RESEARCH ARTICLE

Title: Epitranscriptome changes triggered by ammonium nutrition regulate the proteome response of maritime pine roots.

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34 **ABSTRACT**

35 Epitranscriptomic modifications constitute a gene expression checkpoint in all living
 36 organisms. As nitrogen is an essential element for plant growth and development, a
 37 reasonable hypothesis is that changes in the epitranscriptome may regulate nitrogen
 38 acquisition and metabolism. In this study, epitranscriptomic modifications caused by
 39 ammonium nutrition were monitored in maritime pine roots through direct RNA
 40 sequencing using Oxford Nanopore Technology. Transcriptomic responses mainly
 41 affected transcripts involved in nitrogen and carbon metabolism, defense, hormone
 42 synthesis/signaling, and translation. Global detection of epitranscriptomic marks was
 43 performed to evaluate this posttranscriptional mechanism in untreated and ammonium-
 44 treated seedlings. Increased m⁶A deposition in the 3'-UTR was observed in response to
 45 ammonium, which seems to be correlated with poly(A) lengths and changes in the
 46 relative abundance of the corresponding proteins. The results showed that m⁶A
 47 deposition and its dynamics seem to be important regulators of translation under
 48 ammonium nutrition. These findings suggest that protein translation is finely regulated
 49 through epitranscriptomic marks likely by changes in mRNA poly(A) length, transcript
 50 abundance and ribosome protein composition. An integration of multiomics data
 51 suggests that the epitranscriptome modulates responses to developmental and
 52 environmental changes, including ammonium nutrition, through buffering, filtering, and
 53 focusing the final products of gene expression.

54

55 **Keywords:** Nitrogen, ammonium, epitranscriptomics, ONT sequencing, maritime pine,

56 INTRODUCTION

57 Discoveries in the nascent field of molecular biology culminated in the central dogma of
58 molecular biology during the 1970s (Crick, 1970). Currently, it is known that
59 transcription and translation processes are not always directly linked. Multiple factors
60 intervene in the gene response and its regulation. Two good examples of this are long
61 noncoding RNAs (lncRNAs) (Liu et al., 2015) and microRNAs (miRNAs) (Paul et al.,
62 2015). These types of RNA regulate important aspects of both development and
63 response to external stimuli in plants (Liu et al., 2015; Paul et al., 2015). However, these
64 studies represent only some aspects of overall RNA metabolism. In recent years, an
65 increasing interest has focused on determining the biological role of RNA chemical
66 modifications emerging as a new field of study under the term *epitranscriptomics*.
67 These modifications are found in all RNA types, such as transfer RNAs (tRNAs),
68 ribosomal RNAs (rRNAs), messenger RNAs (mRNAs) and small RNAs (sRNAs)
69 (Xiong et al., 2017). To date, more than 160 different modifications have been
70 identified in RNA (Shen et al., 2019). In *Arabidopsis thaliana*, m⁷G, m⁶A, m¹A, m⁵C,
71 hm⁵C, and uridylation have been identified as modifications in mRNA (Shen et al.,
72 2019). The marriage between classical detection techniques and high-throughput
73 sequencing has allowed to determine N⁶-methyladenosine (m⁶A) as the most prevalent
74 chemical modification in mRNAs, both in animals and plants (Fray and Simpson,
75 2015). Transcriptome-wide analysis revealed that the m⁶A mark in transcripts is
76 predominantly localized near the stop codon and throughout the 3' untranslated region
77 (UTR) (Shen et al., 2019). An m⁶A methylation motif (RRACH [R = A/G; H = A/U/C;
78 A = m⁶A]) that is conserved between plants and other eukaryotic organisms has been
79 described (Shen *et al.*, 2019). m⁶A deposition, recognition and elimination are carried
80 out by different proteins (Shen et al., 2019). Several cellular functions have been
81 observed to be affected by m⁶A modification, such as mRNA stability (Wei et al., 2018)
82 or translational efficiency (Luo et al., 2014). In addition, proper m⁶A deposition has
83 been reported to be essential during *Arabidopsis* embryo development (Zhong et al.,
84 2008) and to take part in biotic and abiotic plant stress responses (Martínez-Pérez et al.,
85 2017; Anderson et al., 2018), fruit ripening (Zhou et al., 2019), flowering transition
86 (Duan et al., 2017), leaf morphogenesis (Arribas-Hernández et al., 2018), trichome

development (Bodi et al., 2012) and apical shoot meristem development (Shen et al., 2016).

Nevertheless, little is known about the potential role of the epitranscriptome in the regulation of nitrogen (N) nutrition, with only the involvement of m⁶A in the regulation of nitrate assimilation and signaling having been studied (Hou et al., 2021). However, N is an essential element for plant growth and development and a key component of cellular constituents such as nucleic acids, proteins, and chlorophylls (Hawkesford et al., 2012). In soils, plants can take up N inorganic forms such as nitrate (NO₃⁻) or ammonium (NH₄⁺) and organic forms such as urea, peptides, or amino acids (Jia and von Wirén, 2020). Plants such as rice, tea or maritime pine prefer NH₄⁺ over NO₃⁻ as the main N source (Sasakawa and Yamamoto, 1978; Ruan et al., 2016; Ortigosa et al., 2020). In many plants, important changes in the transcriptome, proteome and metabolome have been described in relation to nitrogen nutrition and have mainly focused on the supply of NO₃⁻ and NH₄⁺ (Patterson et al., 2010; Yang et al., 2018; Ravazzolo et al., 2020). In this way, it has been observed that these N forms trigger both shared and differential responses involving different pathways and many result in phenotypic differences such as specific changes in the root system architecture (RSA) and growth (Jia and von Wirén, 2020). Therefore, it is reasonable to hypothesize that some of these described responses to N nutrition may be influenced by epitranscriptome regulatory processes.

Oxford Nanopore Technology (ONT) is a third-generation sequencing platform that is currently the only option for direct sequencing of RNA samples without the requirement of reverse transcription and amplification steps (Parker et al., 2020). These characteristics are of great relevance in transcriptomics since they allow the reduction of sequencing biases and maintenance of nucleoside modifications that facilitate epitranscriptomics studies (Gao et al., 2021).

The aim of the present work is to shed light on the short-term response of maritime pine roots to NH₄⁺ nutrition, elucidating what kind of regulatory relationship exists between transcriptomics, epitranscriptomics, proteomics and metabolite profiles. For this purpose, cutting edge and commonly used omics approaches, such as comprehensive transcriptome analyses by direct RNA sequencing (DRS) using Oxford Nanopore Technology (ONT), epitranscriptomic modification detection focused on m⁶A assisted

119 by the ONT platform, and quantitative proteomic and metabolite profiling, were
120 combined in the present study.

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123 **RESULTS**

124 **Metabolite profiling in response to NH_4^+ nutrition**

125 Changes in the amounts of polar metabolites caused by short-term NH_4^+ nutrition were
 126 analyzed in maritime pine roots (Supplemental Dataset 1). A statistically significant
 127 decrease in the amounts of carbohydrates such as sucrose and glucose was observed
 128 (Figure 1; Supplemental Figure 1). Additionally, a statistically significant increase in
 129 shikimate levels was noted. In turn, although the results were not statistically
 130 significant, there was a slight trend of increased levels of glycolysis intermediary
 131 metabolites such as glucose-6 phosphate and glucose-1 phosphate as well as TCA
 132 anaplerotic pathway intermediates such as cis-aconitate and GABA. In parallel, an
 133 increase in the accumulation of L-glutamine, L-glutamate, and L-methionine, as well as
 134 in aromatic amino acids (L-phenylalanine and L-tryptophan), was observed in NH_4^+ -
 135 treated plants. In contrast, metabolites such as L-asparagine, isocitrate, 2-oxoglutarate
 136 and L-malate were observed to decrease.

138 **Direct RNA sequencing (DRS)**

139 The global sequencing results are shown in Supplemental Table 1. The mean read sizes
 140 were between 908 bp to 1059 bp (Figure 2A). The longest reads ranged from 10298 bp
 141 to 14299 bp. Differential expression analyses identified 350 differentially expressed
 142 (DE) transcripts. From the total DE transcripts obtained, 106 were upregulated and 244
 143 were downregulated (Figure 2B; Supplemental Dataset 2). Singular enrichment analysis
 144 (SEA) was performed individually for each gene expression regulation (up- and
 145 downregulated) to classify the biological functions under NH_4^+ nutrition. The SEA
 146 global results are shown in Supplemental Dataset 3. Upregulated transcripts were
 147 significantly enriched with GO terms for Biological Process (BP) such as “ammonia
 148 assimilation cycle” (GO:0019676), “protein glutathionylation” (GO:0010731),
 149 “response to cadmium ion” (GO:0046686) and “developmental growth” (GO:0048589);
 150 for Cellular Component (CC) such as “chloroplast stroma” (GO:0009570) and
 151 “cytosolic ribosome” (GO:0022626); and for Molecular Function (MF) such as
 152 “glutamate synthase (NADH) activity” (GO:0016040), “acylglycerol lipase activity”
 153 (GO:0047372) and “phospholipase activity” (GO:0004620) (Figure 2C; Supplemental
 154 Figure 2). The downregulated transcripts were enriched in the BP terms “response to

155 heat” (GO:0009408), “response to water deprivation” (GO:0009414), “protein folding”
156 (GO:0006457) and “ethylene-activated signaling pathway” (GO:0009873) and the MF
157 terms “calcium ion binding” (GO:0005509), “transcription coactivator activity”
158 (GO:0003713), “mRNA binding” (GO:0003729), “chaperone binding” (GO:0051087)
159 and “transcription factor activity, sequence-specific DNA binding” (GO:0003700). A
160 more detailed study of the upregulated transcriptomic response revealed that *PpGS1b*
161 (pp_68481) and *PpNADH-GOGAT* (pp_238920) were upregulated. Interestingly,
162 transcripts for genes involved in defense-related response were upregulated: *PpAMP1*
163 (pp_58005, pp_58008), different class IV chitinases (pp_239593, pp_239598,
164 pp_239600, pp_117809), different splicing coding forms of patatin-like protein 2
165 (pp_71017, pp_71018, pp_71019, pp_71020, pp_71022), a PR-1 pathogenesis-related
166 protein (pp_87427), a defensin coding transcript (pp_92119) and an RPW8 domain-
167 containing protein (pp_142311) (Supplemental Dataset 2). Cell wall-related transcripts
168 were also upregulated, such as those encoding expansin-A18 (pp_134987), nonclassical
169 arabinogalactan protein 30 (pp_66323), probable prolyl 4-hydroxylase 4 (pp_235715),
170 xyloglucan:xyloglucosyl transferase (pp_68519) and different forms of xyloglucan
171 endotransglucosylase/hydrolase (pp_66707, pp_66708, pp_68517), was also observed.
172 In contrast, the downregulation of different transcription factors (TFs) was observed
173 (Supplemental Dataset 2), such as ethylene response factors (ERFs) (pp_58625,
174 pp_58626, pp_86737, pp_96228, pp_96234), a trihelix transcription factor (pp_59947),
175 and a MYB coding transcript (pp_202778). The repression of different splicing forms of
176 an auxin-repressed protein/dormancy-auxin associated protein coding transcript
177 (pp_58457, pp_58458, pp_58459, pp_58461, pp_58462, pp_58463), the SnRK1
178 regulator FCS-like zinc finger transcript (pp_202096), and transcripts encoding
179 carbohydrate metabolism enzymes such as pyruvate decarboxylase (pp_78343,
180 pp_123611, pp_126347) and sucrose synthase (pp_144843) was also observed. The
181 differential expressions observed in DRS was confirmed by RT-qPCR analyses of seven
182 transcripts including *SAM synthase*, *MYB5*, *PFK*, *AMP1*, *NADH-GOGAT*, *GS1b*, and
183 *ASPG*. The results showed the same trend between the DRS and RT-qPCR results for
184 all DE transcripts, with some differences in the logFC values (Figure 2D).

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187 **Differential DRS epitranscriptomics**

188 To determine the differential epitranscriptomic marks, DRS results were explored to
 189 identify the chemically modified nucleosides in the mRNAs using Tombo software.
 190 After analysis, 513 statistically significant putative modified nucleosides were obtained
 191 in 283 transcripts (Figure 3A; Supplemental Dataset 4). There were 221 undermodified
 192 positions in 161 transcripts and 292 overmodified positions in 184 transcripts. Among
 193 them, 58 transcripts had significant over- and undermodified positions, including
 194 *PpGS1b* (pp_68474) and *translationally-controlled tumor protein (TCTP, pp_72505)*
 195 (Supplemental Dataset 5). The percentage of modifications for each kind of nucleoside
 196 was similar for adenosine, guanosine, and uridine (28%, 29% and 25%) but lower for
 197 cytidine (17%), without any obvious difference between sample conditions (Figure 3B).
 198 When the global set of modification ratios and transcript amounts were compared
 199 (Figure 3C), significant and negative correlations were found to be slightly stronger
 200 under NH_4^+ supply (-0.36) than under the control (-0.30) (Figure 3C). Modification
 201 ratios of individual nucleosides compared to transcript amounts were also similar
 202 (Supplemental Figure 3).

203 The functions of the transcripts with DE modifications were analyzed using SEA
 204 (Figure 3D; Supplemental Figure 4; Supplemental Dataset 6). A total of 116 significant
 205 GO terms were obtained. At the BP level, the main functions were related to the terms
 206 “ribosome biogenesis” (GO: 0042254); “translation” (GO:0006412), “proteolysis”
 207 (GO:0006508), “protein ubiquitination” (GO:0016567), “response to stress”
 208 (GO:0006950), “response to external biotic stimulus” (GO:0043207) and “glutamine
 209 metabolic process” (GO:0006541). Similarly, the “ribosome” term (GO:0005840) was
 210 the main function at the CC level. Finally, the terms “GTPase activity” (GO:0003924),
 211 “mRNA binding” (GO:0003729), “translation factor activity, RNA binding”
 212 (GO:0008135), “ubiquitin protein ligase binding” (GO:0031625), “ubiquitin
 213 conjugating enzyme activity” (GO:0061631) and “endopeptidase inhibitor activity”
 214 (GO:0004866) were the main functions at the MF level.

215 The differential modifications were verified using SELECT. This qPCR-based
 216 technique was initially designed to determine differential deposition of m^6A in total
 217 RNA mixtures. However, nucleoside modifications putatively detected in cytidine and
 218 uridine by Tombo were also detected with SELECT (Figure 3E). The obtained results

for each position showed a similar trend between the differential epitranscriptomic results from SELECT and Tombo (Figure 3E).

m⁶A identification

The bioinformatic pipeline Nanom6A was used to specifically identify m⁶A modifications in the RRACH sites from DRS data (Supplemental Dataset 7). The distribution of m⁶A sites along the full-length transcripts showed a higher accumulation of marks over two-thirds of the relative length in control samples while a higher accumulation of marks in the final portion of the transcripts was observed in NH₄⁺-treated samples (Figure 4A). A more detailed distribution study showed that m⁶A sites were more abundant in the 5'-UTR and coding (CDS) regions under the control condition, while m⁶A sites tended to be more abundant in the 3'-UTR regions of transcripts isolated from NH₄⁺-treated seedlings (Figure 4B). The m⁶A frequency was higher in the CDS, mainly in the central portion, and at the beginning of the 3'-UTR regions, but it was lower at the transcript ends (Figures 4A and 4B).

The most predominant RRACH sequence was AAACA (>15%), while GGACC was the least abundant (< 5%) (Figure 4C). The comparison between m⁶A modification ratios and transcript amounts showed significant negative correlations for the control (-0.42) and NH₄⁺ conditions (-0.46) (Figure 4D). The lengths of the poly(A) tails were determined from DRS data (Figure 4E). As expected, most of the poly(A) tails had a size between 40-250 nt with similar means in both conditions, 91.01 nt and 91.8 nt. The poly(A) tail lengths had significant positive correlations with m⁶A ratios in both conditions (0.2) (Figure 4F). Significant but lower positive correlations were found when poly(A) tail lengths were compared with global and nucleoside modification ratios obtained with Tombo (Supplemental Figure 5). Finally, the poly(A) tail lengths and transcript amounts exhibited significant negative correlations for the control (-0.17) and NH₄⁺ conditions (-0.18) (Figure 4G).

Differential proteomics

A total of 2,385 proteins were identified in the shotgun proteomics analysis (Supplemental Dataset 8). Among the identified proteins, 114 were differentially

regulated by NH_4^+ : 38 were more abundant, while 76 were less represented (Figure 5A; Supplemental Dataset 8). To elucidate the biological roles of the identified proteins, SEA analyses were performed (Figure 5B; Supplemental Figure 6; Supplemental Dataset 9). The upregulated proteins showed as representative BP terms “cell redox homeostasis” (GO:0045454), “protein complex assembly” (GO:0006461), “translation” (GO:0006412), “cellular response to oxidative stress” (GO:0006979) and “defense response to bacterium” (GO:0042742). At CC level, “ribosome” (GO:0005840), “nucleolus” (GO:0005730) and “chloroplast” (GO:0009507) were the enriched terms. Finally, “structural constituent of ribosome” (GO:0003735) and “enzyme regulator activity” (GO:0030234) were the significant MF terms. Among the downregulated proteins, the representative enriched functions were “ribosome assembly” (GO:0042255), “ATP metabolic process” (GO:0046034), “translation” (GO:0006412), “response to metal ion” (GO:0010038) and “oxidation-reduction process” (GO:0055114) among the BP terms. At the CC level, the significant terms were “cytosol” (GO:0005829), “apoplast” (GO:0048046), “chloroplast” (GO:0009507) and “ribosome” (GO:0005840). “Oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor” (GO:0016620), “structural constituent of ribosome” (GO:0003735), “GTPase activity” (GO:0003924) and “GTP binding” (GO:0005525) were the enriched MF terms.

The putative relationship between the m^6A modification ratio and protein abundance was determined through Pearson correlation analysis (Figure 5C). In NH_4^+ -treated seedlings, a significant positive correlation was observed (0.18), while there was no correlation in control seedlings. The same effect was observed between nucleoside modification ratios from Tombo and protein amounts (Supplemental Figure 7). In addition, similar correlation results were obtained when poly(A) tail lengths and protein amounts were compared, and only NH_4^+ -treated roots had a significant positive correlation (0.12) (Figure 5D).

Integration of data from omics approaches

The results of the different omics approaches employed in the present work were integrated to explore possible regulatory steps in response to NH_4^+ supply in maritime pine. The global results showed 30 different elements/genes with significant results

based on at least two approaches at least; 14 of them were significant in DRS and
epitranscriptomics, 5 in DRS and proteomics, 9 in epitranscriptomics and proteomics,
and 2 in all approaches (Figure 6A; Supplemental Dataset 10). Altogether, the
genes/proteins identified were involved in N metabolism (*PpASPG*, *PpGS1b*, alanine-
glyoxylate aminotransferase and isocitrate dehydrogenase), defense (*PpAMPI*, a
ginkbilobin and a chitinase), oxidative stress response (alcohol dehydrogenase, aldehyde
dehydrogenase, peroxidase and glutaredoxin), translation (ribosomal proteins and
elongation factors) and RNA binding (cold shock proteins and a BURP domain protein
RD22). Among them, the presence of the 1-aminocyclopropane-1-carboxylate (ACC)
oxidase, which had a putative modification at the position 462 on the contig, must be
highlighted. This epitranscriptomic mark was overexpressed (logFC 0.84) under NH_4^+
nutrition, while the ACC oxidase protein was underexpressed (logFC -2.69). Similarly,
changes in transcript level, protein level and transcript modification ratio can show
opposite trends with variations between genes. For the aldehyde dehydrogenase, the
transcript expression and modification were overexpressed (0.58 and 0.73,
respectively), but protein expression was underexpressed (logFC -1.20). However, for
the CSP/GRP (pp_211512 and pp_211516), transcript accumulation was
underexpressed (-0.43 and -0.7) and the epitranscriptomic modifications and protein
expression were overexpressed (0.88-0.73 and 2.11 respectively).

The comparison between the significant GO terms in the omics approaches revealed that
94 of the 287 GO terms were shared (Figure 6B-6C; Supplemental Dataset 11).
Interestingly, 20 (7%) of them were common to the three sets of results. The
epitranscriptomics and proteomics comparison included the highest number of GO
terms (59, 20.6%), and the transcriptomics and proteomics comparison included the
lowest number, with only 5 (1.7%). The most representative GO terms common to the
three omics datasets were “ribosome” (GO:0005840), “structural constituent of
ribosome” (GO:0003735) and “translation” (GO:0006412). Between the transcriptomics
and epitranscriptomics datasets, “mRNA binding” (GO:0003729) and “glutamine
metabolic process” (GO:0006541) were the main GO terms. In the transcriptomics and
proteomics comparison, “oxoacid metabolic process” (GO:0043436) was the main GO
term. Finally, between epitranscriptomics and proteomics the main GO terms were
“small ribosomal subunit” (GO:0015935), “GTPase activity” (GO:0003924), “ribosome

315 assembly” (GO:0042255), “defense response” (GO:0006952) and “response to external
 316 stimulus” (GO:0009605). According to these functional results, several transcripts
 317 coding for eukaryotic ribosomal proteins (38) and translation factors (8) had differential
 318 epitranscriptomic marks based on the Tombo results (Figure 6D).

319 DISCUSSION

320 The response of maritime pine roots to NH_4^+ nutrition has been studied from a
321 multiomics perspective that includes direct RNA sequencing using the ONT platform,
322 which has allowed a global epitranscriptomics analysis. Although N is an essential
323 nutrient for proper plant growth and development (Xu et al., 2012), little is known about
324 the role of epitranscriptomic marks in the regulation of gene expression in response to N
325 nutrition. The results in the present work highlight the importance of epitranscriptomic
326 marks in the regulation of gene expression.

327

328 *Epitranscriptome changes in response to NH_4^+ nutrition*

329 Correlation of global epitranscriptomics results with transcript abundance (Figure 3C),
330 especially m⁶A modifications (Figure 4D), are consistent with those from previous
331 works in *Populus* (Gao et al., 2021) and *Arabidopsis* (Luo et al., 2014; Wan et al., 2015)
332 and support the role of m⁶A in mRNA turnover, as previously described in mammals
333 (Wang et al., 2014; Li et al., 2019). Additionally, m⁶A identification revealed that in the
334 roots of maritime pine, AAACA and AAACU were the most abundant RRACH 5-mer
335 motifs (Figure 4C), as previously reported in *Arabidopsis*, maize, and poplar (Wan et
336 al., 2015; Miao et al., 2020; Parker et al., 2020; Gao et al., 2021). These findings
337 strongly suggest conservation of the RNA m⁶A methylation machinery in plants.
338 However, pine m⁶A distribution, with similar levels of enrichment in the middle of the
339 CDS and at beginning of the 3'-UTR (Figure 4A-4B), slightly differs from the obtained
340 results in angiosperms, where a greater enrichment of m⁶A was observed in the 3'-UTR
341 (Miao et al., 2020; Parker et al., 2020; Gao et al., 2021). This discrepancy may be
342 attributed to the lack of a well-curated reference genome for maritime pine.
343 Interestingly, the distribution of m⁶A sites in pine transcripts is dynamically regulated
344 by increasing the m⁶A deposition in the 3'-UTR in response to NH_4^+ , which seems to be
345 correlated with poly(A) length and changes in protein abundance (Figure 6C-6D), as
346 well as the identification of transcripts with upregulated and downregulated
347 epitranscriptomic marks (Supplemental Dataset 5). This observation agrees with
348 previous results in other eukaryotic organisms (Meyer et al., 2012; Anderson et al.,
349 2018).

A previous work in *Caenorhabditis elegans* reported that highly expressed transcripts contained a relatively short and well-defined poly(A) tail (Lima et al., 2017), which has been related to translational efficiency (Wu et al., 2020). Our results reveal that increased poly(A) tail lengths correlated with higher m⁶A and lower transcript abundances (Figure 4F-4G). These findings are consistent with the effect of m⁶A modifications on transcript levels (Figure 4D), which follow the same trend as previously published results (Luo et al., 2014; Wan et al., 2015; Parker et al., 2020; Gao et al., 2021). The integration of all these results with the positive correlations of m⁶A ratios and poly(A) tail length with protein amounts (Figure 5C-5D) suggests that m⁶A could affect in the translation efficiency of the differentially modified transcripts, as previously described in mammals (Meyer, 2019). This evidence could also explain, at least in part, the lack of a relationship between the transcriptomic, proteomic and metabolite data.

Epitranscriptomic marks, mainly m⁶A, seem to modulate protein synthesis through mRNA stability and modify the translation processes. This is in line with a previous hypothesis considering that initial gene responses caused by environmental factors are modulated at the level of final products of gene expression to maintain cellular homeostasis (Cañas et al., 2015). Thus, the variable transcriptomic response can be controlled through intermediate steps, such as epitranscriptomic regulation, to generate an appropriate and stable cellular response.

Carbon and nitrogen metabolism

As expected, N assimilation was induced by NH₄⁺, as shown by the significant increase in *GS1b* and *NADH-GOGAT* transcripts and the concomitant accumulation of L-glutamine and L-glutamate (Figure 1 and 7; Supplemental Dataset 3), consistent with previous transcriptomic reports (Li et al., 2017; Sun et al., 2017). However, no upregulation of *GS1b* and *NADH-GOGAT* was observed at the proteomic level (Supplemental Dataset 8), even though a trend towards higher GS activity was observed in the presence of NH₄⁺ (Supplemental Figure 8). Similar proteomics results for NH₄⁺ nutrition have been described before (Marino et al., 2016; Coletto et al., 2019), as well as the lack of correlation between relative expression levels of GS transcripts and GS activity. To explain these discrepancies, it was hypothesized that the response to N

382 nutrition could be regulated through a posttranscriptional (translational or
383 posttranslational) mechanism (Ortigosa et al., 2020). In this way, several nucleosides
384 with differential epitranscriptomic marks, including m⁶A, have been identified in *GS1b*
385 transcripts, which could explain the differences between transcriptomics and proteomics
386 data (Supplemental Dataset 4 and 7). Accordingly, in *Arabidopsis*, m⁶A can regulate the
387 alternative polyadenylation and transcript abundance of the *GLN1;1* and *GLN1;3* genes
388 during nitrate assimilation and signaling (Hou et al., 2021).

389 Additionally, the results of this study suggest the existence of a carbon flux through
390 glycolysis and the TCA cycle to provide carbon skeletons for N assimilation (de la Peña
391 et al., 2019). Interestingly, the fermentation pathway seems to be repressed, as
392 suggested by the downregulation of pyruvate decarboxylase (PDC) transcripts and the
393 decrease in alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH)
394 protein levels (Supplemental Datasets 2 and 7). It is well known that plants under low
395 oxygen conditions regulate their metabolism, inducing the fermentation pathway in
396 which PDC and ADH activities are essential, which results in pyruvate consumption,
397 the production of ethanol and the concomitant oxidation of NADH to NAD⁺ (Mithran et
398 al., 2014). The repression of the fermentative pathway and the overrepresentation of
399 TIM proteins (inner membrane translocase proteins) (Supplemental Dataset 7) highlight
400 the need for organic acids to assimilate NH₄⁺ and produce energy for plant growth.

401

402 *Defense response*

403 Nitrogen status is often related to plant disease emergence and plant immunity (Fagard
404 et al., 2014). It is well known that NH₄⁺ induces the upregulation of defense-related
405 genes *in planta* (Ravazzolo et al., 2020). The results presented here demonstrate the
406 transcriptional activation of defense-related genes, such as those encoding for class IV
407 chitinase, defensin 5.1, beta-lactamase and a thaumatin-like protein, in maritime pine
408 roots in response to the NH₄⁺ supply (Supplemental Dataset 2). A similar tendency was
409 observed at the proteomic level for several ginkbilobin antifungal proteins (Wang and
410 Ng, 2000). This effect highlights the importance of the upregulation of this kind of
411 genes in conifers in their natural environment since these plants have adapted to living
412 in suboptimal environments where nutrient availability is a limiting factor for their
413 growth and development (Farjon, 2018).

414 In this context, the upregulation of *antimicrobial peptide 1* and *pathogenesis-related*
 415 *protein PR-1* transcripts was consistent with previous results in maritime pine roots
 416 (Canales et al., 2010). Pine AMP1 is known to inhibit fungal development (Canales et
 417 al., 2011). It has been proposed that, related to its defensive function, AMP1 can inhibit
 418 the NH_4^+ uptake in maritime pine, possibly through a physical interaction with
 419 ammonium transporters (AMT) (Castro-Rodríguez et al., 2017). Additionally, related to
 420 AMT activity regulation, transcriptomic results showed a wide repression of several
 421 transcripts coding for *calcineurin B-like (CBL)-interacting protein kinases* and *calcium-*
 422 *binding proteins* (Supplemental Dataset 2); in the second case, the transcript also
 423 contained significant modifications (Supplemental Dataset 10). The protein kinase
 424 CIPK23 has been shown to phosphorylate T460 of AtAMT1.1 and AtAMT1.2 in a
 425 CBL-1-dependent manner, repressing NH_4^+ transport (Straub et al., 2017). These results
 426 imply transcriptional regulation of NH_4^+ uptake resulting in proper N acquisition and
 427 assimilation. Accordingly, no statistically significant changes in the AMT proteins were
 428 observed, likely due to the relevance of their posttranslational regulation (Figure 3;
 429 Supplemental Dataset 2).

430

431 *Ethylene*

432 Transcriptomic studies carried out in rice described that under excess NH_4^+ , ethylene
 433 (ET) could be one of the major regulatory molecules in roots (Sun et al., 2017).
 434 Additionally, Li et al. (2013) described in *Arabidopsis* that shoot-supplied NH_4^+
 435 promoted ET biosynthesis only in shoots, resulting in a reduction in the lateral root
 436 formation process due to *auxin transporter 1 (AUX1)* repression. These data might
 437 indicate that ET biosynthesis is involved in detrimental NH_4^+ -related phenotypes, such
 438 as a reduction in the number of lateral roots (LRs). Some reports have described that the
 439 application of inhibitors of the ET biosynthesis improved symptoms of NH_4^+ toxicity
 440 affecting RSA (Li et al., 2013). However, previous results indicated that NH_4^+ promotes
 441 root growth in maritime pine seedlings (Ortigosa et al., 2020).

442 A comparison of the root transcriptomic response and differential proteomics results
 443 revealed that NH_4^+ promotes a decrease in ET-related transcripts and proteins. Several
 444 ET-responsive TFs were downregulated at the transcriptomic level, while proteomic
 445 results revealed that ACC oxidase was downregulated (Supplemental Datasets 2 and 7).

ACC oxidase is the enzyme responsible for the final stage in the biosynthesis of ET in plants (John et al., 1999). Furthermore, ACC oxidase transcripts exhibited a nucleoside putative modification in the 3'-UTR (position 462) that was differentially increased under NH_4^+ treatment (Supplemental Dataset 10). Since the levels of ACC oxidase transcripts had no significant changes, it is possible that this epitranscriptomic mark could be involved in the translational regulation of these transcripts, therefore affecting the ET levels of the organ. Interestingly, it is reasonable to think that this kind of response could be based on conifer preference for NH_4^+ . Additional technical approaches are required to identify the exact modification and validate its biological relevance in the regulation of ACC oxidase and ET synthesis. Finally, the relationship between ET and N nutrition is remarked by the differential expression (negative) of a trihelix family TF that has a very high similarity degree to AT3G54390 in *Arabidopsis* (Figure 7). This TF in *Arabidopsis* can interact with the promoters of genes involved in N metabolism and ET biosynthesis including *glutamine synthetase* (AT1G66200), *nitrite reductase* (AT2G15620), *ET-responsive transcription factor* (ERF012; AT1G21910) and *1-aminocyclopropane-1-carboxylate synthase* (AT5G65800) (Gaudinier et al., 2018).

463

464 *Translation and growth*

The results also indicate a reconfiguration of the ribosomal proteins and elongation factors at all biological levels, although this was more visible in epitranscriptomics and proteomics results (Figure 6D and 7; Supplemental Datasets 2, 4, 7 and 10). This process is clearly related to the correlation between m^6A ratios and protein amounts (Figure 5C), suggesting that NH_4^+ nutrition promotes general translation activation to support the root growth of maritime pine seedlings. This kind of effect on the ribosomal protein composition and proteins involved in translation has been previously observed under different conditions, including plant mineral nutrition (Wang et al., 2013; Prinsi and Espen, 2018; Ferretti and Karbstein, 2019). The data suggest that changes in the profiles of ribosomal proteins can be significantly mediated by epitranscriptomic modifications to their transcripts. These posttranscriptional changes can influence ribosome function and performance. One example is the RACK1 proteins, which are components of the small subunit of the ribosome and are involved in translation quality

control promoting the endonucleolytic cleavage of nonstop mRNA (Ikeuchi and Inada, 2016). These proteins in plants are involved in the response to phytohormones, including ET, and in the regulation of growth and development (Wang et al., 2019). Interestingly, the mRNA of a *RACK1* gene in maritime pine roots has several differential epitranscriptomic marks (Supplemental Datasets 4 and 5). Although it was not significant, RACK1 protein expression was repressed under NH_4^+ conditions, suggesting a change in the quality control mechanisms and even in the integration of the plant signaling pathways. This result agrees with the negative regulation of ACC oxidase and repression of several ERFs (Supplemental Datasets 2 and 7). Accordingly, ET signaling affects gene translation in different ways (Merchante et al., 2015). Additionally, related to the regulation of translation and root growth, among the transcriptomic results, the repression of an *FCS-like zinc finger* was observed (Supplemental Dataset 2). FCS proteins interact with SnRK1 to mediate the interaction of SnRK1 with other proteins (Jamsheer et al., 2018). SnRK1 is a kinase that phosphorylates RAPTOR, a regulatory element of the TOR complex, repressing TOR activity (Fu et al., 2020). Considering the ribosomal protein changes and the increase in root growth under NH_4^+ nutrition in maritime pine seedlings (Ortigosa et al., 2020), it can be hypothesized a decrease of SnRK1 activity over the TOR complex in these conditions. The TOR complex integrates different developmental and environmental signals, including nutritional status, promoting ribosome biogenesis, translation, and plant growth.

Finally, a transcript coding for a TCTP protein had several differential modifications (Supplemental Dataset 5). It is well established in plants that *TCTP* mRNA is synthesized in shoots and transported to roots, where it is translated and promotes lateral root formation (Branco and Masle, 2019). Interestingly, transport of this mRNA is mediated through epitranscriptomic marks (Yang et al., 2019). This suggests additional mechanisms controlling growth and development in response to N nutrition in maritime pine roots.

Therefore, the results presented in this work suggest that protein translation and growth are finely regulated through epitranscriptomic marks, including m^6A , to acquire an optimum response to N supply (Figure 7). More research efforts are required to

509 corroborate this hypothesis and to investigate whether ET could act as a modulator of
510 the integrated response observed due to its effects on the translation process.

511 MATERIALS AND METHODS

512

513 Plant material

514 Seeds from maritime pine (*Pinus pinaster* Ait.) from “Sierra Segura y Alcaraz”
 515 (Albacete, Spain) were provided by from the *Área de Recursos Genéticos Forestales* of
 516 the Spanish *Ministerio de Agricultura, Pesca y Alimentación*. Maritime pine seed
 517 germination was carried out following the protocol described in (Cañas et al., 2006).
 518 Seedlings were grown in vermiculite in plant growth chambers under 16 h light
 519 photoperiod, a light intensity of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$, constant temperature of 25°C
 520 and watered twice a week with distilled water. One-month old maritime pine seedlings
 521 were used for this experiment. Pine seedlings were randomly subdivided into two
 522 different groups, relocated into forestall seedbeds and watered with 80 mL distilled
 523 water. After three days of acclimation, the control group was irrigated with 80 mL of
 524 water (C) and the experimental group with 80 mL of 3 mM NH_4Cl . Root samples were
 525 collected at 24 hours post-irrigation and immediately frozen in liquid N. This
 526 experiment was carried out three independent times. The adequate development of each
 527 experiment was verified through the gene expression analysis by RT-qPCR of two
 528 control genes, *PpAMT1.3* and *PpAMPI* following previous results (Canales et al., 2011;
 529 Castro-Rodríguez et al., 2016).

530

531 Metabolite profile

532 The metabolites for ^1H -NMR analysis were extracted following the protocol previously
 533 described by Kruger et al. (2008). Two hundred milligrams of frozen powder were used
 534 for extractions. The ^1H -NMR analyses were performed on an ASCEND 400 MHz NMR
 535 Spectrometer (Bruker, Billerica, MA, USA) in the Bionand Centre (Malaga, Spain). The
 536 1D- ^1H -NMR spectrum for each sample was obtained as previously described by Cañas
 537 et al. (2015). Quantitative analysis of the NMR spectra was performed using LCMModel
 538 software (Provencher, 1993) and a previously generated reference metabolite spectral
 539 library (Cañas et al., 2015). The internal reference was an electronically generated
 540 signal, ERETIC (Akoka et al., 1999). The metabolite amounts were determined at
 541 millimolar concentrations. The metabolite contents were analyzed with MetaboAnalyst
 542 4.0 (Chong et al., 2018). Data were normalized using the quantile method, then log
 543 transformation and mean centered. MetaboAnalyst 4.0 was used to construct a Heatmap
 544 and perform a t-test with the metabolite data.

545

546 **Total RNA isolation**

547 Total root RNA from maritime pine seedlings was isolated following the protocol
548 described by Liao et al. (2004) and modified by Canales et al. (2012). The RNA
549 concentration and purity were determined *via* spectrophotometry on a Nanodrop ND-
550 1000 (Thermo Scientific, Waltham, MA, USA). Purity was determined through the
551 260/280 and 260/230 ratios. RNA quality was also determined in a Bioanalyzer 2100
552 (Agilent, Santa Clara, CA, USA). The concentration was verified with a Qubit 4
553 Fluorometer (Invitrogen, Paisley, UK) and Qubit RNA BR, Broad-Range, Assay Kit
554 (Invitrogen, Paisley, UK).

555

556 **mRNA isolation and preparation**

557 Samples with a RIN value > 7 were selected to mRNA isolation. The poly(A)-RNA
558 isolation was performed using Dynabeads™ mRNA Purification Kit (Invitrogen,
559 Paisley, UK) following the manufacturer's instructions. This process was carried out
560 twice per sample to avoid rRNA contamination. poly(A)-RNA quality was determined
561 in a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). The concentration was verified
562 with a Qubit 4 Fluorometer (Invitrogen, Paisley, UK) and Qubit RNA HS, High
563 Sensitivity, Assay Kit (Invitrogen, Paisley, UK).

564

565 **Direct RNA sequencing (DRS) and differential epitranscriptomic analysis**

566 Nanopore libraries for DRS were prepared from 1.65 up to 2.18 µg of isolated poly(A)-
567 RNA using the Nanopore Direct RNA Sequencing kit (SQK-RNA001, Oxford
568 Nanopore Technologies, ONT, Oxford, UK) according to manufacturer's instructions.
569 The DRS libraries were loaded onto a R9.4 SpotON Flow Cells (Oxford Nanopore
570 Technologies, Oxford, UK) and sequenced until complete nanopores depletion.
571 Extended method descriptions are in the Supplemental Methods 1. Basecalling was
572 carried out with ONT Guppy software (<https://community.nanoporetech.com>). The
573 resultant reads were filtered by quality (Q>9). Read alignment was made with
574 minimap2 software (Li, 2018) using root transcriptome of maritime pine as reference.
575 The alignment parameters were adjusted for DRS (*-uf* and *-k14*). Differentially
576 expressed (DE) transcripts were identified using the edgeR package for R, the
577 transcripts were normalised by count per million mapped reads (cpm) and filtered (2

cpm in at least 2 samples) (Robinson et al., 2010). The transcripts with False Discovery Rate < 0.05 (FDR < 0.05) were considered as differentially expressed. ONT-DRS reads were used for *de novo* modification detection with the TOMBO software (Stoiber et al., 2017). The total mapped reads per base and the number of modified bases in each position were obtained using the *text_output_browser_file* method with the options *coverage* and *fraction*. Only the positions with at least 50 mapped reads were employed for subsequent analyses. A Fischer exact test was carried out for each transcript position to determine the differential expression of modified bases among control and NH₄⁺ supplied samples. The transcript positions with a *P*-value < 0.05 and an absolute logFC > 0.5 were considered as differentially modified. *Transdecoder* software (<https://github.com/TransDecoder/transdecoder.github.io>) were used to determine modification positions and nucleobases in the transcripts of the reference transcriptome. Identification of m⁶A sites were carried out with the bioinformatic pipeline *Nanom6A* using default parameters (Gao et al., 2021). The length of poly(A) tails were determined using *Nanopolish* 0.11.1 software package (<https://github.com/adbailey4/nanopolish>) with the *polya* function.

Functional annotation and enrichment analyses

The transcriptome was functional annotated with BLAST2GO (Götz et al., 2008) using DIAMOND software with *blastx* option (Buchfink et al., 2015) result against the NCBI's plants-*nr* database (NCBI Resource Coordinators, 2016). Blast results were considered valid with e-value < 1.0E-6. Singular enrichment analysis (SEA) of the GO terms was made in the AGRIGO v2.0 web tool under standard parameters using as GO term reference the whole assembled transcriptome annotation (Tian et al., 2017). Representative enriched GO was determined using REVIGO (Supek et al., 2011).

RT-qPCR

The cDNA synthesis was performed using 1 µg of total RNA and iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) following manufacturer's instructions. The qPCR primers were designed following the MIQE guidelines (Bustin *et al.*, 2009). The primers are listed in the Supplemental Table 2. qPCRs were carried out using 10 ng of cDNA and 0.4 mM of primers and 2X SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA, USA) in a total volume of 10 µL. Relative quantification of gene expression was performed using thermocycler CFX 384™ Real-Time System, Bio-Rad,

Hercules, CA, USA). The qPCR program was: 3 min at 95 °C (1 cycle), 1 s at 95 °C and 5 s at 60 °C (50 cycles) and a melting curve from 60 to 95 °C, to generate the dissociation curve in order to confirm the specific amplification of each individual reaction. The analyses were carried out as described by Cañas et al. (2014) using the MAK3 model in the R package *qpcR* (Ritz and Spiess, 2008). Expression data were normalized to two reference genes, SKP1/ASK1 and SLAP that were previously tested for RT-qPCR experiments in maritime pine (Granados et al., 2016). The qPCR analyses were made with three biological replicates and three technical replicates per sample.

Validation of differential deposition of m⁶A by RT-qPCR

The validation of the differential deposition of m⁶A in the transcripts were made using the SELECT method (Xiao et al., 2018). A differential cDNA synthesis was made per each transcript with 30 ng of total RNA. qPCR determinations were made with 2 µL of cDNA. The expression level of each transcript was determined in parallel by RT-qPCR and their result were used to normalize the SELECT results. The primers are listed in the Supplemental Table 2. Extended method description can be found in the Supplemental Methods 1.

Differential proteomics analysis

The proteins were extracted following the protocol described by González Fernández et al. (2014). The extractions were carried out with 200 mg of sample. Protein content was determined using a commercially kit (Protein Assay Dye Reagent; Bio-Rad, CA, USA) and bovine serum albumin as a standard (Bradford, 1976). Protein extracts were cleaned-up in 1D SDS-PAGE at 10% polyacrilamde as described in Valledor and Weckwerth, 2014. Protein bands were cut off, diced, and kept in water at 4°C until digestion.

Protein digestion and nLC-MS2 analysis were carried out in the Proteomics Facility at Research Support Central Service, University of Cordoba. Nano-LC was performed in a Dionex Ultimate 3000 nano UPLC (Thermo Scientific, Waltham, MA, USA) with a C18 75 µm x 50 Acclaim Pepmam column (Thermo Scientific, Waltham, MA, USA). Eluting peptide cations were converted to gas-phase ions by nano electrospray ionization and analyzed on a Thermo Orbitrap Fusion (Q-OT-qIT, Thermo Scientific) mass spectrometer operated in positive mode. Extended method descriptions are in the Supplemental Methods 1.

Root transcriptome from *Pinus pinaster* was translated into the six open reading frames with *transeq* tool (Madeira et al., 2019). The output peptides chains were filtered by length, deleting those less than 50 amino acids (Romero-Rodríguez et al., 2014). To reduce the redundancy of proteins in the database, CD-HIT-EST with a 99% identity filter was used (Fu et al., 2012). The raw data were processed using Proteome Discoverer (version 2.3, Thermo Scientific). MS2 spectra were searched with SEQUEST engine against the reference proteome database. *In silico* peptide lists were created using the followings settings: trypsin digestion, a maximum of two missed internal cleavage sites per peptide, precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.75 Da per fragment ions. Only peptides with a high confidence ($FDR \leq 0.01$) and minimum XCorr of 2 were selected. The identified proteins were filtered by a minimum of two different peptides. *Minora* algorithm was used to determine relative quantification. The protein identification with redundancy is considered by Proteome Discoverer and SEQUEST software. Proteins sharing peptides were grouped and all those groups without a unique peptide were removed. Only proteins detected in 5 of the 6 samples were considered for subsequent analyses. The resultant proteins were annotated using BLAST with *blastp* option against NCBI's non-redundant database (Camacho et al., 2008) and BLAST2GO software (Götz et al., 2008). Proteins with the same sequence annotation and quantification profile across the samples was considered the same protein selecting the protein with the longest sequence. Normalized data from Proteome Discoverer software were used for a differential expression analysis using the edgeR package for R (Robinson et al., 2010). Only the proteins with *P*-value < 0.05 were considered as differentially expressed.

669

670 Accession Numbers

The sequences of genes examined in this study can be found in Genbank under the following accession numbers: KC807909 (*PpAMT1.3*) and HM210085 (*PpAMPI*). The root transcriptome sequences consulted at <https://fmp.uma.es/sequenceserver/> or can also be obtained on demand from the contact author. The DRS data have been deposited in the NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series with the accession number GSE174830 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE174830>). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium

679 via the PRIDE partner repository (Vizcaino et al., 2013) with the dataset identifier
680 PXD025331 and 10.6019/PXD025331.

681

682

683

684 **AUTHOR CONTRIBUTIONS**

685 F.O. have performed the experiments; C.L.F. has performed the bioinformatic analyses;
686 J.P.C. and F.R.C. have performed the statistical data analysis; F.O. and R.A.C. have
687 wrote the manuscript; C.A. and F.M.C. made additional contributions and edited the
688 manuscript. F.O. and R.A.C. have planned and designed the research. R.A.C, C.A.V.,
689 and F.M.C were the responsible of the funding acquisition.

690

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FIGURE LEGENDS

Figure 1. Main trend of metabolites in the roots of control and NH_4^+ -treated seedlings at 24 hours post-irrigation. Blue columns correspond to control plants; red columns correspond to 3 mM NH_4^+ supply. Differences between treatments were determined with a t-test. Significant differences are indicated with asterisks on top of the columns: * at $P < 0.05$. Error bars show SE with $n = 3$. PEP: phosphoenolpyruvate.

Figure 2. Main DRS transcriptomics results. **A.** Size distribution of reads obtained through DRS-ONT sequencing. **B.** MA plot containing all detected transcripts. Blue points correspond to significantly downregulated transcripts in maritime pine roots at 24 h after the treatment with 3 mM ammonium. Red points correspond to significantly upregulated transcripts in maritime pine roots at 24 h after the treatment with 3 mM ammonium. **C.** Significant GO terms from significant DE transcripts after a SEA analysis. **D.** Comparison between DRS and RT-qPCR results from different transcripts with significant differential expression in the DRS analysis.

Figure 3. Epitranscriptomics modification results from Tombo software analysis. **A.** Volcano plot of the detected epitranscriptomics modifications. Yellow points correspond to modifications with P -values lower than 0.05 but with an absolute logFC value lower than 0.5. Blue points correspond to modifications with an absolute logFC value higher than 0.5 but with P -values higher than 0.05. Red points correspond to significant DE modifications with an absolute logFC value higher than 0.5 and P -values lower than 0.05. **B.** Percentage of detected modifications per nucleobase. Yellow columns correspond to control samples. Purple columns correspond to 3 mM ammonium treated samples. **C.** Scatter plot and correlation between detected modifications using Tombo software and transcript amounts determined by DRS sequencing. **D.** Significant GO terms from significant DE modifications after a SEA analysis. **E.** Comparison between DRS and SELECT results from different transcript modified positions with significant differential modification in the Tombo analysis.

Figure 4. m⁶A identification results using the bioinformatics pipeline Nanom6A. **A.** Distribution of the identified m⁶A all along the transcripts. **B.** Distribution of the identified m⁶A along each part of the transcripts: 5'UTR, CDS and 3'UTR respectively. **C.** Percentage of identified m⁶A for each different variant (kmer) of the consensus sequence RRACH for adenine methylation. **D.** Scatter plot and correlations between m⁶A modification ratio and transcript amount from DRS sequencing. **E.** Poly(A) length of the transcripts identified in the DRS sequencing. **F.** Scatter plot and correlations between poly(A) length and m⁶A modification ratio. **G.** Scatter plot and correlations between poly(A) length and transcript amount from DRS sequencing.

Figure 5. Differential expression proteomics results. **A.** Volcano plot of the identified proteins. Yellow points correspond to modifications with *P*-values lower than 0.05 but with an absolute logFC value lower than 0.5. Red points correspond to significant DE modifications with an absolute logFC value higher than 0.5 and *P*-values lower than 0.05. **B.** Significant GO terms from significant DE proteins after a SEA analysis. **C.** Scatter plot and correlations between m⁶A modification ratios and protein amounts. **D.** Scatter plot and correlations between transcript amounts from DRS sequencing and protein amounts.

Figure 6. Common results among different omics approaches. **A.** Genes with significant expressions in more than one omics approach. Red boxes correspond to upregulated transcripts and proteins. Blue boxes correspond to downregulated transcripts and proteins. Yellow boxes correspond to significant differentially modified positions in the transcripts. **B.** Venn diagram of significant GO terms in the different omics approaches. **C.** Heatmap of significant GO terms shared by at least two omics approaches. **D.** Heatmap of genes that produce proteins involved in ribosome and translation. Ribosomal protein equivalences obtained from Martinez-Seidel et al. (2020). Red boxes correspond to upregulated transcripts and proteins. Blue boxes correspond to downregulated transcripts and proteins. Yellow boxes correspond to significant differentially modified positions in the transcripts.

1090 **Figure 7.** Schematic representation of functional results obtained through the omics
 1091 integrative approach. NH_4^+ triggers carbon and nitrogen metabolism, ethylene
 1092 biosynthesis and signaling and translation responses. Geometric red and blue forms
 1093 indicate upregulation and downregulation respectively of transcripts, RNA nucleoside
 1094 modifications, proteins, and metabolites.

1095 **Supplemental Information**

1096

1097 **Supplemental Table 1.** Direct RNA sequencing information for each sample run.

1098 **Supplemental Table 2.** Primer list.

1099

1100 **Supplemental Methods 1.** Extended Materials and Methods.

1101

1102 **Supplemental Figure 1.** Heatmap of root metabolites. The analysis was done with
1103 Metaboanalyst using the Euclidean distance and Ward clustering algorithm. Class 0
1104 (blue group) corresponds with control samples irrigated with distilled water. Class 3
1105 (red group) corresponds with seedlings irrigated with 3 mM NH_4^+ .

1106 **Supplemental Figure 2.** Significant GO terms after SEA analysis using the DRS
1107 results.

1108 **Supplemental Figure 3.** Scatter plot and correlations between the transcript amounts
1109 and the modification ratios detected using Tombo software for each nucleoside.

1110 **Supplemental Figure 4.** Significant GO terms after SEA analysis using the
1111 epitranscriptomics results obtained from Tombo software.

1112 **Supplemental Figure 5.** Scatter plot and correlations between the poly(A) length and
1113 the modification ratios detected using Tombo software for each nucleoside and global
1114 results.

1115 **Supplemental Figure 6.** Significant GO terms after SEA analysis using the proteomics
1116 results.

1117 **Supplemental Figure 7.** Scatter plot and correlations between the protein amounts and
1118 the modification ratios detected using Tombo software for each nucleoside and global
1119 results.

1120 **Supplemental Figure 8.** Glutamine synthetase activity in maritime pine roots under
1121 ammonium nutrition. The results are the mean of three independent experiments using
1122 biological pools. Error bars correspond to SE. Enzymatic determinations were described
1123 in Supplemental Methods 1.

1124

1125 **Supplemental Dataset 1.** Normalized data from root metabolite profiling.

- 1126 **Supplemental Dataset 2.** Whole root DRS differential expression results. DE
1127 transcripts upregulated are in highlighted red. DE transcripts downregulated are in
1128 highlighted blue.
- 1129 **Supplemental Dataset 3.** SEA whole root DRS differential overexpressed transcripts
1130 results.
- 1131 **Supplemental Dataset 4.** DRS differential modification results in pine roots under
1132 ammonium nutrition. DE transcripts overmodified are in highlighted red. DE transcripts
1133 undermodified are in highlighted blue.
- 1134 **Supplemental Dataset 5.** Transcripts with upregulated and downregulated
1135 modifications at the same time.
- 1136 **Supplemental Dataset 6.** SEA of transcripts with significant epitranscriptomic
1137 modifications in pine roots under ammonium nutrition.
- 1138 **Supplemental Dataset 7.** Identified differential m⁶A positions coincident with DRS
1139 differential modification detected by Tombo software in the three close positions
1140 context. DE transcripts overmodified are in highlighted red. DE transcripts
1141 undermodified are in highlighted blue.
- 1142 **Supplemental Dataset 8.** Whole root differential proteomics results. DE proteins
1143 upregulated are in highlighted red. DE proteins downregulated are in highlighted blue.
- 1144 **Supplemental Dataset 9.** SEA results of proteins overexpressed in pine roots under
1145 ammonium nutrition.
- 1146 **Supplemental Dataset 10.** Elements with significant changes at least in two
1147 experimental approaches.
- 1148 **Supplemental Dataset 11.** Commons significant GO terms between omics analyses.

A **B** **C** **D**