their targets are yet to be extensively answered (Luo *et al*., 2013).

Although, there is tremendous amount of research being conducted in all the frontiers of coding and non-coding RNA molecules using point mutations, artificial miRNAs engineering or inhibition of miRNA activity by target mimicry (Debat and Ducasse, 2014) .We anticipate more research work to deliver and improve the precision of important agronomic traits like climate-resilience and high nutritional value which will go a long way in helping malnutrition in the world (Ye, 2000).

Following cutting-edge innovations and technology improvements, intensive training of rice miRNAs scientists should be initiated for reliable utilizations of miRNAs as robust tool for enhancing rice productivity. Reported issues like accidental mRNA silencing because of sequence similarity (Qiu *et al*., 2005) or the controversial ingested plant mir168 that was purported to be regulating animal`s gene expression (Zhang *et al*., 2012), should never occur again.

Coherent functional annotation and documentation of many predicted plant miRNA should urgently be addressed, studies on specific actions between mRNA degradation and repression owed to be enhanced not to mention the localization of miRNAs activities in the cell. If miRNA antibodies will be easily available gene functional characterization can be confirmed effortlessly.

Diligent use of gene conservation knowledge should also be observed keenly while doing miRNA research, this is necessary since studies have shown contrasting findings on similar miRNAs, there is likely a diverse outcomes between dicots and monocots plants innate immunity regulation as well as other biological processes(Li *et al*., 2014; Li *et al*., 2010).

Never has miRNA research been more exciting with the extensive characterization of miRNA/targets in non-model plants using in-silico techniques coming up with promising results. MiRNA, siRNA and other non-coding RNAs based applications will definitely be of great use if optimized fully as gene resource factory for crop improvement.

In order to achieve compressive understanding of the evolution of miRNA-mediated regulatory pathways under physiology and stresses, we anticipate many future studies focused in examining the molecular mechanisms and regulatory roles of miRNAs in stress tolerance and relationship to other biological processes.

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