MicroRNA-mediated host defense mechanisms against pathogens and herbivores in rice: balancing gains from genetic resistance with trade-offs to productivity potential

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Abstract

Background: Rice (Oryza sativa L.) is the major source of daily caloric intake for more than 30% of the human population. However, the sustained productivity of this staple food crop is continuously threatened by various pathogens and herbivores. Breeding has been successful in utilizing various mechanisms of defense by gene pyramiding in elite cultivars, but the continuous resurgence of highly resistant races of pathogens and herbivores often overcomes the inherent capacity of host plant immunity. MicroRNAs (miRNAs) are endogenous, short, single-stranded, non-coding RNA molecules that regulate gene expression by sequence-specific cleavage of target mRNA or suppressing target mRNA translation. While miRNAs function as upstream regulators of plant growth, development, and host immunity, their direct effects on growth and development in the context of balancing defenses with agronomic potential have not been extensively discussed and explored as a more viable strategy in breeding for disease and pest resistant cultivars of rice with optimal agronomic potentials.

Results: Using the available knowledge in rice and other model plants, this review examines the important roles of miRNAs in regulating host responses to various fungal, bacterial, and viral pathogens, and insect pests, in the context of gains and trade-offs to crop yield. Gains from R-gene-mediated resistance deployed in modern rice cultivars are often undermined by the rapid breakdown of resistance, negative pleiotropic effects, and linkage drags with undesirable traits. In stark contrast, several classes of miRNAs are known to efficiently balance the positive gains from host immunity without significant costs in terms of losses in agronomic potentials (i.e., yield penalty) in rice. Defense-related miRNAs such as Osa-miR156, Osa-miR159, Osa-miR162, Osa-miR396, Osa-530, Osa-miR1432, Osa-miR1871, and Osa-miR1873 are critical in fine-tuning and integrating immune responses with physiological processes that are necessary to the maintenance of grain yield. Recent research has shown that many defense-related miRNAs regulate complex and agronomically important traits.
Conclusions: Identification of novel immune-responsive miRNAs that orchestrate physiological processes critical to the full expression of agronomic potential will facilitate the stacking of optimal combinations of miRNA-encoding genes to develop high-yielding cultivars with durable resistance to disease and insect pests with minimal penalties to yield.

Keywords: Rice, MicroRNA, Plant immunity, Defense-yield trade-off, Genome editing

Background

The world population is increasing at an alarming rate, with projections of 10 billion by 2050 [1]. At least 60% more food grains will have to be produced to ensure that the global needs for staple foods are secured in the twenty-first century and beyond [1]. Rice (Oryza sativa L.) is one of the major staple food crops that provide calories to more than one-third of the human population. Rice productivity is constantly challenged by the negative impacts of pathogens, insect herbivores, and other parasites. These biotic stresses, particularly pathogens, account for 20–30% of losses in global rice yields [2].

Pathogens and their host plants continuously compete for dominance in a co-evolutionary battle. Plants have evolved multi-layered defense strategies against pathogen invasion [3, 4]. Host plants induce complex defense mechanisms by activating or suppressing a large array of genes in response to pathogen attacks [5]. Such mechanisms are facilitated by the ability of a host plant to recognize a myriad of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) during the initial stages of pathogen or herbivore invasion [6]. The pathogen-triggered PAMP or DAMP is usually recognized by pattern-recognition receptors (PRRs) on the surface of host cells. The PAMPs or DAMPs, in turn, activate the PAMP-triggered immunity (PTI) response by inducing many types of defense-related genes [7, 8].

Host-plant immunity is dependent on the successful activation of defense-related genes. Successful virulent pathogens often overcome the PTI by mediating effector-triggered susceptibility (ETS), which leads to disease development [9]. In response, host plants develop a secondary immune response known as effector-triggered immunity (ETI), mediated by intracellular receptor proteins encoded by R-genes [10]. The product of R-genes (R-proteins) binds to specific pathogen effectors producing a more complex and robust hypersensitive response (HR), which further mediates cell death to restrict the growth of the pathogen at the sites of infection [3]. In rice, many R-genes against pathogens and insect pests have been identified and characterized. Most of these R-genes are effectively utilized to enhance resistance through introgression breeding. However, their efficacies are often overcome within a certain period of time due to the evolution of new resistant races that can no longer be recognized by the R-gene products. Moreover, unregulated expression of R-genes imposes a substantial demand on cellular resources, which negatively affects plant growth with trade-offs to productivity in terms of penalty to grain yield [11–13]. Therefore, in addition to R-genes, exploring other types of genetic defenses, including those that are mediated by other regulatory molecules such as microRNAs (miRNAs) is a potentially important strategy for balancing efficient disease and pest management with sustainable rice production.

MicroRNAs (miRNAs) are short (~22 nucleotides), endogenous, single-stranded, non-coding RNAs that act as crucial modulators of various cellular and biological processes, including plant growth, development, reproduction, and responses to biotic and abiotic stresses [14–16]. Plant miRNAs are transcribed as primary miRNA (pri-miRNA) from miRNA-encoding genomic loci (MIR loci) by RNA polymerase II [17]. The long stem of the looped pri-miRNAs is subsequently processed by RNAse III enzymes called DICER-like 1 (DCL1) proteins in association with hyponautic leaves 1 (HYL1) and serrate (SE) into double-stranded miRNA-miRNA* (*passenger strand of miRNA) duplex [18, 19]. The miRNA/miRNA* duplex is transported from the nucleus into the cytoplasm and subsequently methylated by HUA ENHANCER 1 (HEN1) to prevent degradation [20]. The guide miRNA is loaded into ARGONAUTE1 (AGO1) to form a functional RNA-induced silencing complex (RISC) [21]. Complementarity between the silencer miRNA and its target transcript allows the RISC complex to trigger complete inhibition of protein synthesis either through the degradation of the mRNA or inhibition of its translation.

In the past few years, the rapid development of next-generation sequencing (NGS) technologies and powerful algorithms for the prediction and modelling of interactions at the genetic, genomic, and molecular levels opened new paths in miRNA discovery. Molecular genetic approaches like 5’RACE, degradome sequencing, stem-loop RT-PCR, reporter gene analysis, loss-
gain-of-function mutation experiments have led to the discovery and unraveling of the function of the large number of miRNAs [22]. In rice, numerous miRNAs that fine-tune host immune response and also those that regulate plant growth have been identified, cloned, and functionally validated through loss-of-function or gain-of-function mutation experiments. In this mini-review, we specifically focus on the regulatory impacts of functionally characterized defense-related miRNAs in rice and their roles in fine-tuning complex traits of agronomic importance. Cautionary aspects of innovative strategies for miRNA manipulation towards understanding the balance and trade-offs between defense and productivity in terms of yield are discussed. In the subsequent sections, we highlight the role of miRNAs in mediating resistance to various pathogens and herbivores (Fig. 1, Table 1).

**MicroRNAs against fungal pathogens**
In rice, the two-layered immune system (PTI and ETI) has been shown to play important roles in defense against fungal pathogens such as *Magnaporthe oryzae* [53]. Pattern Recognition Receptors (PRRs) such as CEBiP, LYP4, and LYP6 are known to recognize the pathogen-associated molecular patterns (PAMP) and induce the PAMP-triggered immunity [54, 55]. Whereas the products of R-genes recognize divergent pathogen effectors that activate effector-triggered immunity (ETI), their functionality for recognizing the target effectors depends on several structural features. However, resistance conferred by a single R-gene is quickly overcome by the emergence of new pathotypes that can evade the effects of the R-gene products. Therefore, pyramiding multiple R-genes in the same genetic background represents a more robust approach to develop rice cultivars with broad-spectrum resistance. The caveat to this approach is that R-gene pyramiding by conventional breeding (even with a marker-assisted approach) requires multiple rounds of hybridization and selection that are often confounded by the negative effects of linkage drags. A random combination of multiple R-genes in the same genetic background may not always produce positive or optimal effects, thus the trade-off effects between resistance and yield have been a major challenge in maximizing and optimizing the gains from such an approach for yield improvement. The use of miRNAs provides an alternative strategy to develop broad-spectrum resistance against fungal pathogens in rice cultivars. The increasing number of evidence support that miRNAs also regulate the ETI and PTI [56, 57]. In particular, it has been shown that certain miRNAs fine-tune the expression of innate immunity in certain

![Fig. 1](image-url)
cultivars through the integration of R-gene regulation, hormone signaling, callose deposition, and production of reactive oxygen species (ROS) such as superoxide radicals (O$_2^-$), hydroxyl radicals (OH·) and hydrogen peroxide (H$_2$O$_2$) (Fig. 2).

**MicroRNAs as positive regulators of immunity against blast disease in rice**

Several miRNAs, including Osa-miR159, Osa-miR160, Osa-miR162a, Osa-miR166h, Osa-miR398b, and Osa-miR7695 have been shown to function as positive regulators of defenses against the rice blast disease caused by fungus *Magnaporthe oryzae* [26]. For instance, the Osa-miR159a fine-tunes host plant growth and immunity by inhibiting its three target genes, OsGAMYB, OsGAMYBL, and OsZF. The OsGAMYB and OsGAMYBL are transcriptional regulators of gibberellic acid signaling, while OsZF is a C3HC4-domain-containing zinc finger protein involved in ubiquitin-protein transferase activity. Transgenic rice plants overexpressing a short tandem target mimic (STTM) to inhibit the Osa-miR159a showed enhanced host susceptibility to the disease. In contrast, the knock-down mutation of the target genes conferred resistance to *M. oryzae*.

### Table 1 Comprehensive list of miRNAs involved in rice immunity against pathogens and herbivores

| Organisms | MicroRNAs | Target genes | Regulation | Pathogens/ herbivores | References |
|-----------|-----------|--------------|------------|-----------------------|------------|
| Fungus    | Osa-miR156-fhl-3p | OsSPL14    | Negative   | M. oryzae             | [23]       |
|           | Osa-miR159a | OsGAMYB     | Positive    | M. oryzae             | [24]       |
|           | Osa-miR160a | ARF16       | Positive    | M. oryzae             | [25]       |
|           | Osa-miR162a | OsDCL1      | Positive    | M. oryzae             | [26]       |
|           | Osa-miR164a | OsNAC60     | Negative    | M. oryzae, R. solani  | [27]       |
|           | Osa-miR166h | EIN2        | Positive    | M. oryzae, F. fujikuroi | [28]       |
|           | Osa-miR167d | ARF12       | Negative    | M. oryzae             | [29]       |
|           | Osa-miR169  | NF-YA       | Negative    | M. oryzae             | [30]       |
|           | Osa-miR319  | OsTCP21     | Negative    | M. oryzae             | [31]       |
|           | Osa-miR396  | OsGRFs      | Negative    | M. oryzae             | [32]       |
|           | Osa-miR398b | SOD         | Positive    | M. oryzae             | [33]       |
|           | Osa-miR439a | Defense-related genes | Negative | M. oryzae             | [34]       |
|           | Osa-miR444  | MADS        | Negative    | M. oryzae             | [35]       |
|           | Osa-miR530  | HDRF-TS     | Negative    | M. oryzae             | [36]       |
|           | Osa-miR1432 | OsEH1       | Negative    | M. oryzae             | [37]       |
|           | Osa-miR1871 | OsMFAP1     | Negative    | M. oryzae             | [38]       |
|           | Osa-miR1873 | LOC, Os05G01790 | Negative | M. oryzae             | [39]       |
|           | Osa-miR7695 | OsNramp6    | Positive    | M. oryzae             | [40]       |
| Bacteria  | Osa-miR156  | OsSPLs      | Negative    | X. oryzae             | [41]       |
|           | Osa-miR159b | OsGAMYB     | Positive    | X. oryzae             | [42]       |
|           | Osa-miR164a | OsNAC60     | Negative    | X. oryzae             | [42]       |
|           | Osa-miR167d-5p | OsWD40-174 | Negative    | X. oryzae             | [42]       |
|           | Osa-miR169a | NF-YA       | Negative    | X. oryzae             | [43]       |
|           | Osa-miR396f | OsGRFs      | Positive    | Dickeya zeae          | [44]       |
| Viruses   | Osa-miR168  | AGO1        | Positive    | RSV                   | [45]       |
|           | Osa-miR171b | OsSCL6-L    | Negative    | RSV                   | [46]       |
|           | Osa-miR319  | OsTCP21     | Negative    | RRSV                  | [47]       |
|           | Osa-miR444  | MADS-box    | Positive    | RSV                   | [48]       |
|           | Osa-miR528  | L-ascorbate oxidase | Negative | RSV                   | [49, 50]  |
| Herbivores| Osa-miR156  | OsMPKs      | Negative    | BPH                   | [51]       |
|           | Osa-miR396  | OsGRF8      | Negative    | BPH                   | [52]       |

OsSPL- SQUAMOSA promoter-binding protein-like transcription factor; ARF- Auxin responsive factor; DCL- Dicer-like, EIN- Ethylene insensitive; NF-YA- Nuclear Factor Y-A; OsTCP- Teosinte branched/Cycloidea/Proliferating cell factor; OsGRF- Growth regulating factors; SOD- Superoxide dismutase, DHFR-TS - Dihydrofolate reductase/thymidylate synthase; OsEH1- EF-hand family protein 1; OsMFAP1- Microfibrillar-associated protein 1; OsNramp- Natural resistance-associated macrophage pathogen, AGO- ARGONAUTE 1; OsSCL- Scarecrow-like; RSV- Rice stripe virus; RRSV- Rice ragged stunt virus; BPH- Brown planthopper
In addition, the Osa-miR159-GAMYB module orchestrates the reproductive developmental process by its direct role in the regulation of flower, pollen, and seed development [24]. It is apparent that Osa-159a must be precisely spatiotemporally regulated to coordinate plant development and immunity. Similarly, leaf and seed development in rice has been shown to be regulated by Osa-miR160 via auxin signaling, which also acts as a positive regulator of rice immunity against M. oryzae. Transgenic plants overexpressing Osa-miR160 displayed significantly stronger resistance to blast disease. The Osa-miR160a enhances resistance to blast by suppressing the AUXIN RESPONSE FACTOR 16 (ARF16), which in turn silences the indole-3-acetic acid (IAA) signaling [25].

Plants produce H$_2$O$_2$ in response to biotic and abiotic stresses. It has been shown that Osa-miR162a fine-tunes the host’s innate immunity against M. oryzae by targeting the Dicer-like 1 (OsDCL1) gene through the accumulation of intracellular H$_2$O$_2$ thereby facilitating cell death at the infection site. This mechanism also regulates other physiological processes that are critical to yield maintenance. Overexpression of Osa-miR162a showed enhanced resistance to M. oryzae by positively regulating many other defense-related genes [26]. Growing evidence suggests that loss-of-function of OsDCL1 showed developmental defects in rice at the seedling stage, including dwarfism, root and shoot abnormality, and wilting of leaves. All of these effects indicate that Osa-miR162a optimizes growth and immunity without any yield penalty. ROS homeostasis is regulated by several key enzymes, including superoxide dismutase (SOD). Consistent with earlier reports, elevated levels of cellular H$_2$O$_2$ has been observed with the suppression of the Cu/Zn superoxide dismutase (SOD) genes by the Osa-miR398b. This process positively regulates host immunity.
against blast by negatively regulating the components of ROS production and homeostasis [25].

Upon *M. oryzae* infection, higher activity of SOD is associated with accumulation of H$_2$O$_2$, which leads to enhanced resistance to blast [33]. ROS is also produced by an excess of iron (Fe), an essential micro-element required for photosynthesis and chloroplast maintenance. This process is critically regulated upon pathogen infection as the host and pathogen compete for available Fe. Recently, *Osa-miR7695* has been shown to function as a positive regulator of rice immune response by mediating the trade-off between defense and iron homeostasis. Rice plants overexpressing *Osa-miR7695* showed increased resistance to *M. oryzae* and stronger Fe accumulation at the site of infection. It was proposed that upon infection with *M. oryzae*, expression of *Osa-miR7695* leads to the suppression of the target gene OsNramp 6 (Natural resistance-associated Macrophage Protein 6), which encodes an iron transporter [40, 60].

**Negative regulation of defenses against rice blast by miRNAs**

The *Osa-miR156f*hl-3p, *Osa-miR164a*, *Osa-miR167d*, *Osa-miR169a*, *Osa-miR319*, *Osa-miR396*, *Osa-miR439a*, *Osa-miR530*, *Osa-miR1432*, and *Osa-miR1873* have been identified as negative regulators of rice innate immunity against *M. oryzae*. These miRNAs have been reported to control rice innate immunity by targeting critical transcription factors. The *miR156* belongs to a conserved family that regulates plant growth, development, and yield by targeting the SQUAMOSA promoter-binding protein-like transcription factor 14 (SPL14) and WRKY45 transcription factor. In rice, overexpression of *Osa-miR156f*hl-3p in a target mimic mutant showed stronger resistance to blast by virtue of enhanced expression of the target genes SPL14 and WRKY45 transcription factors [23]. Expression of the OsNAC60 transcription factor is negatively regulated by the suppression of *Osa-miR164a* upon *M. oryzae* infection, which leads to the enhancement of defense responses [27]. Abolition of the entire *Osa-miR164a/OsNAC60* regulatory module has been shown to result in a susceptible phenotype. The *Osa-miR164a* has also been involved in controlling the sheath blight-causing fungus *Rhizoctonia solani* by activating the salicylic acid (SA) signaling pathway and expression of associated defense-related genes. Expression of *Osa-miR169a* has been shown to condition a strong resistance against *M. oryzae* by suppressing its target gene nuclear factor Y-A (NF-YA). Significant accumulation of *Osa-miR169a* has been documented in a susceptible genotype of rice with a somewhat decreased resistance level. Transgenic rice plants overexpressing *Osa-miR169a* have been shown to exhibit a higher level of susceptibility, which was associated with the down-regulation of target genes and reduced accumulation of intracellular H$_2$O$_2$ [30]. The *Osa-miR530* controls H$_2$O$_2$ production by regulating the expression of dihydrofolate reductase/thymidylate synthase (DHFR-TS). It is noted that DHFR-TS participates in the maintenance of redox balance by producing the nicotinamide adenine dinucleotide phosphate (NADPH) and ROS. Subsequently, ROS is converted into H$_2$O$_2$ by SOD. Perturbation of *Osa-miR530* enhances resistance to blast disease through its effects on H$_2$O$_2$ accumulation. It is important to mention that blocking the *Osa-miR530* also positively affects flowering and seed maturation [36]. The *Osa-miR439a* negatively affects immunity by inhibiting the expression of defense-related genes and H$_2$O$_2$ production Suppression of the *Osa-miR439a* using target mimic mutants has been shown to compromise susceptibility to *M. oryzae* by induction of H$_2$O$_2$ [34].

The *Osa-miR319* modulates host immune response in the rice-*M. oryzae* interaction in a negative manner. Accumulation of *Osa-miR319* has been observed in susceptible genotypes as indicated by the suppression of the target gene OsTCP21 upon *M. oryzae* infection. Blocking the conversion of α-linoleic acid (LnA) to hydroxy-octadecadienoic acid (HPODE) has been shown to inhibit the jasmonic acid (JA) signaling pathway [31]. The *Osa-miR444b* has also been identified among the many negative regulators of rice immunity against *M. oryzae*. Overexpression of *Osa-miR444b* enhances susceptibility to *M. oryzae* but with little impact on its target transcription factors MADS27b and MADS57. Altered expression of the target mimicry of *Osa-miR444b* resulted in enhanced resistance to blast [35]. The *Osa-miR444a* has been shown to positively regulate immunity against the rice stripe virus (RSV) by upregulating OsRDRI expression, which is facilitated by suppressing target MADS-box genes [48].

The *Osa-miR1432* has been shown to fine-tune resistance to disease as well as yield potential by targeting the OsEFH1 (EF-hand family protein 1). Overexpression of *Osa-miR1432* has been shown to compromise resistance to *M. oryzae* with concomitant adverse effects on yield. On the other hand, inhibition of *Osa-miR1432* expression has enhanced resistance with positive effects on yield, attributed to the enhancement of PTI responses [37]. During *M. oryzae* infection, immunity and yield in rice are also balanced by the *Osa-miR1871*. Inhibition of *Osa-miR1871* expression greatly enhances resistance and yield, by virtue of the effects on the target OsMFAP1 (Microfibrillar-associated protein 1) gene. In contrast, overexpression of *Osa-miR1871* had been shown to cause susceptibility to blast, with concomitant negative effects on grain yield [38].
The Osa-miR1873 appeared to regulate resistance to rice blast in a negative manner. It has been shown that expression of Osa-miR1873 leads to disease susceptibility through the suppression of a target gene with an unknown function (i.e., LOC_Os05g01790), which encodes a DUF868 domain-containing protein. Suppression of Osa-miR1873 through the expression of the target gene mimicry mutant has been shown to enhance defense response against blast by fine-tuning host plant immunity and growth [39]. The Osa-miR167d modulates plant developmental and stress responses by targeting auxin response factor (ARF) genes. The Osa-miR167d also seemed to play a negative role in rice immunity against M. oryzae by blocking ARF12 genes. Suppression of Osa-miR167d by overexpressing a target disease mimic mutant led to enhanced resistance to blast [29]. Characterization of Osa-miR396 revealed its negative effect on defense response against M. oryzae by silencing multiple OsGRF genes. Overexpression of Osa-miR396 promotes susceptibility to blast through the downregulation of OsGRFs genes. Suppression of Osa-miR396 by expressing the target disease mimics mutant enhanced resistance to blast with concomitant improvement in yield [32].

MicroRNAs regulating responses to bacterial pathogens

Five classes of miRNAs have been shown to modulate resistance against bacterial blight (BB) caused by Xanthomonas oryzae pv. oryzae (Xoo). Another class of miRNAs has been shown to positively regulate resistance against the bacterial foot rot disease caused by Dickeya zeae. Of the miRNAs involved in responses to BB, the Osa-miR159b has been shown to function as a positive regulator, while the others function as negative regulators. The Osa-miR159b enhances resistance to BB by repressing its target transcription factor OsGAMYB involved in gibberellic acid (GA) signaling. This process leads to negative effects on nitrogen assimilation as an outcome of the repression of the GA signaling pathway [42].

The Osa-miR167 has also been shown to be involved in rice immunity to bacterial pathogens [57, 61]. For instance, Osa-miR167d abates immunity upon Xanthomonas oryzae pv. oryzae (Xoo) infection by suppressing the target gene OsWD40–17A, which downregulates the lignin biosynthetic gene OsKNOX during leaf development [42]. Downregulation of KNOX hinders lignin biosynthesis [62]. Transgenic plants overexpressing Osa-miR164a showed enhanced susceptibility to Xoo by virtue of the downregulation of the OsNAC60 transcription factor [42]. The role of the miR164a/OsNAC60 regulatory module in blast resistance has been discussed earlier in this review. The Osa-miR169o represents the coordinated crosstalk regulation between BB and Nitrogen-use efficiency (NUE) in rice. Overexpression of the miR169o enhances NUE and promotes susceptibility to BB by suppressing the nuclear factor Y-A (NF-YA), which in turn causes significant downregulation of several defense-related genes, including PR10b, PR1b, PR10a, and PAL [43]. The Osa-miR156 negatively regulates immunity against bacterial blight [41]. Transgenic plants overexpressing the target gene IPA1 (Ideal Plant Architecture1) under the control of a pathogen-inducible promoter of OsHEN1 have been shown to enhance both disease resistance and yield.

More recently, Osa-miR396f was shown to play a positive role in conferring resistance against bacterial foot rot disease caused by Dickeya zeae. Overexpression of Osa-miR396f in the susceptible rice variety Nipponbare showed enhanced immunity to D. zeae by suppressing the target gene OsGRFs (Growth-Regulating Factors). However, the precise molecular mechanism underpinning the immunity against bacterial foot roots mounted by OsGRFs is still unknown [44].

MicroRNAs control resistance to viral pathogens

The miRNA-mediated gene regulation has emerged as a novel strategy to enhance antiviral defenses in crop plants, including rice [63]. Plants have evolved different RNA silencing mechanisms to respond to diverse classes of viral infections. Antiviral RNA silencing is triggered by virus-derived double-stranded RNA (dsRNA) that are directly recognized and processed by the host’s dicer-like proteins (DCL) to form virus-derived small-interfering RNAs (vsiRNAs) [64]. The vsiRNAs are incorporated into the ARGONAUTE (AGO) proteins that form the core component of the RNA-induced silencing complex (RISC). The functional RISC either cleaves the viral RNA or arrests the viral protein translation. Single-stranded viral RNAs require endogenous RNA-dependent RNA polymerases (RDRs) to synthesize dsRNA that serves as the substrate for DCLs to produce secondary vsiRNAs. To counteract the defense response of host plants, most viruses have developed specialized proteins known as viral suppressors of RNA silencing (VSRs) that impede the antiviral RNA silencing pathway and suppress the defense response [65].

MicroRNAs provide an additional layer of intrinsic defense against viral attacks. In rice, the Osa-miR528 is well characterized and known to be involved in regulating defenses against the rice stripe virus (RSV). In cleavage-defective AGO18 mutants of rice, the Osa-miR528 is sequestered away from AGO1, upon RSV infection. These, in turn, prevent the formation of a functional RNA-induced silencing complex. These events further lead to the enhanced expression of downstream gene AO, encoding an L-ascorbate oxidase and functions in the
initiation of defense response against RSV through the accumulation of ROS [49]. Further research suggested that the transcription factor OsSPL9 specifically regulates the expression of the Osa-miR528-AO module by binding to the promoter of the Osa-miR528. Mutation of OsSPL9 has been shown to cause the dramatic downregulation of Osa-miR528, thereby inducing AO expression that leads to enhanced resistance to RSV [50].

The AGO18 also sequesters the Osa-miR168 upon RSV infection. It has been shown that AGO1 competes with AGO1 for binding to Osa-miR168, resulting in an elevated level of AGO1-mediated resistance [45]. RSV infection triggers the expression of Osa-miR444, which positively regulates immune response. Transgenic plants overexpressing the Osa-miR444 displayed a broad-spectrum resistance to RSV by inducing OsRDR1 and by silencing several MADS-box transcription factors [48].

RSV infection of host rice plants perturbs the expression of Osa-miR171. Such impaired expression has been shown to severely affect plant height and chlorophyll content, thereby causing RSV-like symptoms. It has been demonstrated that plants expressing miR171b were less susceptible to RSV characterized by attenuated RSV symptoms [46]. Similarly, the Osa-miR319 acts as a negative regulator of immunity against the ragged stunt virus (RRSV) in rice. Transgenic plants overexpressing the Osa-miR319 have exhibited severe disease-like symptoms by negatively affecting the target gene Osa-miR168 using a target mimic mutant that competed with AGO1 for binding to Osa-miR168, resulting in an elevated level of AGO1-mediated resistance [45].

**MicroRNA-mediated regulation of R-genes: lessons from rice and other models**

Modern cultivars are being developed by pyramiding multiple R-genes as well as other types of genes involved in quantitative mechanisms as a major strategy to avoid substantial losses to crop yield due to diseases and pests. However, it has been shown that constitutive expression of R-genes often imposes high fitness costs, mainly with deleterious consequences to plant growth and development. Therefore, the expression of R-genes must be optimized in the right spatial and temporal manner to minimize trade-offs.

Emerging evidence suggests that miRNAs and secondary siRNA play important roles in the regulation of R-gene function by silencing the immune-response receptors when the host plant is not under attack by the pathogen. This mechanism is important in stabilizing basal transcript levels to limit the fitness costs of an overactive immune response. Plants have evolved specific miRNAs that can target the conserved domains of the R-gene in two major pathways, either by direct targeting of R-gene or indirect targeting of R-gene via phasiRNA (Fig. 3). In the pathway for direct targeting of R-genes, a mature miRNA produced from MIR loci interacts with AGO1 protein. The AGO1-miRNA complex binds and cleaves the R-gene transcripts in a sequence-specific manner and prevents the R-gene mediated autoimmunity in the absence of the pathogen.

The miR472 was the first to be involved in the direct targeting of the CC-NBS-LRRs domain-containing immune receptor genes in Arabidopsis [68]. Since its discovery, many other miRNAs involved in R-gene regulation that optimize the defense-fitness trade-off have been reported. For example, the sequence-specific cleavage of the TIR-NB-LRR immune receptor of N gene transcripts that conditions resistance to tobacco mosaic virus (TMV) has been shown to be mediated by nta-miR6019 and nta-miR6020 modules in the Solanaceae family [69]. The miR482/2118 super-family targeting NB-LRR genes.
have also been well characterized in tomato, and its prime importance in the regulation of immune response is well documented [70]. A recent report suggested that the miR1885 is involved in a dynamic balance between plant growth and immunity to viral pathogen in *Brassica* by direct silencing the R-gene [71]. More recently, in rice, Osa-miR1876 has been shown to epigenetically regulate the expression of the NBS8R gene encoding an NB-ARC domain protein that confers resistance to *X. oryzae* [72].

Besides the direct targeting of NLRs, miRNAs have also been shown to indirectly regulate R-genes by targeting other genes that are part of the R-gene networks [73]. For example, the NBS-LRR genes, which are not primarily recognized by miRNA, trigger the production of a phased array (in a sequential, head-to-tail manner, according to the miRNA cleavage site) of 21-nt secondary small interfering RNAs (phasiRNAs) to amplify the silencing effects [74]. These phasiRNAs act in trans to silence the addition of R-gene transcripts. In rice, no phasiRNA that regulates R-genes has so far been characterized. However, phasiRNAs regulating reproductive development has been reported.

The phasiRNA targeting R-genes remains unknown in rice. The phasiRNA that targets the conserved motifs of CC-NBS-LRR has been explored in diverse plant species, including spruce, grapevine, poplar, cotton, *Arabidopsis*, and citrus [75, 76]. General observations indicate that the highly conserved siRNAs may be important in optimizing the expression of NBS-LRR genes, which may compromise plant fitness. However, the precise role of phasiRNA in regulating NBS-LRR immune receptors in rice is yet to be fully understood [77]. Furthermore, Fine-tuning of NBS-LRR protein expression by phasiRNA inhibits the constitutive expression of many other
R-genes, which could potentially be detrimental to plant growth, leading to trade-offs to productivity when not regulated optimally. In the model legume *Medicago truncatula*, 22-nt miRNAs including miR2275, miR2109, and miR2118 have been shown to trigger the production of phasiRNA that are specifically associated with the regulation of other genes encoding NBS-LRR immune receptors [77, 78]. These miRNAs are highly conserved in both legumes and non-legume plant species.

**MicroRNAs fine-tune immunity and trade-offs to yield**

High-yielding rice cultivars with resistance to multiple pathogens and pests are paramount to sustainable production. With major accomplishments by breeding, the caveat appeared to be that strong defense responses often come with unintended trade-offs in terms of significant losses to yield as the photosynthetic source-sink dynamics that should favor plant growth tend to be diverted towards defense-related processes [13]. Modern approaches in plant breeding such as marker-assisted selection, transgenics, and genome-editing are being applied to develop resistant cultivars with the minimal penalty to yield. In the past few decades, many R-genes in rice have been tagged with molecular markers for efficient selection and use in pyramiding. However, a limited set of genes are deployed in crop improvement programs since many of them exhibit pleiotropic effects, unwanted linkage drags that undermine agronomic traits, and low heritability [16]. Furthermore, because of the rapid breakdown of R-gene-mediated mechanisms along with their associated fitness costs, the potential use of miRNAs with major roles in regulating the immunity of host rice plants has gained more interest as a more effective means to develop resistant cultivars with optimal balance between resistance and trade-offs to plant vegetative and reproductive growth.

Recent reports have shown that many miRNAs facilitate the maintenance of fitness and resistance with minimal or no penalty to yield. General observations indicate that miRNAs enhance the immunity to pathogens and herbivores with significant positive effects on yield maintenance in most cases. For example, the *Osa-miR159, Osa-miR162, Osa-miR396, Osa-miR530, Osa-miR1432*, and *Osa-1871* have been shown to have positive effects on yield component traits even when the plant is challenged by pathogens [24, 26, 32, 36–38]. For instance, the miRNA, *Osa-miR156*, fine-tunes resistance without significant penalty to yield, while *Osa-miR1873* confers resistance with only a minor penalty to yield [23, 39].

The *Osa-miR162* synergizes the mechanisms involved in resistance to blast with the genetic potential for yield through the *OsDCL1* that causes the accumulation of intracellular H$_2$O$_2$. Nevertheless, overexpression of *Osa-miR162* showed significantly narrower grains, lower seed weight, and poor seed set, leading to a significant penalty to grain yield. Silencing of *Osa-miR162* showed positive effects on yield by increasing the number of grains per panicle during *M. oryzae* infection [26].

Similarly, blocking the expression of *Osa-miR530* induces early flowering and seed maturation, with positive effects on grain number per panicle and grain weight. The underlying regulatory networks that balance yield-related processes and immunity is an area that is currently under investigation [36].

Likewise, downregulation of *Osa-miR1432* enhances both immunity and yield by targeting the *OsEFH1* protein [37]. The resistance and yield trade-off are also fine-tuned by the *miR1871-MFAP1* module. Transgenic rice overexpressing *Osa-miR1871* as well as the *mfap1* mutants exhibit significant reductions in grain yield. Conversely, downregulation of *Osa-miR1871* displayed the opposite effects. These results suggested that *Osa-miR1871* regulates yield-related traits and immunity through the function of MFAP1 [38]. Suppression of *Osa-miR396* induces multiple growth-regulating factor (GRF) genes that enhance resistance to blast and BPH [32, 52]. The *Osa-miR396* also plays important roles in the regulation of cellular processes that are critical in the maintenance of inherent potentials for grain size, grain yield, inflorescence development, panicle branching, as well as tolerance to saline and alkaline soil and water [79–81].

The *Osa-miR156* negatively regulates host immunity against blast, bacterial blight, and BPH by targeting the *IPA1* (Ideal Plant Architecture 1), *OsSPL14* (SQUAMOSA Promoter-binding protein-like transcription factors), and several *OsWRKY* genes. Sequestering *Osa-miR156* by target mimicry led to enhanced resistance to all three biotic stressors by affecting the accumulation of target transcripts, including the products of defense-related genes [23, 41, 51]. However, overexpression of modified *OsSPL14* promotes the panicle branching, which leads to improvements in the number of spikelets per panicle, stronger culm, and tiller reduction. These effects contributed to a significant enhancement of grain yield [82, 83].

Transgenic studies showed that inducible expression of the *IPA1* gene under the control of bacterial effector-induced promoter leads to enhanced resistance to BB, with concomitant improvement in grain size, plant architecture, and grain yield [41]. The *Osa-miR1873* has been shown to balance the cellular processes involved in the expression of resistance to blast and maintenance of plant growth. Overexpression of *Osa-miR1873* has been shown to compromise resistance with additional negative effects to plant growth and developments as manifested by a significant reduction in yield potential due to the reduction of seed-set as indicated by the proportion of
filled grains per panicle. In contrast, studies that seques-
tered Osa-miR1873 showed that proper regulation causes no si-
gnificant effects on yield [39]. Other miRNAs such as Osa-miR159, Osa-miR160, Osa-miR164, Osa-miR167, and Osa-miR398 have been shown to affect different traits that are a critical component of yield potential, independently.

MicroRNAs coordinate immune response with other cellular processes that determine agronomic potential

MicroRNAs rarely work independently. Single miRNA-encoding loci often function in the regulation of multiple cellular processes, hence more than one trait. Recent studies in rice showed that the role of miRNAs is not limited to the regulation of defense response mechanisms, but they are also involved in the regulation of complex traits (Table 2). For example, the Osa-miR156 is known to regulate a total of eleven (11) SPL genes involved in diverse biological and developmental processes in rice. The Ideal Plant Architecture 1, which encodes a SQUAMOSA Promoter-binding protein-like transcription factors is known to be targeted by Osa-miR156. Over-
expression of OsSPL14 enhances resistance to bacterial blight accompanied by a substantial improvement in yield caused by the reduction in the number of unproductive tillers and enhancement of panicle branching [41]. Mutation in OsSPL14 perturbs Osa-miR156-mediated regulation of OsSPL14, leading to positive gains in yield maintenance as indicated by the reduction of unproductive tillers and stronger culm [82]. Furthermore, overexpression of OsSPL14 also enhanced the number of grains per panicle by increasing the panicle branching [83].

It was recently shown that downregulation of Osa-
miR156fhl-3p enhances host plant immunity to M. ory-
zae by positively affecting OsSPL14 and WRKY45 [23]. Furthermore, recent reports also showed that Panicle blast 1 (Pb1), a panicle blast resistance gene encoding a coiled-coil, nucleotide-binding site, leucine-rich repeat (CC-NBS-LRR) protein, interacts with a WRKY45 transcription factor, which plays a critical role in the expression of induced resistance through the salicylic acid signaling pathway regulated by the ubiquitin-proteasome system [121]. Evidence also supports that suppression of Osa-miR156 could promote seed dormancy by inducing the expression of OsSPL14 and repressing the GA pathway [84]. Expression of OsSPL13 promotes yield in rice by improving grain size and panicle length [122]. The OsSPL16 improves grain size by binding to GW7 [85, 123]. The OsSPL18 binds to the promoter of DEP1 and negatively regulates the cellular process for grain number potential [124, 125]. Overexpression of OsSPL7 negatively affects tiller number and positively affects plant height [86]. In rice, the OsSPL9 is involved in the regulation of Osa-miR528, which promotes flowering under long-day conditions by repressing the Red and Far-red Insensitive 2 (OsRFI2) gene [119]. The OsSPL9 regulates the Osa-miR528/L-Ascorbate Oxidase (AO) transcriptional module, which enhances anti-viral defenses [50]. Moreover, Osa-miR528 regulates pollen intine formation by targeting the uclacyanin gene OsUCL23 [120]. Likewise, in creeping bentgrass, it has been shown that constitutive expression of Osa-miR528 enhances tolerance to salinity and nitrogen-starvation [126].

| miRNAs     | Biotic stresses | Traits                                                                 | References           |
|------------|-----------------|------------------------------------------------------------------------|----------------------|
| Osa-miR156 | Blast, BB, BPH  | Grain size, grain yield, grain quality, panicle branching, Seed germination, tillering, plant architecture | [82–88]              |
| Osa-miR159 | Blast, BB       | Floral development, stem elongation, leaf development, grain size       | [89–91]              |
| Osa-miR160 | Blast           | Rice growth and development, tillering, seed setting rate              | [92, 93]             |
| Osa-miR162 | Blast           | Drought tolerance, plant development                                    | [94, 95]             |
| Osa-miR164 | Blast, BB       | Drought tolerance, plant architecture, grain yield                     | [96–98]              |
| Osa-miR166 | Blast           | Nutrient ion uptake, drought tolerance, cadmium tolerance              | [99, 100]            |
| Osa-miR167 | Blast, BB       | Auxin response, tiller number, grain weight                            | [101–103]            |
| Osa-miR169 | Blast, BB       | Nitrogen-use efficiency, salt stress                                   | [43, 104]            |
| Osa-miR319 | Blast, RRSV     | Leaf morphogenesis, cold tolerance, plant height                       | [105–107]            |
| Osa-miR396 | Blast, BPH, Foot rot | Grain size, grain yield, floral development, stem elongation, salt, and alkali tolerance | [79, 80, 87, 108–112] |
| Osa-miR398 | Blast           | Panicle length, grain number, grain size, abiotic stress               | [88, 113]            |
| Osa-miR444 | Blast, RSV      | Tilling, nitrate signalling, root development                          | [114–116]            |
| Osa-miR528 | RSV             | Flowering time, pollen development, arsenite tolerance, cold tolerance | [117–120]            |

BB Bacterial blight, BPH Brown planthopper, RRSV Rice ragged stunt virus, RSV Rice stripe virus
Hormones are the foundations of cellular signaling that integrate growth-related and defense-related responses [11]. In rice, genes involved in auxin signaling particularly the auxin response factors (ARFs) are known to be targeted by Osa-miR160 and Osa-miR167. The Osa-miR160 is known to positively regulate blast resistance, whereas Osa-miR167 acts in an antagonistic fashion. Furthermore, alteration in auxin signaling in rice through the upregulation of Osa-miR160-resistant OsARF18 has been shown to cause severe defects in overall plant growth as well as reproductive development, with negative effects on seed size and seed set [92, 93]. The Osa-miR167 promotes an efficient response to auxin, which translates to the concomitant enhancement of tiller number and grain weight [101–103]. The growth regulating factors (GRFs) targeted by Osa-miR396 regulates diverse biological processes. It has been shown that manipulating the Osa-miR396-OsGRFs module substantially increases grain size and yield. Specifically, mutation of the Osa-miR396 (GS2) perturbs the function of Osa-miR396 and its regulatory role over OsGRF4 [79, 87, 108]. Additionally, Osa-miR396 promotes panicle branching by regulating OsGRF6 [80].

Several immune-responsive miRNAs play a critical role in regulating cellular processes that determine nutrient uptake as well as responses to other types of abiotic stresses. For example, the Osa-miR164, which is important in processes that control grain yield and other plant architecture traits in rice, has been shown to affect drought tolerance [96, 97]. The Osa-miR166 is involved in the regulation of cellular processes determining nutrient and ion uptake, drought tolerance, and cadmium tolerance [99, 100, 127]. The pathogen-responsive Osa-miR169 has been shown to regulate nitrogen uptake and salinity tolerance in rice [43, 104]. The Osa-miR319 regulates leaf morphogenesis, plant height, and improves cold tolerance [105–107]. The Osa-miR398 not only modulates panicle length, grain number, and grain size but also regulates various abiotic stresses [88, 113]. The Osa-miR444 modulates MADS-box transcription factors to regulate tillering and nitrate signaling [114–116]. The RSV-responsive Osa-miR528 regulates tolerance to arsenite and low temperature in rice [117–120].

**Manipulation of miRNAs as a novel approach for the development of high-yielding disease and insect-resistant rice cultivars**

Knock-out mutation of miRNAs is an important approach to validating cellular functions through the identification of their downstream target genes. Furthermore, miRNAs belong to conserved families comprised of multiple members with potentially redundant functions. Therefore, loss-of-function analysis of miRNA-encoding loci has become a major challenge. Traditional methods of mutagenesis (chemical/radiation/T-DNA insertion) are largely ineffective due to the small size of miRNA molecules. Currently, target mimics (TMs), short tandem target mimics (STTMs), molecular sponges (SPs), and artificial miRNAs (amiRNAs) are the most commonly used techniques for loss-of-function analysis of miRNAs. Among them, the miRNA decoy techniques such as TMs and STTMs have been widely adopted for functional analysis [128]. TMs and STTMs efficiently suppress the endogenous activity of highly abundant miRNA molecules and nullify the suppression effects to the target gene. This in turn changes the phenotype through the accumulation of target transcripts. However, neither of these approaches provides the most efficient silencing effects to the endogenous activity of the cognate miRNA genes.

** Genome editing technologies such as zinc finger nuclease (ZFN), transcription activator-like effector nucleases (TALEN), and clustered regularly interspaced short palindromic repeats (CRISPR)-based system are the recent cutting-edge approaches for the control of endogenous miRNA abundance [129]. These strategies work based on the principle of binding an exonuclease to a target region in the genome, creating a double-stranded break (DSB). To stabilize the genome, these breaks are repaired by one of two methods: Non-Homologous End-Joining (NHEJ) and Homology Directed Repair (HDR), which causes insertions and deletions (indels) or incorporation of a larger sequence at the repair site. When these changes occur within the miRNA coding region, they can reduce the rate of miRNA biogenesis leading to incomplete loss of function or production of new miRNAs. In contrast, large deletions can cause a complete loss of function of specific miRNAs by producing null mutants. Editing the target gene also hinders miRNA activity due to errors in binding.

Zinc-finger nucleases (ZFNs) are artificial restriction enzymes generated by fusing zinc-finger-based DNA-recognition modules with the DNA-cleavage domain. Each zinc finger typically recognizes and binds to a nucleotide triplet, and fingers are often assembled into groups to bind to specific DNA sequences. TALENS is equipped with the same tenet as ZFN with a target specificity from the protein-DNA association. In addition, it can recognize a single nucleotide using specific amino acid repeats to recognize a single nucleotide, thus providing more choices for selecting the target locus. CRISPR-mediated genome editing has revolutionized nuclease targeting [130–133]. In the CRISPR-Cas system, a single guide RNA (sgRNA) or double guide-RNA (dgRNAs) recognizes the target sequence, and the double-strand break caused by Cas9 (CRISPR associated protein 9) or
Cpf1 (CRISPR from Prevotella and Francisella 1) nuclease, induces insertion or deletion through the process of DNA repair. The CRISPR-based system has higher efficiency and efficiency than the other methods.

Recently, CRISPR/Cas9 was efficiently used in rice to introduce heritable mutations in mature miRNAs [134–136]. Knockout mutations in Osa-miR396e and Osa-miR396f using the CRISPR/Cas9 system have been shown to create genetic gains through the enhancing effects to yield component traits such as grain size and panicle branching, particularly under nitrogen deficit conditions [81]. CRISPR/Cas9-mediated mutagenesis of Osa-miR396f has been shown to promote the efficiency of GA signaling, with concomitant enhancement in grain yield contributed by improvements in grain size, leaf blade and sheath anatomy and morphology [112]. Targeted mutagenesis of a single miRNA locus (e.g., Osa-miR408, Osa-miR528) or entire miRNA gene families (e.g., Osa-miR8815, Osa-miR820) has been achieved by a CRISPR/Cas9 mediated strategy in rice. It has been shown that under salinity stress, larger deletion in Osa-miR528 led to elevated transcript levels from the target genes [134]. Similarly, targeted mutagenesis of OsSPL9 using CRISPR/Cas9 led to a significant reduction in Osa-miR528 expression, suggesting a critical role of OsSPL9 in transcriptional regulation of Osa-miR528 [119].

Furthermore, a series of CRISPR/Cas9-mediated mutations in Osa-miR156 have been investigated for their effects on seed dormancy [84]. CRISPR/Cas9 system has also been employed to introduce mutations in multiple superoxide dismutase (SOD) genes to examine their effects on Osa-miR398-mediated resistance to M. oryzae [33]. Targeted mutations were introduced in the target gene (OsARF12) of Osa-miR167d using the CRISPR/Cas9 method to investigate Osa-miR167-ARF12 interaction in immunity against rice blast disease [29].

Conclusion and future directions

Continuously emerging evidence from various experimental systems and approaches established the essentiality of miRNA-mediated regulatory mechanisms in integrating defense-related responses to fungal, bacterial, and viral pathogens, and insect herbivores, with growth and development-related responses. MicroRNA regulatory mechanisms have direct roles in host plant immunity by directly suppressing target genes with either negative or positive effects, or by indirectly inducing signaling pathways with different classes of phytohormones and other small molecules such as ROS. Genomics-enabled biology offers an excellent opportunity to identify more miRNAs involved in fine-tuning resistance to biotic stresses to minimize trade-offs to productivity. Harnessing these miRNAs could be useful in breeding disease and pest-resistant rice cultivars without the drags to the agronomic potential that are typically observed when manipulating R-genes alone.

Identifying new miRNAs associated with defense mechanisms against pests and diseases could be achieved by employing high-throughput sequencing technologies. Specifically, this approach involves the identification of differentially expressed miRNAs under stress via miRNA-Seq followed by in silico analysis and degradome sequencing to predict miRNA targets. Once the specific roles of the miRNAs and target genes are identified, it would be possible to engineer them through genome-editing technologies. Site-specific mutagenesis of target genes impairs the cleavage by miRNA, which allows the development of transgenic plants with disease and insect resistance. In addition to functional analysis, miRNAs are usually conserved in nature, hence limited sequence diversity. The miRNA binds to their target genes in a sequence-specific manner. Variation in miRNAs leads to a variable level of transcript accumulation that causes compounded phenotypes.

Abbreviations

PAMPs: Pathogen-associated molecular patterns; DAMPs: Damage-associated molecular patterns; PRRs: Pattern-recognition receptors; PTI: PAMP-triggered immunity; ETS: Effector-triggered susceptibility; ETI: Effector-triggered immunity; HR: Hypersensitive response; DCL1: DICER-like 1; ROS: Reactive oxygen species; STTM: Short tandem target mimic; ARF: Auxin response factor; IAA: Indole-3-acetic acid; EIN2: Ethylene insensitive 2; SOD: Superoxide dismutase; Nramp 6: Natural resistance-associated macrophage protein 6; SPL: SQUAMOSA promoter-binding protein-like transcription factor; NFD: Nuclear factor Y-A; DHFR-TS: Dihydrofolate reductase/thymidylate synthase; HPODE: Hydroperoxy-octadecadienoic acid; GRFs: Growth-regulating factors; TCP: TEOSINTE BRANCHED, cycloidea and PCF; ZFN: Zinc finger nuclease; TALEN: Transcription activator-like effector nuclease; CRISPR: Clustered regularly interspersed short palindromic repeats; NHEJ: Non-homologous end-joining; HDR: Homology-directed repair; R-genes: Resistance genes; AGO1: Argonaute 1; SG3: Suppressor of gene silencing 3; RDR6: RNA-dependent RNA polymerase 6; RSV: Rice stripe virus; RRSV: Rice ragged stunt virus.

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Authors’ contributions

BGDR and KN conceived the idea and content of this manuscript. KK, SNM drafted the manuscript. KN reviewed and proofread the manuscript. BGDR wrote, reviewed the manuscript and provided the intellectual direction for improving the manuscript. All the authors read and approved this manuscript.

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