

Data-Independent Acquisition for the Detection of Mononucleoside RNA Modifications by Mass Spectrometry

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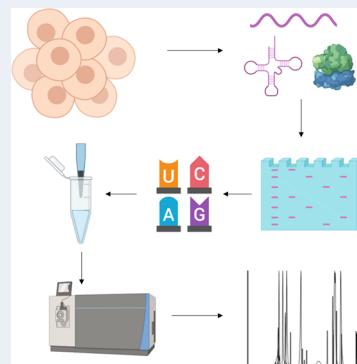
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ABSTRACT: RNA is dynamically modified in cells by a plethora of chemical moieties to modulate molecular functions and processes. Over 140 modifications have been identified across species and RNA types, with the highest density and diversity of modifications found in tRNA (tRNA). The methods used to identify and quantify these modifications have developed over recent years and continue to advance, primarily in the fields of next-generation sequencing (NGS) and mass spectrometry (MS). Most current NGS methods are limited to antibody-recognized or chemically derivatized modifications and have limitations in identifying multiple modifications simultaneously. Mass spectrometry can overcome both of these issues, accurately identifying a large number of modifications in a single run. Here, we present advances in MS data acquisition for the purpose of RNA modification identification and quantitation. Using this approach, we identified multiple tRNA wobble position modifications in *Arabidopsis thaliana* that are upregulated in salt-stressed growth conditions and may stabilize translation of salt stress induced proteins. This work presents improvements in methods for studying RNA modifications and introduces a possible regulatory role of wobble position modifications in *A. thaliana* translation.



INTRODUCTION

The RNA epitranscriptome is defined by the presence of over 140 modifications on RNA across species and RNA types.¹ These modifications decorate both coding and noncoding RNA and play critical roles in biological functions through modulating the RNA structure, RNA–protein interactions, and nucleic acid interactions.^{2–5} Despite RNA modifications being known to exist for over 60 years, it is likely that not all modifications have been discovered. The biological roles of known modifications have been challenging to study due to a limited number of tools to interrogate their functions. Recently, the discovery of modifications such as m⁶A and ac⁴C in mRNA and invention of novel sequencing techniques capable of localizing the marks has led to dramatically increased interest in and investigation of RNA modifications at large.^{6,7} This surge in interest in the biological functions of RNA modifications, which have been implicated in development, cancer, and a multitude of cellular processes, has highlighted the need for additional improvements in quantitative methods of RNA modification analysis.

Much of the recent research on RNA modifications has been performed using sequencing, focused on individual modifications of mRNA.^{8,9} These efforts have yielded important findings on mRNA stability, splicing, and translation; however, the powerful sequencing methods have significant limitations.^{4,9,10} Sequencing provides the best data on modification locations in mRNA but is limited to only a few modifications, and only one can be robustly analyzed at a time. Some of the techniques have high false positive rates or low specificity, such

as bisulfite sequencing, and a number are not quantitative.¹¹ In addition, sequencing approaches are not as well suited for tRNA and rRNA due to the high number of diverse chemical modifications which can inhibit reverse transcription, occluding certain regions from analysis or providing false positives in RT-stop based techniques.^{12,13} Lastly, because modification sequencing is targeted, it requires a priori knowledge of which modification is present.

Analysis of single nucleosides can provide high-quality modification quantitation, produce valuable data, and inform future sequencing efforts. Mononucleoside analysis is best performed by LC–MS, which has been in use since 1990.¹⁴ A variety of different sample preparation techniques are commonly used in combination with mass spectrometry. Generally, a cocktail of multiple enzymes is used to ensure that modified nucleosides do not occlude cleavage of phosphoester linkages. This incubation is sometimes performed at acidic and/or basic pH at 37 °C.¹⁵ In this work, we digest RNA at neutral pH and room temperature.

Mass spectrometric techniques also vary, but the implications of different approaches do not have the same burden of

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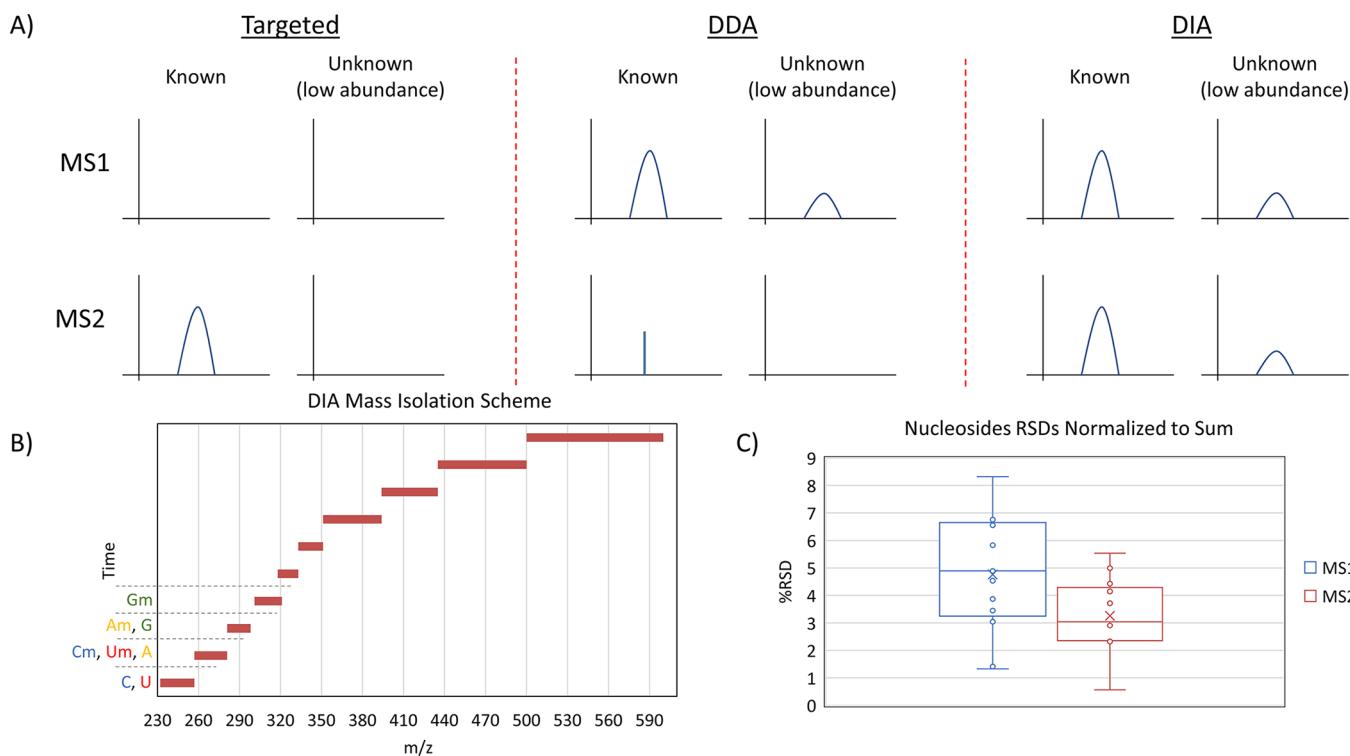


Figure 1. (A) Comparison of MS acquisition approaches. Targeted acquisition is often performed using a QQQ mass spectrometer, which isolates a mass, fragments the molecule, and then isolates and detects a single fragment ion. DDA selects masses for fragmentation based on criteria such as abundance and often excludes previously fragmented masses from selection, resulting in a few MS2 scans of abundant nucleosides and no MS2 scans of low abundance nucleosides. DIA fragments all masses in designated windows in each scan cycle, ensuring adequate points across a peak at the MS1 and MS2 levels for all nucleosides. (B) DIA mass isolation scheme designed in this work. The scan windows prevent different ribose modification states from producing the same fragment ion in the same MS2 scan from different precursors. (C) Comparison of %RSD values for 5 HeLa biological replicates injected at different concentrations (1 \times to 5 \times). The nucleosides used were C, U, Ψ , A, G, m^3 C, m^5 C, Cm, I, m^1 A, m^6 A, Am, and m^1 acp $^3\Psi$, and abundances were normalized to the sum of the included analytes. Within the same runs, MS2 was significantly more consistent than MS1 analysis ($P < 0.05$).

bias that sample preparation differences can induce.¹⁶ These techniques are used largely dependent on the desired data. The common methods include targeted data acquisition, used most commonly with triple quadrupole (QQQ) mass spectrometers, and data-dependent acquisition (DDA), used more commonly with instruments that can acquire full scans rapidly, such as Orbitraps.^{17,18} As many proteomics laboratories lack QQQ instruments and seek to expand their analytical technologies, development of easily adopted RNA modification analysis methods for high-resolution instruments is of value. These methods come with distinct advantages. Targeted QQQ acquisition provides the highest quantitative accuracy and sensitivity, and DDA allows for identification of unknowns; however, each method comes with limitations. Targeted acquisition requires prior knowledge of analytes and must be performed carefully, as QQQ instruments lack the mass accuracy to differentiate, for example, ac⁴C from isotopes of guanosine. Depending on acquisition parameters, DDA can acquire too few MS2 scans to use for quantitation, which can introduce more noise in measurements by requiring quantitation at the MS1 level. Decreasing dynamic exclusion can overcome this issue; however, it is at the cost of missing very low abundance nucleosides. The limitations of these methods provide an opportunity for a recent MS technique, data-independent acquisition (DIA), to be used. DIA allows for both the potential detection of unanticipated molecular species and MS2-based quantitation, addressing the biggest

disadvantages of targeted acquisition and DDA, respectively. In this work, we demonstrate that DIA is a viable approach for mass spectrometry-based nucleoside analysis, allowing us to better detect nucleosides and quantify their abundances.

RESULTS AND DISCUSSION

Data Independent Acquisition (DIA) for Nucleosides.

Different data acquisition techniques and parameters can be used to obtain MS data, and this is mainly determined by the experiment type and downstream analysis. The three common MS techniques that can be used for nucleoside analysis are data-dependent acquisition (DDA), data-independent acquisition (DIA), and targeted data acquisition (Figure 1A). All three approaches have their strengths and weaknesses, some aspects of which are depicted in Figure 1A. Specifically in our studies, we compare DDA and DIA data acquisition approaches using an Orbitrap Fusion mass analyzer (Thermo Electron). In short, the ideal MS nucleoside data acquisition method needs (1) the ability to detect low abundance species, (2) sufficient MS2 scans for quantitation, (3) minimal signal interference, and (4) an unbiased approach that allows for analysis of any known and unexpected modified nucleosides of interest.

Because of the inability to establish a rapid QQQ-based DIA method, the difference between DIA and targeted or DDA methodology is best compared on the basis of Orbitrap technology. Indeed, when designing a targeted method, careful

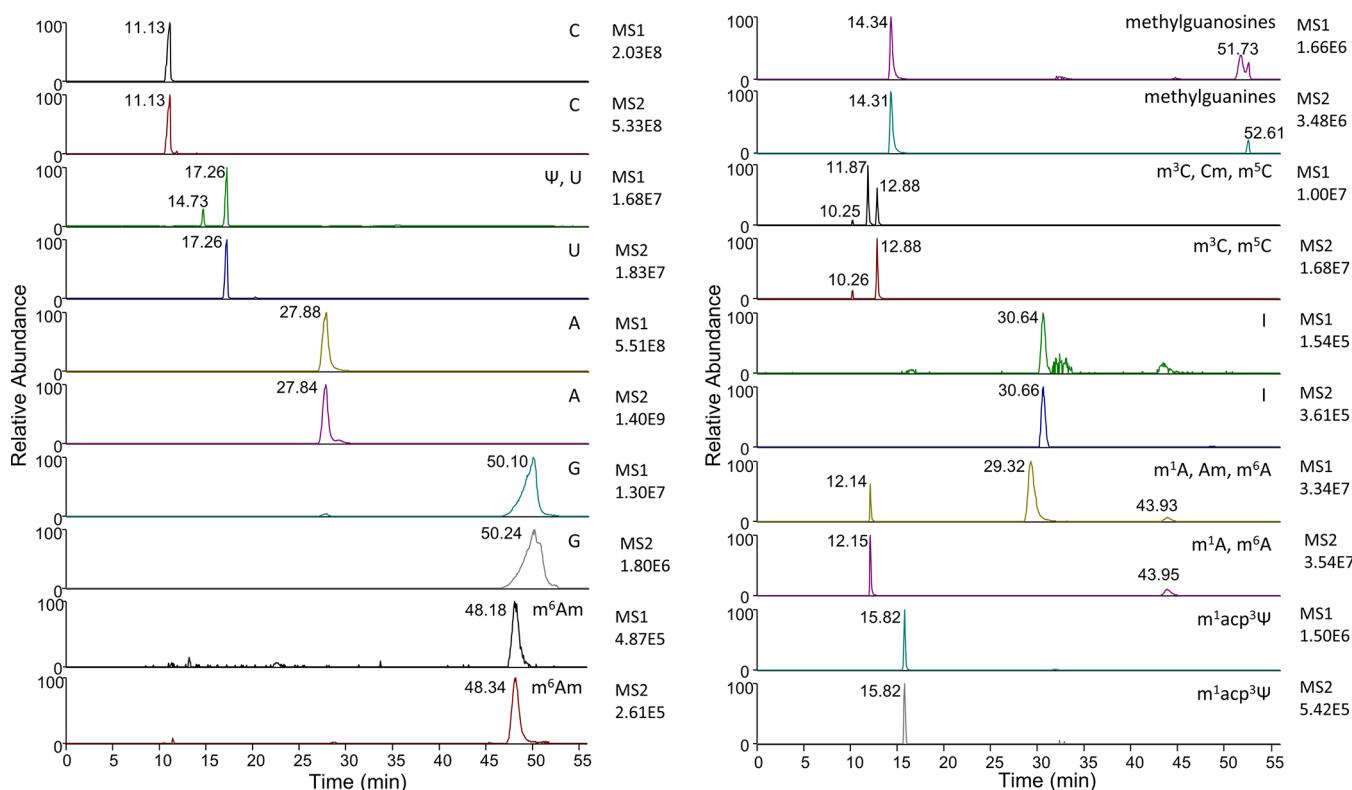


Figure 2. MS1 and MS2 chromatograms of HeLa nucleosides using DIA. Modifications are listed in order of appearance, with retention times denoting which peaks correspond to the listed modifications as ordered, respectively. A reduction in noise can be observed in the MS2 scans, most clearly for U, m^6 Am, and I. MS2 peaks for Ψ , Cm, and Am are not present as their fragment masses differ from the nucleobases of U and the base modifications of C and A.

consideration of which modifications are of interest is important, as not all of the ~160 known marks can be included in an optimized single rapid scan cycle. Many of the modifications are known to be restricted to certain species or RNA types, which can inform method design, but does not completely resolve the issue.¹⁹ Additionally, preliminary work can establish retention times for known modifications, allowing a method to have segments for targeted analyses which increase the number of targets that can be included in a single method. To perform each of these steps and design a method idealized for the modified nucleosides of interest, and additional nucleosides of possible interest, is a burden that can be overcome trivially with DIA. Moreover, DIA has the benefits of potential modification discovery and of remining old data after modifications are discovered. For example, in HeLa-extracted RNA, we were able to identify hm^5Cm , a modification that was described shortly after our data were acquired (Figure S1).²⁰ In this run, the hm^5Cm precursor was never more than the 452nd most abundant m/z , making it very unlikely to have been selected for fragmentation in high-resolution DDA scans.²¹ Thus, DIA occupies a space between targeted acquisition and DDA as, in this application, DIA retains practically none of the limitations of the other two methods (Figure 1B).

In order to facilitate discovery of modifications and accurate quantitation simultaneously, we sought to design a DIA instrument method that would provide unbiased mononucleoside data. The DIA isolation window scheme was designed to minimize interference and misidentifications even under poor chromatographic conditions: no more than 16 known unique nucleoside masses were included in a single window (326–351

m/z), and methylribose nucleoside fragmentation windows were separated from their respective canonical nucleoside fragmentation windows as many of their fragment ions are identical (Figure 1B). This is due to the lability of the glycosidic bond; with the exception of pseudouridine, nucleosides fragment favorably into intact bases corresponding to ribose losses.²² The DIA scan window optimization was performed without accounting for retention time effects, making the isolation window scheme applicable for any stationary phase and any chromatographic gradient runtime. This approach differs from a very recently published DIA method by using larger, nonuniform isolation windows designed to account for potential isobaric fragment ions.²³ Compared to small, uniform isolation windows, this allows for greater points across a peak and/or higher AGC targeting, enhancing quantitative precision and/or sensitivity.

To further compare DIA and DDA, we highlight the difficulty of DDA for discovery of post-transcriptional modifications and analysis of very low abundance modifications, as they can be lost in noise at the MS1 level. Moreover, DDA suffers the limitation of lacking MS2 quantitation unless dynamic exclusion is short enough to obtain several measurements across a peak (typically eight or more). Short dynamic exclusion exacerbates the discovery problem by limiting the number of low abundance peaks that can be selected for fragmentation. DIA resolves both issues by fragmenting all ions in each scan cycle regardless of their abundance and ensuring that sufficient points across a peak will be obtained for quantitation. Uridine and pseudouridine demonstrate the benefits of performing DIA, as the mass of these nucleosides has higher noise than other common nucleosides. We

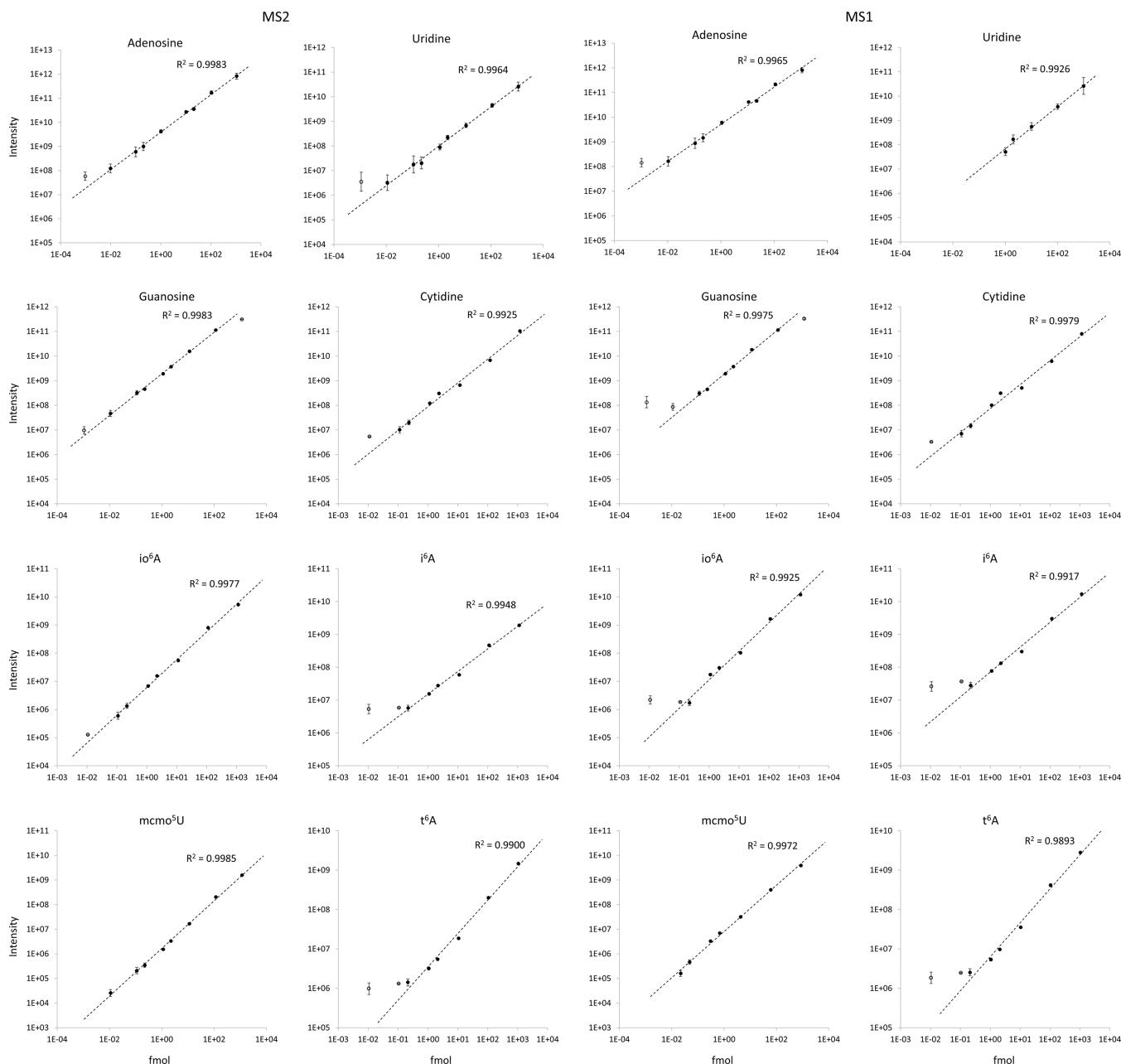


Figure 3. Calibration curves of nucleoside standards in a matrix of ^{13}C -glucose treated HeLa RNA. Unfilled points were not included in linear curves. All MS2 curves yielded superior R^2 values to MS1 curves and lower limits of quantitation (Table S1). Some data are absent from the uridine and mcmo ^5U MS1 curves but were present in the MS2 scans.

quantitated C, U, Ψ , A, G, m^3C , m^5C , Cm, I, m^1A , m^6A , Am, and $\text{m}^1\text{acp}^3\Psi$ using MS1 and MS2 scans from the same five biological replicate runs of extracted total HeLa RNA. Our MS2 quantitated results showed m^5C and Cm at 0.32% and 0.56% of C, respectively, and m^6A and Am at 0.10% and 1.3% of A, respectively, in the range of previously reported values.^{24–27} Compared to MS1 quantitation, MS2 analysis of all listed nucleosides yielded significantly lower relative standard deviation (RSD) (Figure 1C). The greatest difference in %RSD was observed in the uridine analysis. This can be observed qualitatively in chromatograms, which show decreased noise in MS2 scans when compared to MS1 scans. In particular, uridine, inosine, and m^6Am have substantially less relative noise in their respective MS2 scans (Figure 2).

We then generated calibration curves for A, U, G, C, io^6A , i^6A , mcmo ^5U , and t^6A in a background of $^{13}\text{C}_6$ glucose treated HeLa RNA using MS1 and DIA MS2 quantitation (Figure 3). The DIA method was modified to ensure that any heavy ribose containing HeLa nucleosides without at least one ^{13}C atom in the nucleobase were fragmented separately from the standards. Peaks were integrated in Skyline, manually validated, and exported for processing.²⁸ The calibration curves consistently yielded better linearity at the MS2 level and a lower limit of quantitation values (Table S1) as determined by the curves. In particular, MS2 quantitation allowed for inclusion of lower levels of uridine, guanosine, io^6A , and mcmo ^5U in the linear range of the curves. This was most pronounced for mcmo ^5U , in which the lowest level injection was not observable at the MS1 level, and for uridine, in which the lowest four injections

were not observable. These data show that DIA is capable of high quality MS2 quantitation without the need for targeted fragmentation or low dynamic exclusion and has the potential to identify nucleosides that are not observable at the MS1 level. Combined with Skyline data processing, this workflow simplifies MS nucleoside analysis from method design to results and can accommodate the preferred LC method of the user.

When using new LC methods, it is not always necessary to know expected nucleoside retention times beforehand, as many nucleosides have unique precursor and fragment ion m/z values, and even some isobaric species have unique fragments. Notably, HCD has previously been used to generate unique fragments from isobaric nucleosides, and these fragments were recently characterized in depth.²⁹ Using HCD fragmentation for DIA provided some fragments matching the literature at low levels (Table S2). In an hour-long method, high-energy HCD or targeted MS3 can be used for isobaric nucleosides without resulting in fewer than eight MS2 scans of the targeted isomers. These experiments were performed using standards to validate methylcytidine isomer retention times and fragmentation spectra (Figure S2). A direct comparison to QQQ methodology cannot be easily performed, but DIA is an attractive method for quantitation in Orbitrap instruments, as it is similar to targeted Orbitrap methods but has fewer limitations.³⁰

As nearly all known nucleosides fragment into the intact base, searching data can be performed by attempting to identify ribose loss, methylribose loss, or canonical nucleobases in the event a modification is fragmented as well. This allows for postacquisition data searches for neutral losses and nucleobases, both of which can be performed rapidly in MS-DIAL, and the latter of which can easily be applied in the Thermo Xcalibur Qual Browser. For example, searching for adenine across different DIA MS2 windows can identify fragment ions for which the respective precursor can be identified by the process of elimination within the range of the fragmentation window (Figure S3). DIA is not without limitations, as there is a higher potential for artifacts or coelutions to appear to be bona fide nucleosides.³¹ This issue only differs from DDA in that precursor ions can be misattributed to product ions; therefore, one of the first validation steps to perform in the event of modification discovery is targeted fragmentation. Internal standards can also be used with this DIA method provided that stable isotopes are present on the base. An alternative fragmentation scheme can also be used to ensure all modifications and their standards fragment in the same window (Table S3), ensuring AGC target matched quantitation. Overall, DIA decreases MS2 cycle time, ensures adequate points across each peak, fragments nucleosides regardless of abundance, and enables discovery of previously unobserved modifications in studies that may otherwise have missed new findings.

Wobble Position Modifications in *Arabidopsis thaliana*. RNA in *A. thaliana* has previously been reported to undergo modification changes in salt stress. Previous analyses have reported modification changes in mRNA that altered structure and transcription; however, not all translational changes could be attributed to mRNA modification and structure.³² To analyze another potential translation regulation mechanism, we used our DIA method with MS2 level nucleobase quantitation to examine RNA modifications, focusing on wobble position uridine modifications, in salt-

stressed *A. thaliana* (Table S4). Our results showed significant increases in 5-aminomethyluridine (nm⁵U), 5-carbamoylmethyluridine (ncm⁵U), 5-carbamoylhydroxymethyluridine (nchm⁵U), and 5-methoxycarbonylmethyluridine (mcm⁵U) (Figure 4), but no significant changes in tRNA modifications

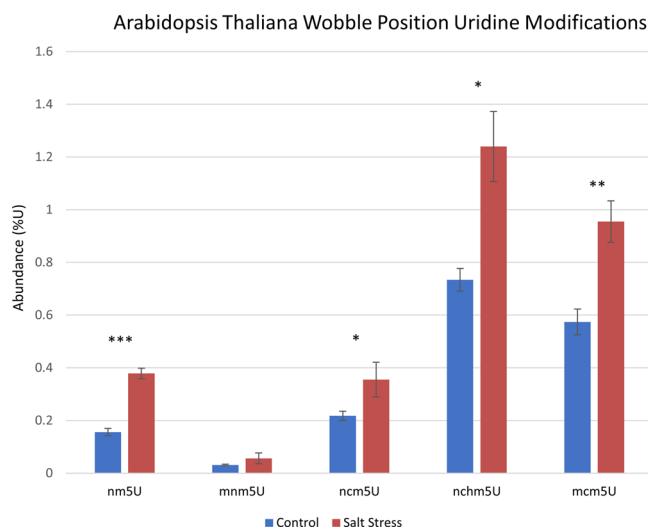


Figure 4. Uridine modifications upregulated in salt-stressed *A. thaliana*. All modifications have been previously observed in only the wobble position of different organisms. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

D and io⁶A. Common rRNA modification ac⁴C also did not show changes, nor did mRNA cap modification m⁷G. The significantly altered ncm⁵U modification is found at the wobble position of tRNA^{Pro}(UGG) in multiple species.^{33–37} Additionally, it has been reported that ncm⁵U or a modification in the same deposition pathway is likely to be modified at U34 in *A. thaliana* tRNA^{Pro}(UGG) as well; however, it has not been proven.^{38–40}

The biogenesis of ncm⁵U, nchm⁵U, and mcm⁵U has previously been shown to be TRM9 and TRM11 dependent, and ncm⁵U also required AtELP1 activity. Consistent with our RNA modification data, TRM11 was shown to be upregulated in transient salt stress in *A. thaliana*.⁴¹ Although our previously published proteomics experiments did not detect TRM9 or TRM11 due to dynamic range limitations, our previously published RNA-seq data did show increases in AtELP1 and in two isoforms each of TRM9 and TRM11 in salt stress (Table 1).³²

Because of the common identification of these uridine modifications in the anticodon loop, we sought to investigate

Table 1. mRNA Levels of tRNA Modifiers^a

gene	control (RPM)	salt (RPM)
TRM11 Isoform 1: AT3G26410	5.60	8.85
TRM11 Isoform 2: AT4G23020	5.14	7.00
TRM9 Isoform 1: AT1G31600	10.28	14.55
TRM9 Isoform 2: AT1G36310	24.05	25.57
AtELP1: AT3G52850	69.49	119.35

^a*A. thaliana* mRNA reads per million for RNA modifying enzymes. The listed modifications catalyze deposition of identified upregulated wobble position uridine modifications. All mRNA transcripts were upregulated in salt stress.

the possibility that they stabilize salt stress response translation.^{42,43} Proline tRNAs were selected for analysis due to the similarity between *A. thaliana* tRNA^{Pro}(UGG) and the same tRNA in modification annotated sequences. Codon enrichment for tRNA^{Pro}(UGG) was calculated as the quantity of CCA codons in an mRNA transcript divided by sum of CCT, CCC, and CCG codons, each of which encodes a different proline tRNA. This enrichment was compared to a measure of translation efficacy, normalized fold change in protein abundance over normalized fold change in mRNA transcript abundance, $\Delta\text{MS}/\Delta\text{mRNA}$ (Figure 5). The results show that

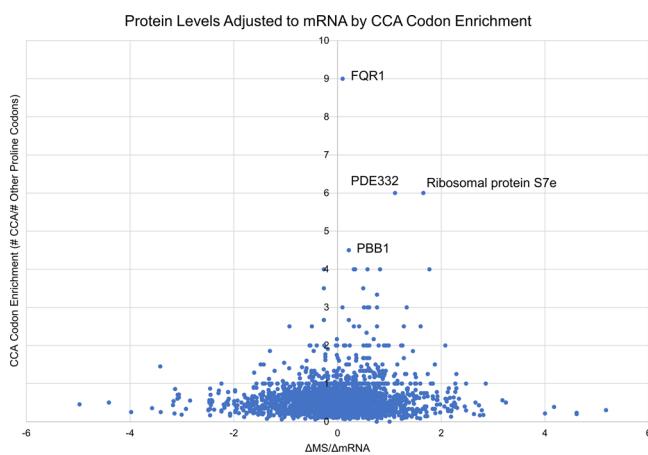


Figure 5. Log₂-normalized $\Delta\text{MS}/\Delta\text{mRNA}$ values plotted based on CCA-enrichment of the mRNA transcript. The highest CCA-enriched transcripts all show positive $\Delta\text{MS}/\Delta\text{mRNA}$ values, suggesting translation stabilization for these proteins. Both FQR1 and PBB1 have been reported to be upregulated in salt stress conditions.

highly CCA-enriched proteins more often had increases in $\Delta\text{MS}/\Delta\text{mRNA}$ than decreases. Two of the most CCA-enriched proteins, FQR1 and PBB1, have previously been reported to be upregulated in salt stress conditions.^{44–47} These two mRNA transcripts were stabilized; however, the stability was not m⁶A induced, suggesting an alternative mechanism. The transcript with the most CCA codons, At4G22485, had a moderate CCA enrichment but yielded a $\Delta\text{MS}/\Delta\text{mRNA}$ value of 1.44 and was previously reported to be upregulated in salt stress.⁴⁸ Finally, we performed the same analysis for tRNA^{Pro}(AGG) (Figure S4), which did not show a positive trend in $\Delta\text{MS}/\Delta\text{mRNA}$ at high CCT codon enrichment. These data provide an example of how RNA modification analysis can be integrated with RNA-seq and proteomic data sets to identify correlations in biology. However, additional work is required to determine if the wobble position uridine modifications are causative in salt-stress response translation stabilization.

CONCLUSIONS

In this work, we present DIA as an improvement to Orbitrap MS based RNA mononucleoside analysis. The fragmentation windows were designed to ensure that different precursor ions with isobaric fragment ions are isolated in different MS2 scans. For any chromatographic method, this is a simple approach that guarantees fragmentation of all knowns and unknowns while simultaneously allowing for MS1- and MS2-based quantitation. We used ¹³C glucose labeling to generate MS1- and MS2-quantitated calibration curves in a relevant matrix,

demonstrating the advantages of MS2 quantitation. Using this DIA method, we established that tRNA modifications in *A. thaliana* undergo changes in response to salt stress and suggest a possible regulatory mechanism for salt stress protein translation. We also demonstrate that DIA is superb at identifying modifications without a priori knowledge and showcase an example of mining old data with hm⁵Cm. These efforts represent a step forward in advancing RNA mononucleoside modification detection by mass spectrometry.

METHODS

Plant Growth and Harvesting. Plant growth took place in a chamber with a controlled cycle of 16 light hours and 8 dark hours at 22 °C. The ecotype of *Arabidopsis thaliana* was UPQ10:NTF/ACT2p:BirA Columbia-0. Salt concentrations were based on fresh weight decreases as previously determined.³²

Rosette leaves were crushed in liquid N₂, and RNA was extracted using Qiazol lysis reagent. QIAshredders (QIAGEN) were used to homogenize samples, and miRNeasy mini columns (QIAGEN) were used to extract RNA. Samples were treated with RNase-free DNase (QIAGEN) for 30 min at room temperature, and then RNA was ethanol precipitated.

RNA Purification. HeLa RNA was purified using TRIzol as previously described.⁴⁹ In short, cells were suspended in TRIzol/TriPure and centrifuged. The supernatant was mixed with chloroform and centrifuged. The aqueous phase was collected, and RNA was precipitated by adding 2-propanol. RNA was washed with ethanol and then dried in a Savant SpeedVac.

Cell Culture. HeLa cells were grown in Dulbecco's Modified Essential Medium (DMEM) lacking glucose, supplemented with 10% FBS, 1% glutamax, 1% penicillin/streptomycin, 1% sodium pyruvate, and 4.5 g/L ¹³C₆ glucose. Cells were cultured until the ribose moieties were completely ¹³C labeled and the bases contained at least one ¹³C. RNA was extracted using TRIzol as previously described.

RNA-Sequencing and Mass Spectrometry Data Analysis. RNA-sequencing (GEO: GSE108852) and mass spectrometry raw data were obtained from previous work.³² RNA-sequencing data were normalized to reads per million (RPM) as previously described. Mass spectrometry data were processed in MaxQuant and iBAQ values were used to normalize protein abundances relative to average abundance within runs. Both sets of data were log₂ transformed for salt stress fold change over wild type. The $\Delta\text{MS}/\Delta\text{mRNA}$ values were calculated by subtracting the resultant RNA log₂ fold change from the resultant mass spectrometry log₂ fold change.

RNA Digestion and Sample Processing. Purified RNA samples were resuspended in water and quantitated by nanodrop, and 500 ng of RNA was added to the reaction mixture: 10 μL of 1 mM ZnCl₂, 30 mM sodium acetate, and digestion enzymes. As it was previously determined that basic conditions can alter modifications, digestion was performed at pH 7.2.^{15,50,51} Four enzymes were used to digest the RNA to mononucleosides: 5 mU/ μL of nuclease P1, 500 $\mu\text{U}/\mu\text{L}$ of phosphodiesterase I, 6.25 $\mu\text{U}/\mu\text{L}$ of phosphodiesterase II, and 5 mU/ μL of shrimp alkaline phosphatase. Digestion was performed at room temperature for 4 h, 8 h, or overnight. Because *A. thaliana* samples had the potential to contain excess salt, postdigestion protein precipitation and sample dilution were not considered adequate for analysis.^{50,52,61,62,53–60} Indeed, we observed salt matrix effects in salt-added standards

(Figure S5). Therefore, digested samples were desalted using in-house Hypercarb PGC (Thermo Scientific) stagetips, similar to previously used commercial kits.¹⁷ In short, digested RNA was loaded onto the resin in 0.1% formic acid, washed with 0.1% formic acid, and eluted in 0.1% formic acid in 80% acetonitrile. For runs using weakly acidic HPLC buffers, ammonium acetate can be used instead of formic acid.

Liquid Chromatography. Online nanoflow PGC runs were performed using 0.1% formic acid as buffer A and 0.1% formic acid in acetonitrile as buffer B at 600 nL/min. Gradients were run as 0% B to 16% B over 5 min, to 32% B over 50 min, to 55% B over 16 min, and finally to 100% B over 14 min. 100% B was held for 5 min, totaling a 90 min run. The same gradient was proportionally adjusted to a 60 min run, the only exception being that the 5 min 100% B wash was retained. Microflow C18 runs were performed using the same buffers at 0.3 mL/min. The gradient was run as 0% B for 3 min followed by an increase to 2% B over 8 min, then to 15% B over 5 min, and finally, a 5 min 100% B wash. Nanoflow C18 runs were performed using 0.1% formic acid as buffer A and 0.1% formic acid in 80% acetonitrile as buffer B with a flow rate of 300 nL/min. Separation was achieved using a 34 min gradient from 0% B to 10% B, and then a 10 min gradient from 10% B to 30% B.

Mass Spectrometry. The DIA method used one full MS scan followed by 11 DIA MS2 scans. The full MS scan was from 200 to 800 *m/z* at 60k resolution with an AGC target of 400k and a maximum injection time of 50 ms. DIA fragmentation used 24% HCD energy and scan windows were, in order, 232–257, 257–271, 271–285, 285–298, 298–312, 312–326, 326–351, 351–394, 394–435, 435–500, and 500–600 *m/z*. All Orbitrap methods were paired with the nanoflow PGC LC method except for the calibration curve. Targeted QQQ methods were paired with microflow chromatography and used nucleoside monoisotopic masses for Q1 selection and corresponding nucleobase monoisotopic masses for Q3 selection except for pseudouridine, for which the nucleobase detection was at 125.035 *m/z*.

Nucleoside Calibration Curve. Nucleoside standards were mixed into a background of ¹³C-labeled HeLa RNA and injected at levels of 1 pmol, 100 fmol, 10 fmol, 2 fmol, 1 fmol, 200 amol, 100 amol, 10 amol, 1 amol, and 100 zmol. Calibration curves were performed using C18 nanoflow chromatography.

Data Analysis. Mass spectrometry data were analyzed manually and by using Skyline.²⁸ Layout templates in the Thermo Xcalibur Qual Browser were generated for MS1 scans using predicted *m/z* values based on chemical formulas in MODOMICS database with a tolerance of 10 ppm. Layouts for MS2 scans were designed based on expected ribose or methylribose loss and determined empirically for modified pseudouridines. Peaks were identified and integrated automatically and then inspected and adjusted by manually as necessary. Nucleoside identities were confirmed by reference standards, MS2 fragmentation patterns, and/or by MS3 (when applicable).

Rapid automated data analysis was performed using Skyline with an inclusion list for all known RNA modifications (see the Supporting Information). Expected retention times were varied by stationary phase and gradients, manually adjusted as necessary. Peaks were integrated automatically and inspected manually.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jasms.2c00065>.

Supplemental Figures S1–S5 and Supplemental Tables S1–S4 (PDF)

Skyline inclusion list (TXT)

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Notes

The authors declare no competing financial interest.

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REFERENCES

- (1) Boccaletto, P.; Machnicka, M. A.; Purta, E.; Piatkowski, P.; Baginski, B.; Wirecki, T. K.; de Crécy-Lagard, V.; Ross, R.; Limbach, P. A.; Kotter, A.; Helm, M.; Bujnicki, J. M. MODOMICS: A Database of RNA Modification Pathways. 2017 Update. *Nucleic Acids Res.* **2018**, *46* (D1), D303–D307.
- (2) Shi, H.; Wei, J.; He, C. Where, When, and How: Context-Dependent Functions of RNA Methylation Writers, Readers, and Erasers. *Mol. Cell* **2019**, *74* (4), 640–650.
- (3) Jung, Y.; Goldman, D. Role of RNA Modifications in Brain and Behavior. *Genes. Brain. Behav.* **2018**, *17* (3), e12444.
- (4) Thapar, R.; Bacolla, A.; Oyeniran, C.; Brickner, J. R.; Chinnam, N. B.; Mosammaparast, N.; Tainer, J. A. RNA Modifications: Reversal Mechanisms and Cancer. *Biochemistry* **2019**, *58* (5), 312–329.
- (5) McCown, P. J.; Ruszkowska, A.; Kunkler, C. N.; Breger, K.; Hulewicz, J. P.; Wang, M. C.; Springer, N. A.; Brown, J. A. Naturally Occurring Modified Ribonucleosides. *WIREs RNA* **2020**, *11*, e1595.
- (6) Fu, Y.; Dominissini, D.; Rechavi, G.; He, C. Gene Expression Regulation Mediated through Reversible M6A RNA Methylation. *Nat. Rev. Genet.* **2014**, *15* (5), 293–306.
- (7) Arango, D.; Sturgill, D.; Alhusaini, N.; Dillman, A. A.; Sweet, T. J.; Hanson, G.; Hosogane, M.; Sinclair, W. R.; Nanan, K. K.; Mandler, M. D.; Fox, S. D.; Zenguya, T. T.; Andresson, T.; Meier, J. L.; Coller,

J.; Oberdoerffer, S. Acetylation of Cytidine in mRNA Promotes Translation Efficiency. *Cell* **2018**, *175* (7), 1872–1886.e24.

(8) Safra, M.; Sas-Chen, A.; Nir, R.; Winkler, R.; Nachshon, A.; Bar-Yaacov, D.; Erlacher, M.; Rossmanith, W.; Stern-Ginossar, N.; Schwartz, S. The M1A Landscape on Cytosolic and Mitochondrial mRNA at Single-Base Resolution. *Nature* **2017**, *551* (7679), 251–255.

(9) Motorin, Y.; Helm, M. Methods for RNA Modification Mapping Using Deep Sequencing: Established and New Emerging Technologies. *Genes (Basel)* **2019**, *10* (1), 35.

(10) Li, X.; Xiong, X.; Yi, C. Epitranscriptome Sequencing Technologies: Decoding RNA Modifications. *Nat. Methods* **2017**, *14* (1), 23–31.

(11) Helm, M.; Motorin, Y. Detecting RNA Modifications in the Epitranscriptome: Predict and Validate. *Nat. Rev. Genet.* **2017**, *18*, 275.

(12) Torres, A. G.; Reina, O.; Stephan-Otto Attolini, C.; Ribas de Pouplana, L. Differential Expression of Human tRNA Genes Drives the Abundance of tRNA-Derived Fragments. *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *116* (17), 8451–8456.

(13) Shi, J.; Zhang, Y.; Tan, D.; Zhang, X.; Yan, M.; Zhang, Y.; Franklin, R.; Shahbazi, M.; Mackinlay, K.; Liu, S.; Kuhle, B.; James, E. R.; Zhang, L.; Qu, Y.; Zhai, Q.; Zhao, W.; Zhao, L.; Zhou, C.; Gu, W.; Murn, J.; Guo, J.; Carroll, D. T.; Wang, Y.; Chen, X.; Cairns, B. R.; Yang, X.; Schimmel, P.; Zernicka-Goetz, M.; Cheloufi, S.; Zhang, Y.; Zhou, T.; Chen, Q. PANDORA-Seq Expands the Repertoire of Regulatory Small RNAs by Overcoming RNA Modifications. *Nat. Cell Biol.* **2021**, *23* (4), 424–436.

(14) Pomerantz, S. C.; McCloskey, J. A. Analysis of RNA Hydrolyzates by Liquid Chromatography-Mass Spectrometry. *Methods Enzymol.* **1990**, *193*, 796–824.

(15) Miyauchi, K.; Kimura, S.; Suzuki, T. A Cyclic Form of N6-Threonylcarbamoyladenosine as a Widely Distributed tRNA Hypermodification. *Nat. Chem. Biol.* **2013**, *9* (2), 105–111.

(16) Hu, A.; Noble, W. S.; Wolf-Yadlin, A. Technical Advances in Proteomics: New Developments in Data-Independent Acquisition. *F1000Research* **2016**, *5*, 419.

(17) Sarin, L. P.; Kienast, S. D.; Leufken, J.; Ross, R. L.; Dziergowska, A.; Debiec, K.; Sochacka, E.; Limbach, P. A.; Fufezan, C.; Drexler, H. C. A.; Leidel, S. A. Nano LC-MS Using Capillary Columns Enables Accurate Quantification of Modified Ribonucleosides at Low Femtomol Levels. *RNA* **2018**, *24* (10), 1403–1417.

(18) Thüring, K.; Schmid, K.; Keller, P.; Helm, M. *LC-MS Analysis of Methylated RNA BT - RNA Methylation: Methods and Protocols*; Lusser, A., Ed.; Springer: New York, 2017; pp 3–18. DOI: [10.1007/978-1-4939-6807-7_1](https://doi.org/10.1007/978-1-4939-6807-7_1).

(19) Lane, B. G. Historical Perspectives on RNA Nucleoside Modifications. *Modification and Editing of RNA* **2014**, No. April28, 1–20.

(20) Huber, S. M.; van Delft, P.; Tanpure, A.; Miska, E. A.; Balasubramanian, S. 2'-O-Methyl-5-Hydroxymethylcytidine: A Second Oxidative Derivative of 5-Methylcytidine in RNA. *J. Am. Chem. Soc.* **2017**, *139* (5), 1766–1769.

(21) Davies, V.; Wandy, J.; Weidt, S.; van der Hooft, J. J. J.; Miller, A.; Daly, R.; Rogers, S. Rapid Development of Improved Data-Dependent Acquisition Strategies. *Anal. Chem.* **2021**, *93* (14), 5676–5683.

(22) Wetzel, C.; Limbach, P. A. Mass Spectrometry of Modified RNAs: Recent Developments. *Analyst* **2016**, *141* (1), 16–23.

(23) Espadas, G.; Morales-Sanfrutos, J.; Medina, R.; Lucas, M. C.; Novoa, E. M.; Sabidó, E. High-Performance Nano-Flow Liquid Chromatography Column Combined with High- and Low-Collision Energy Data-Independent Acquisition Enables Targeted and Discovery Identification of Modified Ribonucleotides by Mass Spectrometry. *J. Chromatogr. A* **2022**, *1665*, 462803.

(24) Lin, X.; Chai, G.; Wu, Y.; Li, J.; Chen, F.; Liu, J.; Luo, G.; Tauler, J.; Du, J.; Lin, S.; He, C.; Wang, H. RNA M6A Methylation Regulates the Epithelial Mesenchymal Transition of Cancer Cells and Translation of Snail. *Nat. Commun.* **2019**, *10* (1), 2065.

(25) Roundtree, I. A.; Luo, G.-Z.; Zhang, Z.; Wang, X.; Zhou, T.; Cui, Y.; Sha, J.; Huang, X.; Guerrero, L.; Xie, P.; He, E.; Shen, B.; He, C. YTHDC1 Mediates Nuclear Export of N6-Methyladenosine Methylated mRNAs. *Elife* **2017**, *6*, e31311.

(26) Yu, Y.; Zhu, S.-H.; Yuan, F.; Zhang, X.-H.; Lu, Y.-Y.; Zhou, Y.-L.; Zhang, X.-X. Ultrasensitive and Simultaneous Determination of RNA Modified Nucleotides by Sheathless Interfaced Capillary Electrophoresis–Tandem Mass Spectrometry. *Chem. Commun.* **2019**, *55* (53), 7595–7598.

(27) Fu, L.; Amato, N. J.; Wang, P.; McGowan, S. J.; Niedernhofer, L. J.; Wang, Y. Simultaneous Quantification of Methylated Cytidine and Adenosine in Cellular and Tissue RNA by Nano-Flow Liquid Chromatography–Tandem Mass Spectrometry Coupled with the Stable Isotope-Dilution Method. *Anal. Chem.* **2015**, *87* (15), 7653–7659.

(28) MacLean, B.; Tomazela, D. M.; Shulman, N.; Chambers, M.; Finney, G. L.; Frewen, B.; Kern, R.; Tabb, D. L.; Liebler, D. C.; MacCoss, M. J. Skyline: An Open Source Document Editor for Creating and Analyzing Targeted Proteomics Experiments. *Bioinformatics* **2010**, *26* (7), 966–968.

(29) Jora, M.; Burns, A. P.; Ross, R. L.; Lobue, P. A.; Zhao, R.; Palumbo, C. M.; Beal, P. A.; Addepalli, B.; Limbach, P. A. Differentiating Positional Isomers of Nucleoside Modifications by Higher-Energy Collisional Dissociation Mass Spectrometry (HCD MS). *J. Am. Soc. Mass Spectrom.* **2018**, *29* (8), 1745–1756.

(30) Quinn, R.; Basanta-Sanchez, M.; Rose, R. E.; Fabris, D. Direct Infusion Analysis of Nucleotide Mixtures of Very Similar or Identical Elemental Composition. *J. Mass Spectrom.* **2013**, *48* (6), 703–712.

(31) Tyagi, S. K.; Dryhurst, G. Electrochemical Oxidation of 9β-D-Ribofuranosyluric Acid in Basic Solution. *J. Electroanal. Chem. Interfacial Electrochem.* **1987**, *223* (1), 119–141.

(32) Kramer, M. C.; Janssen, K. A.; Palos, K.; Nelson, A. D. L.; Vandivier, L. E.; Garcia, B. A.; Lyons, E.; Beilstein, M. A.; Gregory, B. D. N6-Methyladenosine and RNA Secondary Structure Affect Transcript Stability and Protein Abundance during Systemic Salt Stress in Arabidopsis. *Plant Direct* **2020**, *4* (7), e00239.

(33) Hoffman, K. S.; Berg, M. D.; Shilton, B. H.; Brandl, C. J.; O'Donoghue, P. Genetic Selection for Mistranslation Rescues a Defective Co-Chaperone in Yeast. *Nucleic Acids Res.* **2017**, *45* (6), 3407–3421.

(34) Huang, B. O.; Johansson, M. J. O.; Byström, A. S. An Early Step in Wobble Uridine tRNA Modification Requires the Elongator Complex. *RNA* **2005**, *11* (4), 424–436.

(35) Hayashi, S.; Mori, S.; Suzuki, T.; Suzuki, T.; Yoshihisa, T. Impact of Intron Removal from tRNA Genes on *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **2019**, *47* (11), 5936–5949.

(36) Chou, H.-J.; Donnard, E.; Gustafsson, H. T.; Garber, M.; Rando, O. J. Transcriptome-Wide Analysis of Roles for tRNA Modifications in Translational Regulation. *Mol. Cell* **2017**, *68* (5), 978–992.e4.

(37) Johansson, M. J. O.; Esberg, A.; Huang, B.; Björk, G. R.; Byström, A. S. Eukaryotic Wobble Uridine Modifications Promote a Functionally Redundant Decoding System. *Mol. Cell. Biol.* **2008**, *28* (10), 3301–3312.

(38) Chen, P.; Jäger, G.; Zheng, B. Transfer RNA Modifications and Genes for Modifying Enzymes in *Arabidopsis thaliana*. *BMC Plant Biol.* **2010**, *10*, 201.

(39) Warren, J. M.; Salinas-Giegé, T.; Hummel, G.; Coots, N. L.; Svendsen, J. M.; Brown, K. C.; Drouard, L.; Sloan, D. B. Combining tRNA Sequencing Methods to Characterize Plant tRNA Expression and Post-Transcriptional Modification. *RNA Biol.* **2021**, *18* (1), 64–78.

(40) Nakai, Y.; Horiguchi, G.; Iwabuchi, K.; Harada, A.; Nakai, M.; Hara-Nishimura, I.; Yano, T. tRNA Wobble Modification Affects Leaf Cell Development in *Arabidopsis thaliana*. *Plant Cell Physiol.* **2019**, *60* (9), 2026–2039.

(41) Jülke, S.; Ludwig-Müller, J. Response of *Arabidopsis thaliana* Roots with Altered Lipid Transfer Protein (LTP) Gene Expression to

the Clubroot Disease and Salt Stress. *Plants (Basel, Switzerland)* **2016**, *5* (1), 2.

(42) Endres, L.; Dedon, P. C.; Begley, T. J. Codon-Biased Translation Can Be Regulated by Wobble-Base tRNA Modification Systems during Cellular Stress Responses. *RNA Biol.* **2015**, *12* (6), 603–614.

(43) Gu, C.; Begley, T. J.; Dedon, P. C. tRNA Modifications Regulate Translation during Cellular Stress. *FEBS Lett.* **2014**, *588* (23), 4287–4296.

(44) Bach-Pages, M.; Homma, F.; Kourelis, J.; Kaschani, F.; Mohammed, S.; Kaiser, M.; van der Hoorn, R. A. L.; Castello, A.; Preston, G. M. Discovering the RNA-Binding Proteome of Plant Leaves with an Improved RNA Interactome Capture Method. *Biomolecules* **2020**, *10* (4), 661.

(45) Xu, F.-Q.; Xue, H.-W. The Ubiquitin-Proteasome System in Plant Responses to Environments. *Plant. Cell Environ.* **2019**, *42* (10), 2931–2944.

(46) Ditt, R. F.; Kerr, K. F.; de Figueiredo, P.; Delrow, J.; Comai, L.; Nester, E. W. The *Arabidopsis* Thaliana Transcriptome in Response to *Agrobacterium* Tumefaciens. *Mol. Plant. Microbe. Interact.* **2006**, *19* (6), 665–681.

(47) Witzel, K.; Matros, A.; Strickert, M.; Kaspar, S.; Peukert, M.; Mühlung, K. H.; Börner, A.; Mock, H.-P. Salinity Stress in Roots of Contrasting Barley Genotypes Reveals Time-Distinct and Genotype-Specific Patterns for Defined Proteins. *Mol. Plant* **2014**, *7* (2), 336–355.

(48) He, Z.; Li, Z.; Lu, H.; Huo, L.; Wang, Z.; Wang, Y.; Ji, X. The NAC Protein from *Tamarix Hispida*, ThNAC7, Confers Salt and Osmotic Stress Tolerance by Increasing Reactive Oxygen Species Scavenging Capability. *Plants (Basel, Switzerland)* **2019**, *8* (7), 221.

(49) He, C.; Bozler, J.; Janssen, K. A.; Wilusz, J. E.; Garcia, B. A.; Schorn, A. J.; Bonasio, R. TET2 Chemically Modifies tRNAs and Regulates tRNA Fragment Levels. *Nat. Struct. Mol. Biol.* **2021**, *28*, 62.

(50) Kasai, H.; Murao, K.; Nishimura, S.; Liehr, J. G.; Crain, P. F.; McCloskey, J. A. Structure Determination of a Modified Nucleoside Isolated from *Escherichia Coli* Transfer Ribonucleic Acid. *Eur. J. Biochem.* **1976**, *69* (2), 435–444.

(51) Dominissini, D.; Nachtergael, S.; Moshitch-Moshkovitz, S.; Peer, E.; Kol, N.; Ben-Haim, M. S.; Dai, Q.; Di Segni, A.; Salmon-Divon, M.; Clark, W. C.; Zheng, G.; Pan, T.; Solomon, O.; Eyal, E.; Hershkovitz, V.; Han, D.; Doré, L. C.; Amariglio, N.; Rechavi, G.; He, C. The Dynamic N(1)-Methyladenosine Methylome in Eukaryotic Messenger RNA. *Nature* **2016**, *530* (7591), 441–446.

(52) Dudley, E.; Bond, L. Mass Spectrometry Analysis of Nucleosides and Nucleotides. *Mass Spectrom. Rev.* **2014**, *33* (4), 302–331.

(53) Synold, T.; Xi, B.; Wuenschell, G. E.; Tamae, D.; Figarola, J. L.; Rahbar, S.; Termini, J. Advanced Glycation End Products of DNA: Quantification of N2-(1-Carboxyethyl)-2'-Deoxyguanosine in Biological Samples by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry. *Chem. Res. Toxicol.* **2008**, *21* (11), 2148–2155.

(54) Castleberry, C. M.; Rodicio, L. P.; Limbach, P. A. Electrospray Ionization Mass Spectrometry of Oligonucleotides. *Curr. Protoc. nucleic acid Chem.* **2008**, Chapter 35, Unit 10.2. 35 DOI: 10.1002/0471142700.nc1002s35.

(55) Cai, W. M.; Chionh, Y. H.; Hia, F.; Gu, C.; Kellner, S.; McBee, M. E.; Ng, C. S.; Pang, Y. L. J.; Prestwich, E. G.; Lim, K. S.; Babu, I. R.; Begley, T. J.; Dedon, P. C. A Platform for Discovery and Quantification of Modified Ribonucleosides in RNA: Application to Stress-Induced Reprogramming of tRNA Modifications. *Methods Enzymol.* **2015**, *560*, 29–71.

(56) van Dongen, W. D.; Niessen, W. M. A. Bioanalytical LC-MS of Therapeutic Oligonucleotides. *Bioanalysis* **2011**, *3* (5), 541–564.

(57) Thüring, K.; Schmid, K.; Keller, P.; Helm, M. Analysis of RNA Modifications by Liquid Chromatography–Tandem Mass Spectrometry. *Methods* **2016**, *107*, 48–56.

(58) Crain, P. F. B. T.-M. in E. [42] Preparation and Enzymatic Hydrolysis of DNA and RNA for Mass Spectrometry. In *Mass Spectrometry*; Academic Press, 1990; Vol. 193, pp 782–790. DOI: 10.1016/0076-6879(90)93450-Y.

(59) Sakaguchi, Y.; Miyauchi, K.; Kang, B.; Suzuki, T. Nucleoside Analysis by Hydrophilic Interaction Liquid Chromatography Coupled with Mass Spectrometry. *Methods Enzymol.* **2015**, *560*, 19–28.

(60) Noestheden, M.; Hu, Q.; Tonary, A. M.; Tay, L.-L.; Pezacki, J. P. Evaluation of Chemical Labeling Strategies for Monitoring HCV RNA Using Vibrational Microscopy. *Org. Biomol. Chem.* **2007**, *5* (15), 2380–2389.

(61) Rose, R. E.; Pazos, M. A., 2nd; Curcio, M. J.; Fabris, D. Global Epitranscriptomics Profiling of RNA Post-Transcriptional Modifications as an Effective Tool for Investigating the Epitranscriptomics of Stress Response. *Mol. Cell. Proteomics* **2016**, *15* (3), 932–944.

(62) Kellner, S.; Ochel, A.; Thüring, K.; Spenkuch, F.; Neumann, J.; Sharma, S.; Entian, K.-D.; Schneider, D.; Helm, M. Absolute and Relative Quantification of RNA Modifications via Biosynthetic Isotopomers. *Nucleic Acids Res.* **2014**, *42* (18), e142.

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