The plant epitranscriptome: revisiting pseudouridine and 2′-O-methyl RNA modifications

Muthusamy Ramakrishnan1,2,†, K. Shanmugha Rajan3,4,†, Sileesh Mullasseri5, Sarin Palakkal6, Krishnan Kalpana7, Anket Sharma8, Mingbing Zhou8,9, Kunnummal Kurungara Vinod10, Subbiah Ramasamy11,∗ and Qiang Wei1,2,∗

1Co-Innovation Center for Sustainable Forestry in Southern China, Nanjing Forestry University, Nanjing, Jiangsu, China
2Bamboo Research Institute, Nanjing Forestry University, Nanjing, Jiangsu, China
3The Mina and Everard Goodman Faculty of Life Sciences and Advanced Materials and Nanotechnology Institute, Bar-Ilan University, 52900, Ramat-Gan, Israel
4Department of Chemical and Structural Biology, Weizmann Institute, 7610001, Rehovot, Israel
5School of Ocean Science and Technology, Kerala University of Fisheries and Ocean Studies, Cochin, India
6The Institute for Drug Research, School of Pharmacy, The Hebrew University of Jerusalem, Jerusalem, Israel
7Department of Plant Pathology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, 625 104, Madurai, Tamil Nadu, India
8State Key Laboratory of Subtropical Silviculture, Zhejiang A&F University, Hangzhou, Zhejiang, China
9Zhejiang Provincial Collaborative Innovation Center for Bamboo Resources and High-Efficiency Utilization, Zhejiang A&F University, Hangzhou, Zhejiang, China
10Division of Genetics, ICAR – Indian Agricultural Research Institute, New Delhi, India
11Cardiac Metabolic Disease Laboratory, Department of Biochemistry, School of Biological Sciences, Madurai Kamaraj University, Madurai, Tamil Nadu, India

Introduction

Agricultural production worldwide is facing an unprecedented challenge, as economic growth and population dynamics play a key role in increasing agricultural demand. According to the latest United Nations report, the world population is expected to reach 9.7 billion in 2050 (World Population Prospects, 2019), which could increase agricultural demand by 50% compared to 2013 (FAO, 2017). Regulation of RNA expression is an important mechanism for plant growth and development of plants, especially when plants are exposed to severe environmental stress (Yaish et al., 2017). Many coordinated epigenetic events on nucleosomes alter RNA expression via DNA methylation, post-translational histone modifications (PTMs), and small RNA interference (Baulcombe and Dean, 2014; Thiebaut et al., 2019). Recent evidence supports the notion that RNA modifications are dynamic and serve as key switches for RNA translation efficiency, splicing, and decay (Kadumuri and Janga, 2018). In recent years, post-transcriptional RNA modifications have emerged as an important regulatory network, similar to DNA methylation and PTMs, that help organisms fine-tune gene regulation during developmental responses and environmental stresses (Meyer and Jaffrey, 2014; Saletore et al., 2012). Similar to epigenomics, epitranscriptomics describes RNA modifications (Motorin and Helm, 2021).

Although post-transcriptional modifications of RNA were reported half a century ago, it is only in the last two to three decades that we have begun to better understand the extent of these modifications and their purpose. RNA modification is part of a hierarchy of regulatory processes that help an organism adapt to environmental exigencies. Unlike animals, plants cannot move to a more favourable environment, but have a broader repertoire of regulation through genome modification, as shown by the higher activities of transposable elements (TEs) and DNA methylation in plants (Ramakrishnan et al., 2021; Zhang et al., 2018). Although RNA modifications have attracted considerable attention in cancer and other human diseases, a more comprehensive understanding of the diverse functions of RNA
modifications can be gained by studying plants. In this review, we therefore focus on RNA modifications in plants and use results from animal studies to fill gaps or highlight contrasts.

In eukaryotes, the regulatory role of RNA modifications in development and gene expression has been highlighted by the recent identification of N6-methyladenosine (m6A) (Domissini et al., 2012; Meyer et al., 2012), pseudouridine (Ψ) (Carliet et al., 2014; Li et al., 2015; Lovejoy et al., 2014; Safrà et al., 2017a; Schwartz et al., 2014), 2′-O-methylation (Nm) (Tollervey and Kiss, 1997), N6-acyethylcytidine (acC) (Arango et al., 2018; Sas-Chen et al., 2020), N6, 2′-O-dimethyladenosine (m6Am) (Mauer et al., 2017), N7-methyladenosine (m7G) (Domissini et al., 2016; Grozhik et al., 2019; Li et al., 2016, 2017; Safrà et al., 2017b), 5′-hydroxymethylcytosine (hmC5), 5-methylcytosine (mC5), 8-OHG, 8-NO2-G (Chmielowska-Bąk et al., 2019), and other sparse modifications, such as N7-methylguanosine (m7G) (Zhang et al., 2019b) and 2′-3′-cyclic phosphate (cp) (Shigematsu et al., 2019), which are also present in mRNA (Linder and Jaffrey, 2019).

However, only the roles of m6A and mC5 in almost all aspects of RNA metabolism and physiological functions are better documented, although several other RNA modifications are known in plants (Chmielowska-Bąk et al., 2019; Shen et al., 2019). Recent studies in the model plant Arabidopsis and in agricultural crops such as rice, maize, clover, foxtail millet, and potato have demonstrated the importance of these RNA modifications (especially m6A and mC5) for several vital developmental processes such as embryonic development, leaf initiation, shoot stem cell fate, trichome morphogenesis, flowering, root development, photomorphogenesis, and nitrogen utilization (Arribas-Hernández et al., 2018; Cui et al., 2017; Duan et al., 2017; Hou et al., 2021; Martínez-Pérez et al., 2017; Ruzicka et al., 2017; Scutenaire et al., 2018; Wang et al., 2022; Zhang et al., 2019a; Zuber et al., 2016).

The field of epitranscriptomics has been reviewed by many researchers from various aspects (Hu et al., 2019; Liang et al., 2020; Shen et al., 2019), but the functional significance of these modifications in a site-specific manner is still not well summarized. This lack of knowledge hinders the application of epitranscriptomics in crop improvement. Because RNA modification in plants is a potential strategy for improving crop productivity and addressing the challenge of meeting the nutritional needs of the growing world population, this focus on plants is warranted. Therefore, in this review, we focus on the importance of two abundant RNA modifications, pseudouridine (Ψ) and 2′-O-methylation (Nm), in plants, their biological significance, and the available methods for qualitative and quantitative detection of these modifications.

RNA modifications

Post-transcriptional RNA modification was first observed in the 1950s when pseudouridine (Ψ) was identified in tRNA. At that time, pseudouridine was considered a “fifth nucleotide” (Cohn, 1959, 1960). Later, in the 1970s, the formation of 7- methylguanosine (m7G) in the process of mRNA capping and the methylation of adenosine in N6 position (N6-methyladenosine (m6A)) were detected (Desrosiers et al., 1974; Dubin and Taylor, 1975; Perry and Kelley, 1974; Shatkin, 1976). Methylation in the base and ribose of mRNA was first detected as early as 1974. However, progress in the field of RNA modifications has long been hampered by the lack of appropriate technology. In the last decade, the development of new technologies, such as next-generation sequencing (e.g. Illumina), third-generation sequencing (e.g. Nanopore sequencing and PacBio), and other bioinformatics tools, has greatly improved this field of research. To date, more than 172 unique RNA modifications have been discovered in all kingdoms of life. Most of these RNA modifications occur in mRNA and non-coding RNAs (ncRNAs), such as ribosomal RNA (rRNA), small nuclear RNA (snRNA), and transfer RNA (tRNA). Modified ribonucleotides detected in all types of RNA molecules are deposited in the MODOMICS database (http://genesiilco/modomics/), which provides complete information on modified ribonucleosides (Boccaletto et al., 2021). Despite the discovery of such a large number of RNA modifications, the functions of most of these modified nucleotides are still a mystery.

m6A and m5C RNA modifications in plants

In 2012, the foundation for epitranscriptomics in eukaryotic mRNA was laid with the development of a method for mapping N6-methyladenosine (m6A) (Burgess et al., 2016; Saletore et al., 2012). m6A is the most abundant internal mRNA modification in mammalian and plant cells. The mRNA of almost all plant species studied to date, such as maize, wheat, oats, rice, Populus trichocarpa, and Arabidopsis, carries m6A modifications (Gao et al., 2021; Haugland and Cline, 1980; Kennedy and Lane, 1979; Li et al., 2014; Nichols, 1979; Zhong et al., 2008). Each mRNA molecule carries ~2.2 methyl groups per 1000 nucleotides and consists predominantly of m6A, whereas RNA nucleotides consist of a complex base and sugar methylation profile (Desrosiers et al., 1974; Perry and Kelley, 1974). In flowering plants, m6A is not only the most abundant but also the best-studied mRNA modification (Arribas-Hernández and Brodersen, 2019). m6A has been detected in >5000 mRNA species, enriched not only in 3′-UTRs and stop codons but also in start codons. On average, each mRNA transcript carries ~1–3 m6A modifications (Zaccara et al., 2019).

The m6A motif, RRACH (R = A,G, H = A,U,C), is conserved in yeast, mammals, and plants (Luo et al., 2014). m6A plays a critical role in plant growth, such as organ definition, apical dominance (Bodi et al., 2012), trichome branching (Wei et al., 2018), leaf morphology, root development (Arribas-Hernández et al., 2018), seed development (Shen and Yu, 2021), seed germination (Tan et al., 2021), flower transition (Duan et al., 2017), vascular structure, embryonic development (Ruzicka et al., 2017), fruit ripening (Zhou et al., 2019, 2021), stem cell fate differentiation (Shen et al., 2016), photomorphogenesis and flowering (Wang et al., 2022), nutrient utilization (Hou et al., 2021), and stress development (Anderson et al., 2018; summarized in Figure 1). m6A is also involved in maintaining circadian and seasonal rhythms in plants (Parker et al., 2019). Thus, this type of RNA modification varies from tissue to tissue. For example, the mRNA m6A levels in different Arabidopsis tissues can vary from 0.4% to 1.5% (m6A/A ratio; Zhong et al., 2008).

Methylation at the sixth nitrogen of the adenosine base to form m6A is introduced by methyltransferase enzymes (Fu et al., 2014; Meyer and Jaffrey, 2014). Orthologues of mammalian m6A-reader proteins have been identified in Arabidopsis, including MTA, MTB, FIP37, VIR, HAKAI, ECT1-3, MhYTP1-15, OsYTH1-12, CsYTH1-5, and ALKB10B (Chmielowska-Bąk et al., 2019). m6A-eraser proteins can remove methylation marks on RNAs. Several putative m6A-eraser proteins belonging to the ALKBH family have
been identified in *Arabidopsis*. ALKBH9B and ALKBH10B play key roles in virus infection and flowering transition (Duan et al., 2017; Martínez-Pérez et al., 2017). A recent study suggests that expression of the human m^6^A eraser protein, FTO, in rice and potato resulted in a tremendous increase (~50%) in yield and biomass in field trials (Yu et al., 2021). In recent years, all the above aspects have been addressed in several reviews (Chmielowska-Bażk et al., 2019; Shen et al., 2019).

Like m^5^A, 5-methylcytosine (m^5^C) has also been detected in mRNAs of various plant species (Figure 1). In contrast to m^6^A, the m^5^C level in the mRNA of different *Arabidopsis* tissues can vary from 0.01% to 0.036% (m^5^C/C ratio) (Cui et al., 2017). m^5^C in mRNAs is reported to occur in ~1000 to 4000 transcripts, mostly enriched in coding sequences, downstream of the start codon and upstream of the stop codon. In contrast to m^6^A, the m^5^C

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Figure 1  Schematic diagram summarizing the phenotypes observed when m^6^A and m^5^C RNA modifications were examined in plants.
motifs in plant, HACCR (H = A/U/C, R = A/G) and CTYCTYC (Y = U/C), differ from those in mammals (Yang et al., 2017).

The tRNA-specific methyltransferase TRM4B has been identified as an m5C RNA methyltransferase in Arabidopsis and is involved in root development (David et al., 2017). Recent studies have reported that mRNA is enriched in rRNA, tRNA and snRNA, but also in mRNA, and are also more abundant in the total RNA of a cell, which is 80% rRNA. Interestingly, few homologues of m5C-methylated genes have been identified in Arabidopsis and rice, and yet, m5C is common in both dicotyledons and monocotyledons during plant development (Zhang et al., 2020). The above summary of findings suggests that the contributions of m5A and m5C are critical for plant growth and development and thus have been extensively studied by many groups (Hu et al., 2019; Liang et al., 2020; Shen et al., 2019). Although m5A is the most common internal RNA modification on mRNA of eukaryotic cells, pseudouridine (Ψ) and 2-O-methyl (Nm) RNA modifications are predominantly present in rRNA, tRNA and snRNA, but also in mRNA, and are also more abundant in the total RNA of a cell, which is 80–90% rRNA. However, our knowledge of Ψ and Nm, the two most abundant RNA modifications in absolute numbers in the cell, is still unclear in plants. Therefore, in the following sections, we discuss the role of Ψ and Nm in eukaryotes including plants.

**Pseudouridine (Ψ) RNA modification in plants**

Pseudouridine (Ψ) is a C5 glycose isomer of uridine formed by the breakage of the N1 glycosyl bond and a subsequent 180° base rotation (Figure 2a). Ψ is a common RNA modification first discovered in various non-coding RNAs such as tRNA, rRNA, snRNA (Charette and Gray, 2000; Chikne et al., 2016; De Zoysa and Yu, 2017; Rajan et al., 2019b) and later in mRNAs (Carlile et al., 2014; Li et al., 2015; Lovejoy et al., 2014; Schwartz et al., 2014). Pseudouridylation does not alter the Watson–Crick edge of uridine, but releases the N1 hydrogen to serve as a hydrogen bond donor (Carlile et al., 2015). Ψs lead to increased base stacking and base pairing and make the sugar-phosphate backbone more rigid when incorporated into RNA chains (Machnicka et al., 2013).

**Functional role of Ψ in RNA–protein interaction: reader-like mechanism**

To date, nine studies have reported the functional significance of Ψ in RNA–protein binding (Chen et al., 2010; deLorimier et al., 2014, 2017; Kolev and Steitz, 2006; Levi and Arava, 2021; Rajan et al., 2019b, 2021a; Vaidyanathan et al., 2017; Wu et al., 2016). The first study showing the influence of a single Ψ on snRNP formation was performed with the U7 snRNP (Kolev and Steitz, 2006). This study showed in an artificial system that the introduction of a single Ψ into U7 snRNA 5m binding inhibited snRNP assembly twofold.

Similarly, introduction of a single Ψ into the polyurymidine tract inhibited binding of the splicing factor U2AF65 (Chen et al., 2010). Substitutions of U by Ψ in CUG repeats associated with myotonic dystrophy type I also decreased the binding affinity of the splicing factor MBNL1 by 4- to 20-fold (deLorimier et al., 2014, 2017). Moreover, incorporation of Ψ into the binding site of PUM2 also decreased the binding affinity (Vaidyanathan et al., 2017). All of the above studies indicate that Ψ in protein binding sites decreases the binding affinity. In contrast to the above studies, Wu et al. (2016) suggested that conserved Ψs within the Branch Site Recognition Region (BSRR) are essential for Prp5 protein binding. In vivo structural mapping also showed that pseudouridylated U2 has a different structure than U2 lacking Ψ, suggesting that the Ψ might contribute to Prp5 binding by altering the structure of the binding site rather than directly contributing to RNA–protein interactions. Similarly, our recent study suggests that Ψ in the 3'-UTR of valyl-tRNA synthetase (ValRS) also decreases the binding affinity of an RNA-binding protein, RBSR1 (Rajan et al., 2021a), and Ψs at the binding site of U2A° and U2B° in U2 snRNA enhance the interaction at high temperature (Rajan et al., 2019b). Note that the later results could also stem from the effect of Ψ on the overall structure of U2 snRNA, which could affect protein binding (Rajan et al., 2019b). Reader proteins that recognize individual Ψ sites have also been identified in yeast (Levi and Arava, 2021). The mechanisms of the binding of reader proteins and their relationships with Ψ need to be explored to further clarify our understanding.

**Genome-wide sequencing technologies for mapping Ψ**

To date, there are two (conceptually) different approaches to detect and quantify Ψ genome-wide. The first approach is based on the unique ability of CMC (N-cyclohexyl-N'-(2-morpholinoethyl) carbodimide methyl-p-toluenesulfonate) to bind to the Ψ modification (Ofengand et al., 2001). It is well documented in the literature that Ψ-, U- and G- nucleotides can be chemically modified by CMC. But U-CMC and that G-CMC adducts, but not the Ψ-CMC adduct, can be reversed by mild alkaline hydrolysis (Ofengand et al., 2001).

Because the Ψ-CMC nucleotide is bulky, reverse transcriptase stops or pauses one nucleotide upstream of the Ψ-CMC sites during reverse transcription, allowing detection of the actual Ψ in RNA. This property of Ψ-CMC, together with next-generation sequencing, can be used to detect Ψs in rRNA, mRNA, and other ncRNAs. This technique is referred to as Ψ-seq, pseudo-seq, or CeU-seq by several research groups (Carlile et al., 2014; Li et al., 2015; Lovejoy et al., 2014; Schwartz et al., 2014). The second high-throughput approach used for absolute quantification of Ψ sites is based on random cleavage of uridine nucleotides (but not Ψ nucleotides) in RNA using a combination of hydrazine and aniline treatments, which opens the ring structure of the pyrimidine base and releases S'-phosphorylated RNA fragments suitable for adapter ligation during library preparation for next-generation sequencing (Marchand et al., 2020) (Figure 2cii). A/C/G nucleotides are known to be resistant to hydrazine cleavage and therefore generate only background noise (Peatie, 1979). This method is called HydraPsiSeq (Marchand et al., 2020). Using this method in HelA and HEK-293T cell lines, the authors identified ~20–25 Ψ sites out of 104 known Ψ sites that are variable in human RNA. They also demonstrated the dynamics of multiple Ψ sites during chondrogenic differentiation of bone marrow cells (Marchand et al., 2020).

Detection of Ψ sites by cyanoethylation (Figure 2cii) and other chemical processes was proposed in the early 2000s (Mengel-Jørgensen and Kirpekar, 2002). However, these methods have not yet been reported in genome-wide studies. Recent advances in Oxford nanopore technology (ONT) have allowed researchers to map Ψ on various target RNAs using native RNA sequencing (Begik et al., 2021; Fleming et al., 2021; Huang et al., 2021).
Figure 2  Pseudouridine (Ψ) modification and available technologies for mapping Ψs. (a) Schematic overview of pseudouridine synthase (PUS) mediated pseudouridylation. (b) Structure of the eukaryotic H/ACA snoRNA. The pseudouridylation pocket specifies the nucleotides in the snoRNA for base pairing with its target, and the modified uridine is always 14–16 nt away from the H/ACA box. (c) Methods used to detect Ψs. (i) Ψ-seq, pseudo-seq, or CeU-seq is based on the ability of CMC (N-cyclohexyl-N'-b-(4-methylmorpholinium) ethylcarbodiimide) to specifically modify Ψ residues instead of uridine residues. During reverse transcription, the bulky Ψ-CMC nucleotide leads to premature termination event one nucleotide upstream of the actual Ψ-CMC sites, and can be used to detect the Ψ. (ii) HydraPsiSeq relies on the ability of Ψ sites to resist random cleavage upon treatment with hydrazine and aniline. Unmodified uridine residues are fragmented during this procedure and can be used to deduce the proportion of Ψ at each nucleotide. (iii) Detection of Ψ sites by cyanoethylation. Ψ-ACN adducts can be distinguished from unmodified uridines by mass spectrometric analysis.
Similar to m^A mapping, an antibody-based Ψ mapping technique called photo-crosslinking-assisted Ψ sequencing (PA-Ψ-seq) has recently been developed to map Ψ sites (Martínez Campos et al., 2021). Further studies are needed to evaluate the qualitative and quantitative nature of both ONT and PA-Ψ-seq in detecting Ψs.

Recent advances in mass spectrometry have also accurately and quantitatively detected Ψ sites, but this method requires sophisticated equipment and expertise (Yamaki et al., 2018). tandem mass spectrometry analysis on rRNA-derived from human TK6 cells, the authors found that most Ψ sites in humans are non-variable and static. This discrepancy needs further investigation (Taoka et al., 2018). However, tandem mass spectrometry analysis (LC MS/MS) of RNA derived from X-linked dyskeratosis congenita (X-DC) patients showed that nine specific Ψs were reduced compared with control cells (Bellido et al., 2013).

There are several inconsistencies in studies of Ψs in mRNA, such as the exact number of Ψ sites and the extent of pseudouridylation on mRNAs. For example, three independent groups reported the number of Ψ sites in human mRNA, which ranged from several hundreds to thousands (Carrière et al., 2014; Li et al., 2015; Schwartz et al., 2014). Comparison of all three data sets revealed only 91 common sites (Safra et al., 2017a). Similarly, the newly developed HydraPsiSeq, which is considered as a quantitative method, detected only 23 and 16 Ψ sites in yeast mRNA when compared to (Carrière et al., 2014) and (Schwartz et al., 2014) respectively. Altogether, only 2 Ψ sites are common between all these three studies. These results suggest that there are large differences in the detection of Ψ sites within the same species using different technologies. Further studies are needed to investigate such experimental or other possible variations in Ψ-site mapping.

Pseudouridylation on rRNA

Pseudouridylation of rRNA and snRNA in eukaryotes is the best-studied modification to date. In bacteria, the enzyme pseudouridine synthase (PUS) controls Ψs in rRNA (Gutguss et al., 2001). All known Ψs in yeast, mammalian, archaeal, and plant rRNA are controlled by H/ACA snoRNAs, a family of small nucleolar RNAs (snoRNAs) (Figure 2b). In most eukaryotes, the H/ACA snoRNA pairs with the target sequence flanking the uridine to be converted to Ψ by two short recognition motifs. The interaction domains are discontinuous on the snoRNA and separated by tens of nucleotides. The Ψ is always installed on the 14–16 complementary nucleotides (on the target RNA) upstream from the H or ACA box of the snoRNA (Figure 2b).

Ψs in rRNA are concentrated in functional domains of yeast and human rRNA (Ge and Yu, 2013). These domains include functional domains of LSU, namely helix 69 (H69), the decoding centre, the A-site finger (ASF) and the peptidyl transferase centre (PTC), the decoding centre of small subunit (SSU). The H69 of the large subunit (LSU) is known to interact with helix 44 (H44) in the SSU to form the intersubunit bridge 2a (Yusupov et al., 2001). Reduction of one or two Ψ modifications in yeast H69 had no significant effect on cell growth, whereas simultaneous loss of three to five Ψ modifications affected growth (Liang et al., 2007). It also resulted in decreased amino acid incorporation rates and rRNA levels, increased stop codon read-through and sensitivity towards ribosome-based antibiotics, and altered accessibility to rRNA nucleotides in the ribosome (Liang et al., 2007). Similarly, the functional importance of Ψs has been observed in the decoding centres of SSU rRNAs (Wilson et al., 2005), in the ASF region, and in PTC (King et al., 2003). Depletion of 2–3 Ψ modifications in the A- and P-site regions of the decoding centre resulted in reduced growth and amino acid incorporation rates, increased sensitivity to antibiotics, and a significant deficiency in free small subunits (Liang et al., 2009). The LSU rRNA in eukaryotes contains a dense cluster of eight to ten Ψs in the three-helix structure (H37-H39; Piekná-Przybylska et al., 2008). This domain is characterized by the presence of a long flexible helix (H38) known as ASF. Genetic depletion of Ψs located in the ASF helix and adjacent helices demonstrated that these Ψs are not essential for cell viability, but their presence increases ribosome fitness (Piekná-Przybylska et al., 2008). Deletion of different combinations of Ψs in this region showed that the Ψ pattern is essential. Loss of several Ψs simultaneously reduced the amount of rRNA, impaired polyosome formation and translational activity, and increased sensitivity to ribosome-based antibiotics such as paromomycin targeting SSU and sparsomycin targeting LSU (Piekná-Przybylska et al., 2008).

In yeast, all rRNA Ψ sites are catalysed by the CBFS enzyme, and CBFS is catalytically impaired by the D95A mutation (CBFS-D95A; lacking all Ψs in the rRNAs). Ribosomes from (CBFS-D95A) mutant cells showed decreased affinity for tRNAs and decreased translational fidelity (Jack et al., 2011). Ribosomes isolated from CBFS-D95A cells also have decreased affinity towards tRNA binding to the A and P sites of the ribosome and the Crick Paralysis Virus IRES (Internal Ribosome Entry Site) domain, which interacts with both the P and E sites of the ribosome (Jack et al., 2011). From all the studies described above, it appears that rRNA Ψs affect ribosome-tRNA binding, translational fidelity, and drug susceptibility in yeast. Although studies in yeast suggest that disruption of more than two or more Ψ sites produces a significant phenotype, no studies to date have demonstrated the functional significance of a single Ψ site. Recent studies with the unicellular parasite Trypanosoma brucei (Chikne et al., 2016; Rajan et al., 2019b) and other studies with humans (McCann et al., 2020) suggest that Ψs are regulated during differentiation.

Although the rRNA processing phenotype of yeast snR10 was initially thought to be guided by Ψ at the tip of the peptidyl transferase centre (PTC), a mutational analysis of the pseudouridylation pocket later showed that the chaperonic activity of the guide snoRNA in folding pre-rRNA is essential for this phenotype, thus excluding potential phenotype arising from the Ψ guided by the snoRNA (Liang et al., 2010). To date, only one study has demonstrated the significant functional importance of a single Ψ site. This study in a mouse model suggests that the H/ACA snoRNA SNORA24 that guides two Ψs in the rRNAs) mediates tumour suppression. Using single-molecule fluorescence resonance energy transfer analysis (FRET), the study showed that ribosomes from cells lacking SNORA24 exhibit alterations in aminoacyl transfer RNA (aa-tRNA) selection and in the dynamics of the pre-translocation ribosome complex.

The presence of Ψs in Arabidopsis rRNA was predicted in the early 2000s (Brown et al., 2003). In a recent study, 187 Ψ sites were discovered in Arabidopsis rRNA by genome-wide mapping.
In eukaryotes, small nuclear RNAs (snRNAs) exist as RNP (RNA-protein) complexes known as small nuclear ribonucleoproteins (snRNPs). During the assembly of spliceosomes, these snRNPs are sequentially recruited onto a pre-mRNA substrate forming short stretches of RNA-RNA duplexes that play vital roles in recognizing, specification, and catalysis of chemical reactions, leading to the release of mature mRNAs (Adachi and Yu, 2014). Similar to the presence of Ψ in rRNA, all snRNAs studied to date contain Ψ modifications and play important functional roles in snRNP biogenesis and function. All Ψs studied in the Xenopus oocyte reconstitution system were found to be functionally important for snRNP biogenesis and splicing (Zhao and Yu, 2004). Eukaryotic U1 snRNAs carry two Ψs at the 5' end (Ψ5 and Ψ6), which form a base pair with the 5' splice site during spliceosome assembly. A Ψ-G base pair has been shown to stabilize the interaction of U1 with the 5' splice site of HIV-1 SD4 RNA (Freund et al., 2003). U2 snRNAs carry the largest number of Ψs among all snRNAs (human U2 contains 13 Ψs and T. brucei U2 contains 27 Ψs), and pseudouridylation of U2 snRNA has been extensively studied (Adachi and Yu, 2014; Rajan et al., 2019b).

Three Ψs (Ψ34, Ψ41, and Ψ43) in the mammalian U2 branch site recognition region (BSRR) participate in base pairing with the pre-mRNA branch site. Yeast U2 also interacts with the BSRR using positions Ψ35, Ψ42, and Ψ44. Similarly, both mammalian and yeast U5 have a Ψ (Ψ43 and Ψ99, respectively) in the conserved loop of U5 that is involved in the interaction between the 5' and 3' exon sequences and holds the exons within the spliceosome. Ψs are also found in the U4–U6 duplex regions of vertebrates and in other regions of U6 snRNA. Some of these Ψs are conserved in plant snRNAs and in minor-class snRNAs such as U4atac and U12 (Adachi and Yu, 2014). All three Ψs in yeast U2 snRNA have been thoroughly studied using genetic systems that can manipulate the corresponding pseudouridinyl synthetases. The enzyme PUS7 catalyses the conversion of U2 Ψ35, which interacts with the nucleotide adjacent to the adenosine of the pre-mRNA branch point during splicing. Although PUS7 deletion strains are viable, these cells grow slowly under high salt conditions or in competition with wild-type cells (Adachi and Yu, 2014; Ma et al., 2003). The combination of PUS7 deletion (leading to loss of Ψ35) and a point mutation at U40 (U40Δ) of U2 snRNA resulted in a temperature-sensitive growth defect and accumulation of pre-mRNA (Yang et al., 2005). This study showed that Ψ35 in the U2 BSRR contributes substantially to pre-mRNA splicing in yeast. Similarly, deletion of PUS1 and snR81 (which directly Ψ44 and Ψ42, respectively) also affected pre-mRNA splicing.

Structural studies of the solution structure of yeast U2 snRNA have shown that Ψ35 is preferred over the uridine nucleotide to maintain the bulge of the branch-point adenosine (Newby and Greenbaum, 2002). Similarly, crystal structures of the duplex formed between a pre-mRNA branch site and U2 snRNA carrying Ψ35 have shown that the bulged nucleotide at the branch site prefers an extrahelical conformation that exposes its 2'-OH group for nucleophilic attack during the first step of pre-mRNA splicing (Lin and Kiellkopf, 2008). Studies with yeast U2 snRNA suggest that the conserved Ψ at positions Ψ35, Ψ42, and Ψ44 of the U2 snRNA within the BSRR, which forms base pairs with the pre-mRNA branch site, is essential for PrP5 protein binding (Wu et al., 2011, 2016). Our recent study suggests that Ψ in the U2A' and U2B' binding sites of U2 snRNA also facilitates stronger protein binding at higher temperatures (Rajan et al., 2019b).

In humans, the U4 and U6 snRNAs carry six Ψs and twelve 2'-O-methylation (Nm) residues. Nearly 50% of these modifications are located in domains involved in the U4–U6 base pairing interaction (Karjiojilich and Yu, 2010). Our recent study suggests that Ψs in U4-U6 snRNA strengthens the duplex at higher temperatures (Rajan et al., 2019b). A single Ψ at position 28 on U6 snRNA is induced during yeast filamentous growth and is guided by PUS1. It has been reported that this Ψ28 on the U6 snRNA alters the splicing efficiency of suboptimal introns and contributes to the filamentous growth programme (Basak and Quer, 2014).

Until recently, most snRNA-Ψs were considered to be constitutive and static. However, recent studies suggest that Ψs are dynamic and in some cases conditionally induced. Surprisingly, U2 snRNA from yeast cells deprived of nutrients has two additional Ψs (Ψ at positions 56 and 93) along with three existing Ψs (Ψ at positions 35, 42, and 44). Ψ56 is also induced under stress conditions (Wu et al., 2011). Interestingly, Ψ56 is guided by the enzyme PUS7, which also guides U2 at position 35, and Ψ93 is guided by snR81, which also guides Ψ42 in U2 snRNA and Ψ1051 in 25S rRNA. Notably, snR81 guides Ψ93 by imperfect base pairing, suggesting that stress may induce the formation of additional sites. This altered Ψ on the U2 snRNA impairs splicing under stress conditions (Wu et al., 2011). The Ψs of snRNAs are also developmentally regulated, and loss of a single Ψ on the U2 snRNA affects splicing in Trypanosoma brucei (Rajan et al., 2019b).

Arabidopsis snRNAs also carry Ψs. In a recent study, 13 Ψ sites were detected in Arabidopsis snRNA by genome-wide mapping (Sun et al., 2019). It is currently unknown whether these sites are guided by snRNAs or PUS enzymes and their biological significance remains to be explored.

### Pseudouridylation in mRNA

It is generally believed that mRNAs are regulated either by mRNA–protein interaction or by interference from noncoding RNAs such as miRNAs or siRNAs (Ipsaro and Joshua-Tor, 2015). Recent evidence suggests that base modifications can also alter the stability and function of mRNAs (Zaccara et al., 2019). Ψ is the second most abundant internal modification on mRNA (Li et al., 2015). Advances in mapping Ψs on mRNA and other ncRNAs...
using N-cyclohexyl-N'-b-(4-methylmorpholinium) ethylcarbodiimide (CMC) modification, followed by deep sequencing, have revolutionized the principles by which these modifications can be detected and studied in detail. Recent studies have demonstrated the existence of ~50–300 mRNAs Ψ sites in yeast and ~100–400 in human cells (Carlile et al., 2014; Li et al., 2015; Lovejoy et al., 2014; Schwartz et al., 2014; Figure 2c). Using a novel approach to sequence Ψ-rich transcripts with a chemical pull-down method using biotin-labelled CMC followed by affinity selection on streptavidin beads, Li et al. (2015) also demonstrated that even more Ψ sites can be explored. For example, using this combination technology, the transcriptome of HEK293T cells was found to contain 2084 Ψ sites, whereas 1741 and 1543 Ψ sites were found in mouse brain and liver respectively. A recent mapping of Ψ on the mRNA of the parasite Toxoplasma gondii revealed more than 1669 sites in the mRNA, and TgPUS1 was found to be the major PUS enzyme guiding these Ψ sites (Nakamoto et al., 2017). This study suggests that mRNAs carrying TgPUS1-dependent Ψ are more abundant in TgPUS1-mutant parasites. The mechanism behind this increased expression of mRNA was later attributed to the increased half-life of these mRNAs in TgPUS1 mutant parasites. Mapping of Ψ sites in two different life stages of the Trypanosoma brucei parasite revealed the existence of ~273 Ψ sites, including 21 hypermodified sites. The study also showed that Ψ sites in mRNA differentially affect the binding of an RNA-binding protein and thus the stability of mRNA (Rajan et al., 2021a). Similarly, reader proteins that recognize individual Ψ sites have also been identified in yeast (Levi and Arava, 2021).

Arabidopsis mRNAs also carry Ψ sites internally (Sun et al., 2019). In a recent study, ~451 Ψ sites were detected in Arabidopsis mRNAs by genome-wide mapping (Sun et al., 2019). In contrast to m^A and m^C, Ψ are enriched in position 1 of triplet codons carrying uridine and underrepresented in the 3’ UTR. No Ψ was detected in the stop codon. The gene ontology of mRNAs carrying Ψ sites suggests that these transcripts were enriched for functions other eukaryotes suggest that these transcripts were enriched for functions related to stress responses, energy production, and photosynthesis. As with rRNAs, six HACA snoRNAs were predicted to guide mRNAs carrying Ψ by obeying canonical guidance rules. Further studies are needed to decipher the functional significance of individual Ψ sites in plant development. Previous studies from other eukaryotes suggest that Ψ sites regulate the binding affinity of RNA-binding proteins (Rajan et al., 2019b, 2021a), which are an important determinant of plant development (Cho et al., 2019; Marondedze, 2020). It is currently unknown whether the same mRNA molecule carries the Ψ site, m^A, and m^C and whether all three are functionally independent or dependent on the same mRNA molecule.

2'-O-methylation in plants

2'-O-methylation (also known as 2'-O-Me or Nm) is the second most abundant RNA modification occurring in various RNA species such as rRNA, tRNA, snoRNA, mRNA cap structure, and in the 3’ termini of piRNAs. Methylation of the 2'-OH moieties occurs in the ribose sugar (whereas in Ψ, m^A and m^C, the modification occurs in the base) and is detected in all four nucleotides (Figure 3a-b). Nm on eukaryotic rRNA is coordinated by the action of the methyltransferase fibrillarin/NOP1 together with NOP56, NOP58, SNU13, and C/D snoRNA (Figure 3a; Tollervey and Kiss, 1997). C/D snoRNAs have short-sequence motifs known as C-box (5’-RUGAUGA-3’) and D-box (5’-CUGA-3’). C/D snoRNA pairs with its target RNA via 10–22 nucleotide long complementary sequences. The nucleotide to be methylated is always recognized by the +5 rule, in which the nucleotide base pair to be methylated is located five nucleotides upstream of the D or D’ box (Figure 3c; Rajan et al., 2019a). Recent findings have expanded the function of C/D snoRNA beyond its role in Nm modification (not covered in this review). Although the chemical occurrence of Nms was documented several decades ago, methods for quantitative mapping of this modification have only been developed in the last decade, and the functional significance of this modification remains to be explored.

Genome-wide sequencing technologies to map Nm

Three different high-throughput sequencing methods are commonly used to map Nm: 2'-OMe-seq (Incarnato et al., 2017), RibOmeth-seq (Birkedal et al., 2015; Krogh et al., 2016; Marc-hand et al., 2016) and RibOxi-seq (Rajan et al., 2020; Zhu et al., 2017; Figure 4). Traditionally, Nm residues have been detected using primer extension analysis, which relies on the intrinsic property of reverse transcriptase to stop one nucleotide located prior to the actual Nm site upon treatment with low dNTP (Maden, 2001). This property has been coupled with next-generation sequencing to map Nm and is referred to as 2'-OMe-seq (Incarnato et al., 2017). Although the method has been reliably used to detect fully modified Nm residues, its usefulness in detecting partially modified residues has recently been questioned (Rajan et al., 2020).

To overcome structural artefacts that arise during reverse transcription (RT), a new RT-independent method, called RibOxi-seq (Rajan et al., 2020; Zhu et al., 2017), was developed (Figure 4a). This method relies on the unique ability of Nm residues to resist alkaline hydrolysis and oxidation (Figure 4b). In this method, the first nucleotide available for 3' ligation is an Nm residue that can be inferred from statistical analysis of sequencing reads. This method outperformed all other methods in detecting all known Nm sites on human rRNA (Krogh and Nielsen, 2019). Although both methods are useful for detecting Nm residues, they cannot be used for quantitative purposes due to their quality.

To quantitatively assess the stoichiometry of Nm residues, RibOmeth-seq (Birkedal et al., 2015; Krogh et al., 2016; Marchand et al., 2016) was developed. This method exploits the property of Nm sites to resist alkaline hydrolysis (Figure 4c). The fragmentation profile obtained during RibOmeth-seq analysis is used to evaluate the percentage of modification at each Nm site. The development of the RibOmeth-seq method has significantly advanced the notion that Nm modifications are dynamic and are subject to variation upon various cues.

Ribosome heterogeneity by 2'-O-methylation on rRNA: functional role of Nm

The number and stoichiometric levels of Nm sites in rRNA vary among eukaryotes, ranging from 55 Nms in yeast and 99 Nms in Trypanosoma brucei to 112 Nms in humans (Rajan et al., 2020). 2'-O-methylation of rRNA occurs co-transcriptionally and is required for proper rRNA biogenesis (Kos and Tollervey, 2010). A detailed review of Nm sites in rRNA was recently published by Jaafar et al. (2021), showing the variation of different Nm residues in yeast and humans. The development of RibOmeth-seq has revolutionized the field of ribosome heterogeneity. This method was first used to quantify Nm sites on yeast rRNA and revealed the existence of partially modified sites in a genome-wide manner (Birkedal et al., 2015). Although the existence of a
single partially modified Nm residue (Am100) on a small subunit rRNA was previously detected in yeast (Buchhaupt et al., 2014), RiboMeth-seq also identified several novel partially modified Nm sites (including those on large subunits). Similarly, this method was able to identify variable and partially modified Nm sites on human rRNA (Krogh et al., 2016; Marchand et al., 2016). Since then, several studies have used this technique to quantify Nms to answer various biological questions (Jaafar et al., 2021; Motorin and Marchand, 2021).

Although the recent use of sequencing technologies has increased our knowledge of Nm-bearing residues, the precise function of the single Nm moiety is still unknown. To date, only a handful of studies have addressed the role of a single Nm site on rRNA. In *Escherichia coli*, the Nm sites on rRNA are guided by the enzymes rsmH/rsmL (Cm1402 on SSU), rlmB (Gm2251 on LSU), rlmM (Cm2498 on LSU), and rlmE/rrmJ (Um2552 on LSU). Studies in *E. coli* suggest that Um2552 affects the interaction of aminoacyl-tRNA with the A site in ribosomes and affects the

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**Figure 3** 2′-O-methylation modification. (a) Schematic overview of 2′-O-methylation mediated by methyltransferase enzymes. (b) Chemical structure of all four-ribose nucleotides and their 2′-O-methylated derivatives. (c) Structure of eukaryotic C/D snoRNA. The conserved C/D boxes are labelled. The nucleotide to be methylated on the target RNA is determined by the +5 rule, in which the methyltransferase adds a methyl group to the nucleotide located five nucleotides upstream from the D/D' box of the snoRNA that forms a base pair with its target. (d) Evolutionarily conserved Nm residues in *Arabidopsis* rRNA. Nm residues corresponding to human 80S (PDB ID: 6EKU) and *Triticum aestivum* (wheat) 80S (PDB ID: 4V7E) ribosomes are indicated. Peptidyl transferase centre (PTC), tRNA, and decoding centre (DC) are indicated. Large subunit (LSU) and small subunit (SSU) rRNA are coloured grey and blue respectively. Figures were generated using UCSF Chimera-X software (https://www.rbvi.ucsf.edu/chimerax/).
fidelity of translation (Sergeeva et al., 2015). The high-resolution atomic structure of the Thermus thermophilus ribosome provided insights into the possible role of Nm on ribosome structure. The structure at 2.3–2.5 Å resolution revealed the role of Gm2251 and Um2552 in making contacts with the CCA end of tRNAs (Polikanov et al., 2015). Similarly, the atomic structure of the
Leishmania donovani ribosome at 2.8 Å resolution showed thatNm modifications could help in RNA and protein interactions. For example, Um7 on the 5.8S rRNA maintains base interactions with A456, and Gm1628 on the large subunit (LSU) RNA (chain alpha) interacts with A18 of the LSU beta chain rRNA. In addition, the same Nm residue Gm1628 also maintains electrostatic interactions with the universally conserved ribosomal protein uL23. In a recent study, the function of the snoRNA guiding Cm174 on the small subunit rRNA of the human ribosome was shown to regulate the translation of certain mRNAs by affecting the elongation rates of the ribosome (Jansson et al., 2021). Similarly, a previous study demonstrated the role of Nm in the translation of mRNAs containing the element IRES using a human cell line inhibited by rRNA methyltransferase (Erales et al., 2017). The chemical properties of the 2'-O-methyl group in the Nm site make it resistant to nucleophilic attack due to the lower reactivity of oxygen and prevent H-bonding at the sugar edge. The 2'-O-methyl group promotes C3'-endo sugar conformation which could stabilize base pairing and base stacking (Krogh and Nielsen, 2019). 

Similar to the function of individual Nm residues, the mechanism by which each Nm residue is regulated is not yet clear. For example, Hebras et al. (2020) performed Ribometh-seq and found that there was Gm4593 on LSU rRNA in adult mouse tissues than in developing tissue. This phenotype was attributed to decreased expression of the cognate guide C/D snoRNA, SNORD78, which guides Gm4593. The expression of SNORD78 was indeed regulated by an alternative splicing mechanism of the SNORD78, which guides Gm4593. The expression of SNORD78 to decreased expression of the cognate guide C/D snoRNA, SNORD78, which guides Gm4593. The expression of SNORD78 was indeed regulated by an alternative splicing mechanism of the host gene (Gas5), which harbours nine different C/D snoRNAs.

Recently, two independent groups mapped and quantified rRNA Nm sites in Arabidopsis, finding 117 (Azevedo-Favory et al., 2021) and 111 (Wu et al., 2021) cytoplasmic Nm sites. Together with the previously mapped sites, the authors identified 151 Nm residues on Arabidopsis rRNA (Azevedo-Favory et al., 2021). Of these mapped sites, at least 63 and 36 Nm sites are conserved between human and yeast, respectively. Most rRNA Nm sites have also been associated with C/D snoRNA, that could possibly guide this modification, and have also been linked to the methyltransferase fibrillarin (FIB) (FIB1 and/or FIB2; Azevedo-Favory et al., 2021; Wu et al., 2021).

As reported in human and yeast studies, hypomethylated Nm residues (Um2422 and Am2641) were also present in Arabidopsis rRNA, supporting the notion that these Nm residues can be potentially regulated and rRNA without these Nms exists (Azevedo-Favory et al., 2021). Indeed, five Nm sites have been identified in each chloroplast and mitochondrial rRNA (Wu et al., 2021). Among these sites, at least two sites are universally conserved in cytoplasmic, chloroplast, and mitochondrial rRNAs from Arabidopsis, human, yeast, and E. coli. The localization of these conserved Nm sites on the atomic structures of human and wheat ribosomes suggests that these Nm residues are in close proximity to functional domains such as the decoding centre (DC) and the peptidyl transferase centre (PTC) of the ribosome (Figure 3d). Although these two studies did not address the functional significance of a single Nm residue, Azevedo-Favory et al. (2021) showed that the absence of the nucleolar protein Nur1 (Nucleolin 1) affects the level of specific rRNA Nm sites, suggesting a link between nucleolar organization and rRNA biogenesis.

Nm residues on spliceosomal snRNA: still an enigma

In contrast to Ψ, the functional role of Nm residues on snRNA remains a mystery. No Nm sites have yet been detected in yeast snRNA, whereas human snRNA has 3, 11, 4, 5, and 8 Nm sites on U1, U2, U4, U5, and U6 respectively (Morais et al., 2021b). To date, only the cumulative role of 4–5 Nm sites on U2 snRNAs (Am1, Um2, Gm11, Gm12, and Gm19) has been attributed for efficient splicing (Dönmex et al., 2004; Yu et al., 1998).

Quantitative analysis of known Nm residues in human snRNA using Ribometh-seq shows that these modifications are almost completely methylated in the solid tissues examined and only U4-Cm8 was altered in the T-cell leukaemia model cell line (Jurkat cells). Similarly, multiple Nm sites on Arabidopsis snRNA were also mapped in the Ribometh-seq studies mentioned above (Azevedo-Favory et al., 2021; Wu et al., 2021). Although a total of 25 Nm sites were detected in Arabidopsis snRNA, only 7 sites were common in both studies (Table 1). As with Nm sites in human snRNA, Nm sites in Arabidopsis snRNA await further verification by other Nm detection methods such as mass spectrometry (Yamaki et al., 2020).

Ψ and Nm on other ncRNAs

To date, only a handful of studies have described the existence of internal Ψ and Nm on ncRNAs other than snRNA and tRNAs. Therefore, this review also recommends reading reviews dealing with tRNA modifications (Krutyholowa et al., 2019; Spenkuch et al., 2014; Zhang et al., 2022). In addition to the major spliceosomal snRNAs (U1, U2, U4, U5, and U6), the minor spliceosomal RNAs such as U12, U4atac, and U6atac also harbour both Ψ and Nm modifications (Morais et al., 2021b). Several Ψs have been detected on ncRNAs such as snoRNA, telomerase RNA component (TERC), and RNase MRP by genome-wide mapping of Ψ (Carlile et al., 2014; Schwartz et al., 2014). Similarly, photoreactive nucleotide-enhanced cross-linking and immuno-precipitation (PAR-CLIP) of core proteins associated with snoRNAs enabled detection of these modifications on vault RNA, 7SK, and 7SL RNA (Kishore et al., 2013). Recent advances in mass spectrometry have also allowed detection of these modifications on U3, U8, 7SL, and MRP RNA (Yamaki et al., 2020).
Nevertheless, further studies are needed to decipher the biological significance of RNA modifications on these RNA molecules.

## Conclusion

Plants provide a unique platform to study the biological role and genetic influences of RNA modification in multicellular eukaryotic organisms. Advances in the mapping and quantification of RNA modification have greatly enhanced research in this area. Recent studies show that manipulation of the human RNA modification eraser protein FTO in both rice and potato plants increased yield by 50% in field studies, indicating potential benefits for global food production and drought tolerance (Yu et al., 2021). Although different sequencing technologies use their own unique advantages and disadvantages. For example, RiboMeth-seq quantifies single Nm residues as accurately as mass spectrometry, but the method has a significant signal–signal ratio and is less effective at detecting novel Nms with low stoichiometry (Krogh and Nielsen, 2007).

The ability of 2'-O-methylation (Nm) and Nm modifications to regulate translation (Elliott et al., 2019; Eyler et al., 2019) and RNA stability (Abou Assi et al., 2020) has been highly appreciated in recent years as a new way to regulate gene expression. The global deployment of COVID-19 mRNA vaccines with Ψ independently developed by Pfizer-BioNTech and Moderna Therapeutics (Morais et al., 2021a) has shown that fine-tuning nucleotide composition can dramatically improve outcomes. In summary, our understanding of the mechanistic insights associated with these modified nucleotides is still in its infancy. Future studies will pave the best way for better mechanistic insights to increase global food production and enable climate-resilient agriculture.

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

### Author contributions

MR, KSR, and SM planned, designed, and wrote the review. MR, KSR, SR, and QW outlined and revised the review. MR, KSR, and SM planned, designed, and wrote the review. MR, KSR, and SM planned, designed, and wrote the review. MR, KSR, SR, and QW outlined and revised the review. MR, KSR, SR, and QW outlined and revised the review.

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Muthusamy Ramakrishnan

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