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The m6A pathway protects the transcriptome integrity by restricting RNA chimera formation in plants

Dominique Pontier1,2,*, Claire Picart1,2,*, Moaine El Baidouri1,2, François Roudier3, Tao Xu4, Sylvie Lahmy1,2, Christel Llauro1,2, Jacinthe Azevedo1,2, Michèle Laudié1,2, Aurore Attina5, Christophe Hirtz5, Marie-Christine Carpentier1,2, Lisha Shen6, Thierry Lagrange1,2

Global, segmental, and gene duplication–related processes are driving genome size and complexity in plants. Despite their evolutionary potentials, these processes can also have adverse effects on genome regulation, thus implying the existence of specialized corrective mechanisms. Here, we report that an N6-methyladenosine (m6A)-assisted polyadenylation (m-ASP) pathway ensures transcriptome integrity in Arabidopsis thaliana. Efficient m-ASP pathway activity requires the m6A methyltransferase-associated factor FIP37 and CPSF30L, an m6A reader corresponding to a YTH512-B Homology Domain–containing protein (YTHDC)-type domain containing isoform of the 30-kDa subunit of cleavage and polyadenylation specificity factor. Targets of the m-ASP pathway are enriched in recently rearranged gene pairs, displayed an atypical chromatin signature, and showed transcriptional readthrough and mRNA chimera formation in FIP37- and CPSF30L-deficient plants. Furthermore, we showed that the m-ASP pathway can also restrict the formation of chimeric gene/transposable-element transcript, suggesting a possible implication of this pathway in the control of transposable elements at specific loci. Taken together, our results point to selective recognition of 3′-UTR m6A as a safeguard mechanism ensuring transcriptome integrity at rearranged genomic loci in plants.

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Introduction

N6-methyl-adenosine (m6A) has recently emerged as a prevalent mRNA modification that tends to be enriched in 3′-UTR within the consensus RRm6ACH motif (where R is A/G and H is A/C/U) (Dominissini et al, 2012; Meyer et al, 2012; Schwartz et al, 2013; Shen et al, 2016). m6A mRNA modification occurs co-transcriptionally and is driven by a conserved writer complex composed of methyltransferase-like3 (METTL3/MT-A70), METTL14 (a noncatalytic METTL3-homologous protein), and Wilms tumor 1-associated protein (WTAP/FIP37) (Bokar et al, 1997; Zhong et al, 2008; Liu et al, 2014a; Shen et al, 2016; Meyer & Jaffrey, 2017). Mutation in any of these genes prevents m6A methylation and causes detrimental effects leading to differentiation defects and apoptosis in animal cells (Bastita et al, 2014; Geula et al, 2015), embryonic lethality in plants, and mice (Zhong et al, 2008; Geula et al, 2015; Shen et al, 2016; Růžička et al, 2017), defects in meiosis in yeast (Schwartz et al, 2013), and defects in gametogenesis and sex determination in insects (Hongay & Orr-Weaver, 2011; Haussmann et al, 2016).

m6A is expected to control the fate of mRNA mostly by recruiting specific readers that belong to the YTH domain-containing proteins and bind m6A methyl group through a conserved hydrophobic pocket (Meyer & Jaffrey, 2017; Wu et al, 2017). YTH proteins fall into three major groups defined as the YTHDF- and YTHDC2-type clades whose members are primarily cytoplasmic and the YTHDC1-type clade whose members are expected to act in the nucleus (Meyer & Jaffrey, 2017). Recent studies performed in animal and plant models have connected m6A-dependent recruitment of YTH-type proteins to the control of various aspects of mRNA metabolism, including stability (Wang et al, 2014; Wotjas et al, 2017), splicing (Lencse et al, 2016; Xiao et al, 2016), alternative polyadenylation (APA) (Ke et al, 2015; Yue et al, 2018), nuclear export (Roundtree et al, 2017), and translation initiation (Wang et al, 2015). More recently, m6A modification has been also shown to play a role in XIST-mediated X-chromosome inactivation (Patil et al, 2016), repair of UV-induced DNA damages in mammals (Xiang et al, 2017), and response to abiotic stress in plants (Anderson et al, 2018), confirming its broad spectrum of activities in eukaryotes.

Compared with other eukaryotic genomes, plant genomes show high dynamics with a substantial degree of diversity (Rensing, 2014). Genome and gene/transposon duplication–related processes in plants are now regarded as important evolutionary forces that contribute to genome complexity, generating new genetic material as...
well as nonfunctional sequences that are rapidly eliminated (Rensing, 2014; Panchy et al, 2016; Galindo-González et al, 2017). Studies of naturally occurring genomic rearrangements in plants revealed that duplicated/translocated loci can impact initial gene regulatory networks in cis or trans, resulting either in beneficial, deleterious, or neutral effects depending on the genetic context. Whereas trans-acting effects on original genes generally entail gene silencing through natural epigenetic variation (Bender & Fink, 1995; Durand et al, 2012), cis-acting effects on a neighboring gene vary among cases, leading to epigenetic control (Zheng & Cheng, 2014; El Baidouri et al, 2018), chimeric gene formation (Thimmappuram et al, 2005; Shahmuradov et al, 2010), polyadenylation defects (Tsukamoto et al, 2010), transcriptional interference (Kashkush et al, 2003), and gene/exon transduction (Xiao et al, 2008; Zhu et al, 2016).

There is compelling evidence that plants develop specific control mechanisms to neutralize the adverse effects that can result from loci rearrangements. For instance, genetic studies revealed that the cell cycle regulator gene BONSAI (BNS) is insulated from the DNA- and histone-dependent repressive effects of an adjacent LINE retroelement by the action of INCREASE IN BONSAI METHYLATION1/1BM1, a jmjC domain-containing histone demethylase (Saże et al, 2008). Likewise, a pathway comprising plant-specific RNA- and chromatin-binding proteins has been implicated in the control of transcriptome integrity by counteracting premature transcription termination defects caused by the presence of an intronic transposable element (TE) (Wang et al, 2013a; Saże et al, 2013). Given the importance of gene duplication/translocation mechanisms for gene expansion in plants, it is likely that plants develop additional mechanisms to neutralize potential detrimental effects of such gene rearrangements. Here, we show that mA RNA modification and a network of conserved and plant-specific proteins constitutes a post-transcriptional regulatory pathway that enforces polyadenylation and 3′-end formation at loci that exhibit transcriptional readthrough, cryptic intergenic splicing, and chimeric mRNA formation in Arabidopsis thaliana. Targets of this newly identified pathway are enriched in rearranged gene pairs (GENE1/GENE2) that present duplicated or translocated paralog/pseudogene at GENE2 position, suggesting that gene rearrangements at targeted loci could be responsible for the observed termination/polyadenylation defects. We show that GENE1 transcripts are mA-tagged and that mA-assisted polyadenylation at these genes requires the concerted action of the mA methyltransferase auxiliary factor FIP37, and CPSF30L, a plant-specific YTH-domain containing variant of the conserved 30-kd subunit of cleavage and polyadenylation specificity factor (CPSF30). Finally, we provide evidence that the m-ASP pathway can also restrict the formation of potentially deleterious chimeric GENE-TE transcripts. Taken together, this study establishes a strong link between mA methylation and the control of transcriptome integrity in plants, expanding the repertoire of functions performed by this type of RNA modification in eukaryotes.

Results

NERD mutations lead to chimeric mRNA formation in A. thaliana

We previously reported that NERD gene mutations relieve DNA methylation-dependent repression of a subset of newly acquired loci in A. thaliana, including a pseudogene named pseudo-ORF (psORF) (Pontier et al, 2012). Investigating further NERD’s function, we found evidence of cotranscriptional readthrough and chimeric RNA formation between psORF and a Copia-type transposon (AT5TE50260) located downstream (Fig S1A). The accumulation of chimeric RNA species in nerd-1 was validated by semi-quantitative RT–PCR assay using primers anchored on both sides of the psORF/AT5TE50260 genomic junction as well as by real-time qRT–PCR assay with primers specific for psORF and AT5TE50260 loci (Fig S1B and C). Interestingly, although the expression of a flag-tagged version of NERD (nerd-T) did not restore psORF silencing in nerd-1, as previously reported in Pontier et al (2012), it efficiently reverted the read-through phenotype (Fig S1A–C), suggesting a contribution of NERD in transcription termination control at psORF locus.

To investigate whether NERD controls transcription termination and chimeric RNA formation at loci other than psORF, we scrutinized our nerd-1 transcriptome data and observed several cases of mRNA chimera formation. In many instances, as exemplified by AT4G30570 and AT1G71330 (GENE2), increased mRNA levels spread across the upstream intergenic region as a possible result of defective RNA 3′-end formation at upstream AT4G30580 and AT1G71340 genes (GENE1), respectively (Fig S2A). The specific accumulation of chimeric mRNA species in nerd-1 was validated by RT–PCR assay using specific GENE1/2 (F1/R1 and F2/R1) primers and RNA gel blot analysis using GENE1-specific probes (N) (Fig S2B and C). The readthrough phenotype was specific to NERD deficiency as it was reverted in nerd-T plants (Fig S2A–C). Consistent with a role for NERD in transcription termination control at GENE1 rather than transcription control at GENE2, we did not detect any enrichment of H3K4me3, a chromatin mark associated with active promoter regions, in the corresponding intergenic regions in nerd-1 compared with WT or nerd-T plants (Fig S2D).

To evaluate the generality of the observed readthrough pattern, we calculated the variation in strand-specific read density in the 500-bp windows downstream of all annotated genes upon NERD depletion. As an apparent increase in downstream read density might derive from upstream gene deregulation, all genes whose expression showed a significant change in nerd-1 mutant were also excluded from the list of gene candidates. Using a twofold change cutoff, 107 genes showing unaffected levels of expression in nerd-1, but a significant increase in strand-specific read density in their downstream region were identified (Fig 1A and Table S1). Inspection of the candidate loci using the integrative genomics viewer confirmed the existence in nerd-1 mutant of numerous transcription readthrough events leading in most cases to the formation of putative mRNA chimera (GENE1/GENE2) but also of mRNAs with extended 3′-UTR (GENE1e) (Table S2). To ascertain that annotated GENE2s correspond to bona fide genomic units rather than misannotated 3′ region of GENE1s, we assessed the conserved colinearity of A. thaliana GENE1/GENE2 chimeric loci (excluding tandem repeats that have a clear evolutionary origin) in 325 sequenced angiosperms species, including monocots and dicots (Fig 1B). This comparative genomic analysis showed that a colinear GENE1–GENE2 organization was not conserved in most cases, with many species having only one member of the gene pair present in their genome (G1 only or G2 only), or both genes present but at distant genomic locations (Fig 1B), therefore supporting the fact...
that GENE1 and GENE2 represent independent gene units. Interestingly, the few cases in which a contiguous GENE1–GENE2 organization was phylogenetically conserved corresponded to sequences found exclusively in close relatives of *A. thaliana* (Brassicales) (Fig 1B), indicating a recent evolutionary origin.

To characterize further the chimeric/extended readthrough phenotype, several candidate loci in addition to AT4G30580/70 and AT1G71340/30 were selected for analysis. Using primers anchored on both sides of the corresponding chimeric/extended regions (and thus spanning the intergenic region) in RT–PCR assays, we confirmed the specific over-accumulation of readthrough mRNAs in two independent *nerd* mutant backgrounds (Fig 1C). Sashimi plots indicating RNA-seq reads that specifically cross the GENE1–GENE2 junction in *nerd*-1 background are further shown in Figs S2A and S3A, and B. The specific up-regulation of chimeric/extended mRNA forms in *nerd*-1 was further validated by real-time quantitative reverse transcription-polymerase chain reaction (qRT–PCR) assay (Fig S3C), confirming that the readthrough events are not a consequence of unleashed GENE1 transcription. Heat map analysis further confirmed that GENE2 at NERD-dependent chimeric loci are generally silent or expressed at a low level in WT plant organs (Fig 1D), likely owing to the termination-promoting activity of NERD. From now on, we will refer to genes showing termination defects in *nerd* mutant backgrounds as ITN (inefficient termination in NERD) loci.

Prior studies have shown that the split ends protein (SPEN) family proteins FPA/FCA repress the formation of chimeric mRNAs at a subset of *A. thaliana* genomic loci (Sonmez et al, 2011; Duc et al, 2013). However, a limited overlap is currently observed between loci that GENE1 and GENE2 represent independent gene units. Interestingly, the few cases in which a contiguous GENE1–GENE2 organization was phylogenetically conserved corresponded to sequences found exclusively in close relatives of *A. thaliana* (Brassicales) (Fig 1B), indicating a recent evolutionary origin.

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reported to form mRNA chimera in fpa-7 and those observed in nerd-1 mutant plants (Fig S4A) (Duc et al, 2013), an observation that prompted us to evaluate the extent to which these proteins interact in vivo. Using intergenic primers in RT–PCR assays, we confirmed that NERD was not required to control extended/chimeric mRNA formation at most FPA-dependent loci tested (hereafter named as FPA-only) (Fig S4B). Reciprocally, FPA activity was not mandatory for the control of chimeric mRNA formation at most ITN loci (Fig S4B).

While the mechanisms underlying target specificity of NERD and FPA in this process remain unclear, expression analysis indicates that FPA controls mRNA chimera formation preferentially between two expressed loci (Fig S4C). Taken together, our results reveal the specific and largely independent roles of NERD and FPA proteins in the control of transcription termination and chimeric mRNA formation, further highlighting the existence of multiple chimeric mRNA control pathways in A. thaliana.

Rearranged gene pairs are significantly overrepresented among mRNA chimera-producing ITN loci

To uncover common evolutionary trends among mRNA chimera-producing ITN loci, we traced their evolutionary history in A. thaliana and its close relatives A. lyrata and Capsella rubella, after their classification into five categories: single copy genes, segmental/WGD genes, tandemly duplicated genes, translocated duplicates, and finally other duplicates (Fig 2A and Table S3). Translocated duplicates refer to non-syntenic paralogs that have been relocated elsewhere in the genome since the divergence between A. thaliana and A. lyrata and/or C. rubella. They may originate from single gene duplication or from rearrangements that occurred after the paralog duplication. As shown in Fig 2A both GENE1 and GENE2 are represented in all categories indicating that, overall, they have various evolutionary origins, being either single or duplicated genes. However, it is interesting to note that both GENE1 and GENE2 are enriched in tandem duplicates (GENE1, \( P \)-value < 0.0001; GENE2, \( P \)-value < 0.001) in comparison with the total gene set in A. thaliana (Fig 2A). In addition, translocated paralogs are over-represented among GENE2 compared with both GENE1 and total gene sets (\( P \)-value < 0.001) (Fig 2A and Table S3). Figs 2B and S5 give two examples of translocated GENE2. The first one, AT5G60610, corresponds to a translocation that is present in A. thaliana only, whereas AT1G71330 comes from a translocation that occurred in the last common ancestor of A. thaliana and A. lyrata, after the separation from the C. rubella lineage, and which was followed by a...
more recent rearrangement. Altogether, these data suggest that recently rearranged gene pairs are significantly overrepresented among mRNA chimera-producing ITN loci.

Low expression of GENE2 at ITN chimeric loci may be a hallmark of pseudogenization, which is a common outcome of gene duplication/translocation in plants (Rensing, 2014; Panchy et al., 2016; Galindo-González et al., 2017). Thus, we estimated the Ka/Ks ratio of GENE2 in comparison with both total gene set and previously identified pseudogenes (Krishnakumar et al., 2015). A Ka/Ks ratio approaching 1 is indicative of the absence of selective constraint. As shown in Fig 2C, GENE2s have significantly lower Ka/Ks values compared with well-characterized pseudogenes. Despite this clear difference with pseudogenes, GENE2 loci present higher Ka/Ks in comparison with the A. thaliana total gene set that may indicate ongoing pseudogenization.

In addition, we surveyed the chromatin signatures of GENE1/GENE2 at ITN chimeric loci in WT seedlings using the nine prevalent chromatin states (CSs) previously defined in A. thaliana (Sequeira-Mendes et al., 2014; Vergara & Gutierrez, 2017). In agreement with their expression level, GENE1s were associated with CSs characterizing active transcription units, including CS2, 1, 3, 7, and 6 that correspond to proximal promoters, transcription start site, 5’ end of genes, long coding sequences, and 3’ end of genes, respectively (Fig 2D). Remarkably, the corresponding GENE2s were significantly depleted in the CSs bearing the hallmarks of transcription initiation such as H3K4me3 or the presence of the H3.3 variant and were preferentially enriched in CS6 (Fig 2D and Table S4). This CS is found at the 3’ end of genes and characterized by a slight enrichment in the H2AZ variant (Sequeira-Mendes et al., 2014) and by the presence of H3K36me3, a chromatin modification associated with transcriptional elongation (Vergara & Gutierrez, 2017). Such a dichotomous distribution of chromatin signatures at GENE2 was not observed at a genome-wide level when considering all gene pairs harboring a GENE1 in CS1 (13,988 cases: Chi-square = 86.414, P-value = 2.4 × 10−5), whereas GENE2 at FPA-dependent chimeric loci followed the genome-wide trend (Chi-square = 7.498, P-value = 0.440) (Fig S4D and Table S4).

The atypical chromatin and Ka/Ks signatures of GENE2s raise questions about their potential protein-coding capacity. A mass spectrometry (MS)-based proteomics study of proteins extracted from either WT or nerd-1 plants failed to reveal any peptides corresponding to putative GENE2-encoded proteins, possibly because of low expression levels or lack to obtain in-depth protein identification from total extracts (data not shown). To circumvent this limitation, we developed a sensitive reporter system to visualize GENE2 translation in vivo. We generated two transcription readthrough ATN reporters (AT4G30580/70m and AT1G71340/30m) where a Myc-tag sequence was fused in frame at the C-terminal of the GENE2 ORF (Fig S5B). Although showing similar readthrough phenotype in nerd-1 (Fig S2A), both reporters are expected to differ in their GENE2 protein-coding capacity as AT1G71330, in contrast to AT4G30570, loses the ATG initiator codon upon chimeric mRNA formation (Fig S2A). Upon plant transformations and Western blot analysis, only AT4G30580/70m-tag–transformed plants showed a Myc-tagged signal at the size expected for the AT4G30570m-predicted protein (Fig S5B), suggesting that GENE2 may have retained protein-coding capacity. However, primary sequence prediction indicates that AT4G30570m probably encodes a nonfunctional homolog of the GDP-mannose pyrophosphorylase AT2G39770/VTC1 lacking the first 20 amino acids of a conserved and essential N-terminal nucleotidyl transferase domain (Qin et al, 2008; Li et al, 2016) (Fig S5C). This truncation results in a missed start codon that shifts the predicted initiation codon to the next in frame methionine at position 22 (Fig S5C). Altogether, our data suggest that ITN loci are often rearranged and present a predictive chromatin signature in A. thaliana.

The m6A pathway mutant fp37 exhibits mRNA chimera formation at ITN loci

NERD (needed for RDR2-independent DNA methylation) was previously characterized via a computational screen designed to identify RNA silencing–related proteins owing to the presence of glycine-tryptophane/tryptophane-glycine (GW/GW)-rich ARGONAUTE-anchor (Argonaute [AGO]-hook) motifs (Azavedo et al, 2011; Pontier et al, 2012). To assess the potential role of AGO hook motifs in the termination-promoting activity of NERD, we transformed nerd-1 mutant with constructs expressing either a WT (nerd+T) or an AG/GA-mutated version of NERD (nerd+Tag), previously shown to be defective in binding AGO (Fig S6A) (Pontier et al, 2012). Both T and Tag variants rescued termination defects at all loci tested, suggesting that the AGO hook motifs are not required for the termination-promoting activity of NERD (Fig S6B and C).

To get further insights into the mechanisms involved in mRNA chimera control at ITN loci, we took advantage of the observation that GENE2 at ITN chimeric loci are poorly expressed in WT plants (Fig 1D) and surveyed published transcriptome data for mutation(s) that would lead to global GENE2 up-regulation. We found numerous GENE2s up-regulated in the transcriptomes of two mutant plants deficient in N6-adenosine (m6A) mRNA methylation by virtue of a post-embryonic knockout of two conserved players of m6A methyltransferase complex, FKBPT2-interacting protein 37 KD (fp37-4 LEC1:FIP37; hereafter referred to as fp37L; Shen et al, 2016) and MTA (mtaABI3:MTA; Anderson et al, 2018) (Table S2), suggesting the possible role of the m6A pathway in the control of mRNA chimera formation at ITN loci. Visual inspection of RNA-seq alignments, sashimi plots, and intergenic RT–PCR assays further support this conclusion showing readthrough transcription and mRNA chimera formation at ITNs, but not at FPA-only loci in fp37L mutant (Figs 3A and B and S7). Calculating the changes in read density in the 500-bp windows downstream of annotated genes upon FIP37 depletion revealed a set of 174 genes experiencing readthrough transcription in fp37L mutant (Table S5), with a significant (P-value < 0.0001; hypergeometric test) overlap with ITN loci (about 59% of ITN loci and 36% of FIP37L-dependent loci) (Fig 3C). Examination of ITN regulatory circuitry using epistasis analysis confirmed a marked increase in extended/chimeric transcripts in fp37L compared with nerd-1 backgrounds and a nonadditive effect of nerd-1 and fp37L mutations (Fig 3D), indicating that FIP37 is epistatic to NERD in the control of mRNA chimera formation at ITN loci.

Consistent with the finding that WATP/FIP37 is a conserved component of m6A methyltransferase complex in eukaryotes (Meyer & Jaffrey, 2017), FIP37 depletion triggers a transcriptome-wide loss of m6A mRNA modification in A. thaliana (Shen et al, 2016). To get further...
insight into the potential mechanism linking NERD to FIP37-dependent m6A regulation in plants, we assessed the impact of nerd-1 mutation on m6A regulation by performing quantitative measurement of m6A/A ratio on mRNA using liquid chromatography-tandem MS (LC-MS/MS). As a control in this experiment, we also quantified m6A/A ratio in mRNAs extracted from fip37L plants. Interestingly, we found that the global m6A/A levels in mRNA was reduced by 25% in nerd-1 mutant background (Fig 3E), indicating that NERD impacts mRNA m6A dynamics in plants, although to a lesser extent than FIP37 (Fig 3E). To extend this analysis, we surveyed A. thaliana m6A methylome data (Luo et al, 2014; Shen et al, 2016; Anderson et al, 2018) and found that a majority (65%) of ITN GENE1 mRNAs contained a high-confidence m6A peak near the 3'-UTR region (Table S2). Retrieval and analysis of FIP37 transcriptome-wide m6A methylome data (Shen et al, 2016), further indicated that ITN GENE1 mRNAs are methylated in an FIP37-dependent manner (Fig S8A), an observation that prompted us to assess the impact of nerd-1 mutation on m6A methylation at those loci. By performing m6A-IP-qPCR assays on ITN GENE1s and control loci previously shown to be methylated in an FIP37-dependent manner (Fig S8B)( Shen et al, 2016), we confirmed that ITN GENE1 transcripts were m6A-tagged in an FIP37-dependent manner (Fig 3F). More importantly, NERD deficiency led also to a decrease, although to a lower extent, of m6A levels at all loci tested, including control loci.

Figure 3. FIP37-dependent m6A modification controls mRNA chimera formation at ITN loci.
(A) Shown are sashimi plots of two ITN loci forming chimeric mRNA transcripts in fip37L mutant. The transcript structures and annotations are the same as in Fig 1C.
(B) Semi-quantitative RT–PCR analyses at ITN loci in nerd-1, fip37L, and their corresponding WT controls (top part) and at FPA-only loci in fpa-7, fip37L, and their corresponding controls (bottom part). EFT-4α was used as loading control. Minus RT (−RT) reactions are controls for genomic DNA contamination.
(C) Overlap between loci showing an increase in downstream read counts in nerd-1 and fip37L plants. **** denotes P-value < 0.0001 for enrichment in the overlap, hypergeometric statistical test.
(D) qRT–PCR analysis of extended/chimeric ITN mRNAs in WT, nerd-1, fip37L, and nerd-1/fip37L plants. WT and nerd-1 lines used here correspond to segregants harboring the LEC-FIP37 transgene. Relative transcript levels were normalized to actin using the ΔΔCt method. Data represent the means of four replicate experiments and error bars correspond to SD values. AT4G26410 is used as a control.
(E) LC-MS/MS quantification of the m6A/A ratio in mRNA isolated from WT, fip37L, and nerd-1 plants. *P < 0.05; ***P < 0.001; t test.
(F) ITN GENE1 and methylated control mRNAs are m6A-methylated in an FIP37- and NERD-dependent manner. m6A-IP-qPCR analysis was performed on several ITN and methylated control loci in WT, nerd-1, and fip37L seedlings. Error bars, mean ± SD; n = 3 biological replicates.
(G) Scatter plot representation of m6A enrichment score (on the Y-axis) and FC in downstream reads between WT and fip37L conditions (X-axis).
supporting the notion that NERD is required to reach full level of m\textsuperscript{6}A modification on mRNA. However, no general correlation could be observed between the level of FIP37-dependent m\textsuperscript{6}A enrichment and the control of readthrough transcription (Figs 3G and S8B and Table S6), suggesting that ITN loci require m\textsuperscript{6}A modification to restrict chimeric mRNA formation in a qualitative manner.

The m\textsuperscript{6}A pathway restricts mRNA chimera formation at ITN loci by assisting GENE1 mRNA polyadenylation

To gain insight into the mechanism by which FIP37-dependent m\textsuperscript{6}A pathway could restrict mRNA chimera formation at ITN loci, we performed rapid amplification of cDNA 3'-ends (3' RACE) on total RNA isolated from WT and fip37L plants. To do so, total RNAs were reverse-transcribed with an oligo (dT) anchor primer and amplified sequentially with nested primers located in ITN GENE1 sequence together with the anchor primer (Fig 4B). Variable patterns of polyA sites were detected at tested ITN GENE1 loci in WT plants (Fig 4A), including cases in which GENE1 have a prominent polyA site cluster (AT1G05890 and AT2G21440) and cases in which GENE1 shows more complex profiles of polyA sites (AT1G71340 and AT5G07630) (Fig 4A and B). The nature of polyA sites was confirmed by sequencing the corresponding PCR fragments, and, in some cases, by the examination of polyA tags (PATs) database obtained from high-throughput sequencing analysis in A. thaliana (Fig 4B) (Wu et al, 2011; Thomas et al, 2012). Our analysis revealed that the appearance of long polyadenylated chimeric mRNA transcripts at ITN loci (noted as pAc and confirmed by sequencing) was concomitant with the reduced accumulation of polyadenylated GENE1 mRNA forms in the fip37L background (pA highlighted in red in Fig 4A and B). This effect was specific because the accumulation of polyadenylated mRNAs at control loci (AT2G23350 and AT2G18790) was not affected in fip37L (Fig 4A–C). Taken together, our results suggest that the m\textsuperscript{6}A pathway restricts mRNA chimera formation at ITN chimeric loci by assisting polyadenylation of GENE1 mRNA.

m\textsuperscript{6}A-assisted polyadenylation of GENE1 mRNA requires the YTHDC-type domain-containing protein CPSF30L

The most prominent family of m\textsuperscript{6}A-readers corresponds to the YT521-B homology (YTH) protein clade (Meyer & Jaffrey, 2017). A likely candidate gene for linking FIP37-dependent m\textsuperscript{6}A modification to mRNA polyadenylation is the CPSF30 gene because, in plants, it encodes two isoforms (CPSF30S and CPSF30L) of the conserved 30-kD subunit of cleavage and polyadenylation specificity factor complex (CPSF) that differ by the presence of an YT512-B Homology Domain-containing (YTHDC)-type protein domain in CPSF30L (Liu et al, 2014b; Shi & Manley, 2015; Wu et al, 2015; Berlivet et al, 2019) (Figs 5A and S9F). To assess the implication of CPSF30S and CPSF30L in the m\textsuperscript{6}A-assisted polyadenylation of GENE1 mRNA, we characterized two T-DNA insertion lines in CPSF30, the first one lacking both CPSF30 isoforms (cpsf30-1; also known as oxt6) (Liu et al, 2014b; Pontier et al, https://doi.org/10.26508/lsa.201900393 vol 2 | no 3 | e201900393 7 of 17
Delaney et al., 2006), and a novel one that specifically abrogates CPSF30L mRNA production \( (\text{cpsf}30^{-3}) \) (Figs 5A and S9A). We then performed RNA-seq analysis on purified mRNA from WT, \( \text{cpsf}30^{-1} \), \( \text{cpsf}30^{-3} \), and \( \text{fp37L} \) lines and calculated the changes in read density in the 500-bp windows downstream of annotated genes in these plant backgrounds (Table S5). This analysis revealed a significant \( (P\text{-value}<0.0001; \text{hypergeometric test}) \) overlap between genes showing readthrough transcription in \( \text{fp37L} \) and \( \text{cpsf}30^{-1} \) (142 out of 174 i.e., about 81% of FIP37-dependent loci; Fig 5B and Table S5), indicating that CPSF30-dependent activity is likely engaged in the control of readthrough transcription at FIP37-dependent loci. This conclusion was confirmed at ITN loci as exemplified by Integrative Genomics Viewer snapshots and using semiquantitative RT–PCR analysis (Fig S9B and D). However, in agreement with previous analysis (Thomas et al., 2012), \( \text{cpsf}30^{-1} \) mutant exhibits a broader impact on 3′-end formation, defining a large set of 618 loci showing no dependency on FIP37 for readthrough transcription and polyadenylation controls, hereafter named as FIP37-independent.

Figure 5. CPSF30L links m6A methylation to site-specific polyadenylation at ITN loci.

(A) Structure of the CPSF30 gene and mRNA transcripts. Exons and UTRs are shown with thick and thin grey bars, respectively. Introns are shown with black-dashed lines. APA at Intron-2 produces CPSF30S mRNA, whereas alternative splicing of Intron-2 generates CPSF30L mRNA. Location of the YTH domain in CPSF30L is indicated with a brown rectangle. The positions of T-DNA insertions in the \( \text{cpsf}30 \) mutants are indicated. Primers used in the qRT–PCR experiments are indicated.

(B) Overlap between loci showing a significant increase in downstream read counts in \( \text{cpsf}30^{-1} \), \( \text{cpsf}30^{-3} \), and \( \text{fp37L} \) plants. **** denotes \( P\text{-value}<0.0001 \) for enrichment in the overlaps, hypergeometric statistical test.

(C) Boxplot showing the read counts in the 500-bp downstream region for FIP37-dependent and FIP37-independent CPSF30-responsive loci in WT, \( \text{cpsf}30^{-1} \), \( \text{cpsf}30^{-3} \), and \( \text{fp37L} \) plants. ns, not significant; *** \( P<0.001 \); t test.

(D) Relative contribution of CPSF30S and CPSF30L isoforms to mRNA chimera formation control at ITN- and FIP37-independent CPSF30-responsive loci. Data were calculated from the qRT–PCR analysis of ITN- and FIP37-independent CPSF30 chimeric/extended transcripts presented in Fig S9E. Data represent the means of three independent experiments and error bars correspond to SD values.

(E) Iso. Cont. to read. stands for isoform contribution to readthrough and is expressed in % (E) 3′ RACE gel images of ITN loci in WT, \( \text{fp37L} \), and \( \text{cpsf}30^{-1} \) 9-d-old seedlings. RACE assays were performed as previously described. (F) Western blot analyses of \( \text{cpsf}30^{-1} \) mutant plants expressing Myc-tagged WT (30L) and yth mutant (30Lyth) versions of CPSF30L. The corresponding Coomassie staining is represented on the left part. The anti-Myc antibody was used to visualize the tagged proteins (right part).

(g) qRT–PCR analysis of ITN- and FIP37-independent CPSF30-responsive mRNAs accumulation in WT, \( \text{cpsf}30^{-1} \), \( \text{cpsf}30^{-1}+30L \), and \( \text{cpsf}30^{-1}+30Lyth \) plants. Relative transcript levels were normalized to actin using the \( \Delta\Delta\text{Ct} \) method. Data represent the means of four independent experiments and error bars correspond to SD values.
CPSF30-sensitive loci (Figs 5B and C, S9C, and D). Interestingly, by comparing the set of genes showing readthrough transcription in cpsf30-3, we found that the corresponding loci were significantly (P-value < 0.0001; hypergeometric test) included within the set of cpsf30-1–dependent ones (156 out of 167 i.e., about 93% of cpsf30-3–dependent loci; Fig 5B), as expected from the common genomic origin of those mutants. More importantly, we also found a significant overlap (P-value < 0.0001; hypergeometric test) between cpsf30-3– and fip37L–dependent loci (110 loci i.e., about 70% of cpsf30-3– and 66% of fip37L–dependent loci; Fig 5B and Table S5), supporting the idea of a functional convergence between these proteins. This observation was further supported by boxplot and real-time RT–PCR analyses, reporting a more significant contribution of CPSF30L to readthrough control at FIP37–dependent versus FIP37–independent CPSF30–sensitive loci (Figs 5C and D, and S9E).

To extend this analysis, we conducted 3’ RACE procedure on total RNA extracted from fip37L and cpsf30-3 seedlings and observed that these mutations affected polyA sites patterns similarly at ITNs (Fig 5E). To assess the contribution of the YTHDC–type domain in CPSF30L activity, we transformed either a WT (30L) or an YTHDC–mutated version of CPSF30L (30Lyth), altered in the conserved m6A-binding aromatic cage (see W258, W309, and Y318 in Fig 5F) in cpsf30-1 mutant background, and selected transgenic lines expressing closely similar levels of these proteins (Fig 5F). We then tested the ability of these CPSF30L variants to complement readthrough transcription defects at ITN– and FIP37–independent CPSF30–sensitive loci. Although CPSF30L and CPSF30Lyth showed a similar and partial ability to complement readthrough transcription at FIP37–independent CPSF30–sensitive loci, they differed in their ability to restrict chimeric mRNA formation at ITN loci (Fig 5G), suggesting that the YTHDC–type domain of CPSF30L is essential to control chimeric mRNA formation at ITN loci.

m6A–assisted polyadenylation restricts chimeric gene–TE transcript formation

During our analysis of ITN loci, we noticed two cases in which GENE2 correspond to a mis–annotated TE (Table S3), suggesting that m6A–assisted polyadenylation could also exert an inhibitory control over chimeric GENE–TE transcripts. To extend this observation, we focused our analysis on the AT3G47890–AT3G47875 candidate locus, in which the mis–annotated GENE2 corresponds to a non–LTR retrotransposon of the LINE (long interspersed nuclear element, AT3TE71565) family (Table S3). Sequence comparison in A. thaliana and A. lyrata reveals evolutionary conserved regions flanking AT3TE71565 indicating that the LINE1 element got inserted within AT3G47890 3’–UTR posterior to the separation between the A. thaliana and A. lyrata lineages (Figs 6A and S10D). As such, AT3TE71565 contains all of the typical hallmarks of LINE1–mediated retrotransposition, including the presence of target–site duplications, 5’–truncated ORFs, and a short polyA tail confirming that AT3TE71565 is inserted in a tail–to–head orientation relative to the AT3G47890 gene (Figs 6A and S10A). Visual inspection of the mRNA–seq reads and RT–PCR assays further confirmed the accumulation of chimeric AT3G47890–AT3TE71565 transcripts in fip37L– and cpsf30–mutant backgrounds (Figs 6B and S10C). 3’ RACE analysis further revealed that the appearance of polyadenylated chimeric RNA transcripts in both fip37L– and cpsf30–3–mutant backgrounds was concomitant with the reduced accumulation of a long polyadenylated GENE1 mRNA form (pA2 highlighted in red in Figs 4A and B).

Figure 6. m6A–assisted polyadenylation restricts chimeric GENE–TE transcript formation in Arabidopsis. (A) An example of a LINE1–type retroelement translocation that has occurred recently in A. thaliana (top), after the separation from the A. lyrata (middle) and C. rubella (bottom) lineages. (B) Normalized reads mapping to the chimeric AT3G47890–AT3TE71565 loci are shown in WT, fip37L, cpsf30–1, and cpsf30–3 backgrounds. Upper panel shows m6A peaks as revealed by m6A–seq performed in WT and fip37L backgrounds (Shen et al, 2016). Light blue color represents input reads, whereas red color represents IP reads. The transcript structures and annotations are the same as in Fig 1C. Primers used in the RT–PCR and McrBC experiments are shown as green, red, and grey arrowheads, respectively. (C) 3’ RACE gel images of chimeric AT3G47890–AT3TE71565 loci in WT, fip37L, cpsf30–3, and cpsf30–1 plants (top panel). RACE assays were performed as previously described. pA2 corresponds to the AT3G47890 polyadenylated site that decreases in fip37L and cpsf30–3 plants, whereas pA1 indicates the polyadenylated sites corresponding to the mRNA chimera. PolyA sites detected at AT3G47890–AT3TE71565 ITN loci are indicated relative to GENE1 STOP codon (bottom panel). The respective positions of ITN GENE1 and TE loci are indicated with green and blue lines. The primers used in the 3’ RACE are indicated with arrowheads. The oligo (dT) anchor primer is indicated with a broken arrow. (D) Model for m6A–assisted polyadenylation (m–ASP) at ITN loci. NERD and FIP37 are required to reach full m6A deposition at all loci including ITN GENE1s (1). The recognition of m6A by the YTHDC–type domain of CPSF30L promotes cleavage and polyadenylation at GENE1 3’–UTR (2), therefore restricting mRNA chimera formation (3).
and S10D), supporting the idea that m6A-assisted polyadenylation also restricts chimeric GENE-TE transcript formation in plants. Using McBC-PCR analysis, we found no change in AT3TE71565 DNA methylation levels in fp37L and cps30-1/3 mutants (Fig S10B and E), indicating that RNA chimera formation likely accounts for the up-regulation of RNA signal at AT3TE71565. Taken together, our results show that m6A-assisted polyadenylation can also restrict the formation of specific chimeric gene-TE transcript in plants.

Discussion

Our study provides novel insights into the role of m6A pathway in plants, revealing the implication of plant-specific and conserved m6A-related proteins in polyadenylation and transcription termination control at rearranged genomic loci in A. thaliana. The first evidence that m6A could participate in the control of chimeric mRNA formation at specific loci in plants came from our ongoing analysis of NERD, a plant homeodomain finger- and AGO hook-containing protein that, until now, was thought to contribute to the transcriptional silencing of newly acquired genomic sequences via a non-canonical siRNA-dependent DNA methylation pathway (Garcia et al, 2012; Pontier et al, 2012). Surprisingly, our studies point to an unexpected activity in that NERD depletion reduces mRNA m6A abundance at a genome-wide level, resulting in transcription termination defect and chimeric mRNA formation at rearranged genomic loci. We show that the contribution of NERD in mRNA chimera control is independent of its AGO hook–binding platform, indicating that NERD is likely to achieve multiple functions in plants.

Our finding that the NERD depletion decreases m6A deposition on mRNAs provides a mechanistic basis for NERD-mediated regulation of chimeric mRNA formation at ITNs; however, it does not explain the exact role of NERD in m6A regulation in plants. Despite our epistatic genetic interaction data, all attempts made to detect a physical interaction between NERD and FIP37 in vivo failed so far, suggesting that NERD is not likely to be a novel core component of m6A methyltransferase complex. However, recently published Arabidopsis proteomics data indicate that NERD copurifies with SPT4/SPT5 transcription elongation factors and elongating Pol II in vivo (Antosz et al, 2017). In addition, NERD also harbors a specific protein domain, named as Plus3 (Pontier et al, 2012), which has been shown to mediate Pol II-associated factor 1 complex (PaF1C) recruitment to genes by binding to a specific domain within the Pol II elongation factor SPT5 (Mayekar et al, 2013; Wier et al, 2013). This suggests that NERD could potentially act at an early stage of m6A deposition within the elongating RNA polymerase II (Pol II) complex. Together with our previous data showing that NERD binds unmethylated histone H3 lysine 4 (H3K4me0) through a conserved plant homeodomain finger-type domain (Pontier et al, 2012), these observations suggest that NERD could act at the interface between elongating Pol II and chromatin, potentially facilitating the co-transcriptional deposition of m6A on mRNAs, in agreement with the prevailing model for m6A deposition (Ke et al, 2017).

Our study revealed an unexpected function of m6A pathway in the control of transcriptome integrity by guiding site-specific mRNA polyadenylation at a subset of genomic loci that exhibit intrinsic transcription termination and polyadenylation defects (Fig 6D). The reported m6A-assisted polyadenylation (m-ASP) pathway is distinct from the classical mechanisms of APA (Wu et al, 2015). Although several studies have previously found a link between CPSF30 gene and APA in plants, this activity has been mostly attributed to CPSF30S, the YTHDC-less short isoform, previously shown to be sufficient to restore developmental and molecular phenotypes incurred by mutations in CPSF30 (Liu et al, 2014b; Shi & Manley, 2015; Wu et al, 2015). The role of m6A modification in the control of polyadenylation site selection remains more obscure, despite the prevalence of m6A within 3′-UTRs. In animals, m6A has been shown to mediate opposing effects on site selection at genes experiencing APA, favoring either proximal or distal polyadenylation depending on the cell type (Ke et al, 2015; Yue et al, 2018). A direct role for m6A in targeting mRNA polyadenylation in plants is supported by our finding that the concomitant depletion of FIP37 and m6A suppresses specific polyA sites at a subset of genomic loci, in fine leading to transcription readthrough, intergenic splicing, and chimeric mRNA formation. Given that chimeric mRNA formation in plants can also be controlled by m6A-independent mechanisms, as demonstrated at FPA-only loci, our study raises the possibility that the selective recruitment of m-ASP pathway to sustain efficient transcription termination and polyadenylation may depend on the local genomic context. Such hypothesis is supported by our observation that ITN loci are enriched in recently rearranged gene pairs and display an atypical chromatin signature, with GENE1 harboring canonical histone marks of both active transcription initiation and elongation (i.e., H3K4me3 and H3K36me3/H2Bub, respectively), and GENE2 solely exhibiting marks of transcription elongation. We propose that the presence of a dual peak of transcription elongation marks over ITN gene pairs likely reflects the basal level of pervasive readthrough transcription that characterizes these loci in WT plants. Whether the atypical state of chromatin and the presence of m6A at ITNs shape the transcription termination and polyadenylation responses to pervasive readthrough transcription remains to be clarified. Interestingly, evidence for cross-talk between histone modification and RNA methylation machineries has recently emerged in animals (Hu et al, 2019), reinforcing the idea that chromatin- and m6A-related pathways can influence one another.

Our study demonstrates that m6A-assisted polyadenylation at ITN loci requires CPSF30L, a poorly characterized YTHDC-type domain-containing isoform of the A. thaliana gene CPSF30 (Fig 6D) (Berlivet et al, 2019). Although several studies have previously found a link between CPSF30 gene and APA in plants, this activity has been mostly attributed to CPSF30S, the YTHDC-less short isoform, previously shown to be sufficient to restore developmental and molecular phenotypes incurred by mutations in CPSF30 (Liu et al, 2014b; Shi & Manley, 2015; Wu et al, 2015). The role
of CPSF30L could have been underappreciated in previous studies because they were focused on exploring gene-specific APA site distribution (Thomas et al, 2012). Our study also pinpoints the concerted action of both CPSF30 isoforms at ITN loci, indicating that both m6A-dependent and m6A-independent polyadenylation mechanisms contribute to transcription termination at those loci. Such cooperation could be promoted by the presence of a conserved N-terminal zinc-finger domain shared by both CPSF30 isoforms that is believed to self-associate (Delaney et al, 2006).

Concerning the CPSF30L isoform, our data show that its role in chimeric mRNA formation control is contingent on FIP37 activity and on the integrity of the conserved aromatic cage known to bind m6A in other YTH proteins (Wu et al, 2017). These equivalence indicate that the regulatory function of m6A requires CPSF30L, and that CPSF30L is likely an m6A reader whose recruitment is needed to ensure optimal or near-optimal transcription termination and polyadenylation at ITN loci (Fig 6D).

Finally, our results suggest that the m-ASP pathway can also restrict chimeric GENE-TE transcripts produced upon the insertion of TE in the 3’-untranslated region of genes. Although such events are rare, chimeric gene-TE transcripts are a mean by which retrotransposons can mobilize or lead to gene retroposition in plants and animals, potentially interfering with genome function (Kazazian, 2004; Xiao et al, 2008; Eickbush & Eickbush, 2011; Zhu et al, 2016). Although specific DNA methylation-based silencing mechanisms have been developed by plants to restrict TE expression per se (Law & Jacobsen, 2010), our data suggest that the m-ASP pathway may act, at a locus-specific level, as an additional mechanism to limit the inappropriate expression of TE inserted in the vicinity of genes.

Materiel and Methods

Plant methods

A. thaliana Col-0 as well as the following mutants were used in this study: nerd-1 (SALK_093814) (Pontier et al, 2012), nerd-3 (GABI_123G03), fca-9 (Liu et al, 2007), fpa-7 (SALK_138449), cpsf30-1 (Liu et al, 2014b), cpsf30-3 (GABI_477H04, obtained from the Nottingham Arabidopsis Stock), fp37-4 LEC1:FIP37 (Shen et al, 2016). The primers used to genotype new lines are listed in Table S7. nerd-1-T corresponds to the complemented nerd-1 line described in (Pontier et al, 2012). The same construct was introduced into nerd-3 plants by crosses to obtain nerd-3+T. For the epistasis analysis, fp37-4 LEC1:FIP37 heterozygous plants were crossed to nerd-1 mutants. The progenies were screened by PCR (see Table S7) to retrieve WT LEC1: FIP37, nerd-1 LEC1:FIP37, and nerd-1 fp37-4 LEC1:FIP37 lines.

Plants were either grown in soil or cultivated in vitro on plates containing 2.20 g/l synthetic Murashige and Skoog (MS) (Duchefa) medium, pH 5.7, and 8 g/l agar. For transgenic seeds selection, 30 μg/l hygromycin were added. For fp37-4 mutants or derivatives, experiments were carried out on plates containing 4.41 g/l MS medium, pH 5.7, 1% sucrose, and 8 g/l agar; 9-d-old seedlings were collected under a microscope. In growth chambers, the conditions are 20°C, 60–75% hygrometry with a 16-h light/8-h dark cycle and 130 μE m−2 s−1 light (LEDs with white 4500K spectrum, purchased from Vegeled).

Generation of transgenic plants

To generate AT4G0580/70m and AT1G71340/30m reporters, the corresponding genomic sequences were amplified using TL3437(KpnI)- TL3436(BamHI) and TL3438(KpnI)-TL3439(BamHI) primer pairs, respectively. After sequencing, the corresponding fragments were introduced in a binary vector (CTL639) containing a 4cMyc tag in front of a NosT terminator and the hygromycin resistance gene driven by a 35S promoter (CTL235). The obtained plasmids were used to transform nerd-1 plants via Agrobacterium transformation.

To obtain WT (30L) and YTHDC-mutated version of CPSF30L (30Lyth) constructs, we amplified the CPSF30 promoter using TL3400 and TL3401. We also gene-synthesized (Genecust) the cDNAs corresponding to the WT and the YTHDC-mutated versions of CPSF30L (W258 to A, W309 to A, Y314 to A mutations; Fig S9F) with a 4cMyc tag upstream the ATG. Both fragments were introduced upstream of a NosT terminator into a binary vector containing the hygromycin resistance gene driven by a 35S promoter (CTL235). The obtained plasmids were used to transform cpsf30-1 plants via Agrobacterium transformation.

RNA sequencing

Total RNA was extracted from 14-d-old seedlings for Col0, nerd-1, and nerd-1+T plants and from 9-d-old seedlings for Col-0, nerd-1, and cpsf30-1 plants using the RNeasy Plant Mini Kit (Cat. No. 74904; QIAGEN) and treated onto the column with the RNase-Free DNase Set (Cat. No. 79254; QIAGEN). Two replicates were performed per line. Polya purification, library preparation using the Illumina TruSeq Stranded mRNA Kit and library quality controls were performed by Fasteris SA. For Col-0, nerd-1, and cpsf30-1, sequencing was performed by Fasteris SA with paired reads, 2 × 125 pb and 37–53 × 106 reads per library. For Col-0, nerd-1, and nerd-1-T, sequencing was performed by Fasteris SA with single-end reads, 1 × 125 bp and 21–52 × 106 reads per library.

For each library, 20–30 × 106 reads were obtained with 85–90% of the bases displaying a Q-score ≥ 30, with a mean Q-score of 38 as assessed with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Filtering out of reads corresponding to chloro-plastic, mitochondrial, and rRNA genes was performed with Bowtie 2 v2.3.4 in sensitive local mode (Langmead & Salzberg, 2012). Remaining reads were mapped against the TAIR10 genome using gtf annotation file and default parameters of TopHat2 v2.0.7 (Kim et al, 2013). Assembly and transcript quantification were performed with Cufflinks v2.2.1 (Trapnell et al, 2010). Finally, the low-expressed transcripts, less than 1 RPKM (reads per kilobase per million reads per kilobase of transcript per million reads mapped) transcripts.
mapped reads), in one of the libraries were filtered out. The differential analysis was conducted by Cuffdiff software, belonging to the Trapnell suite. Quantification over 500–bp windows was made with BEDTools software (Quinlan & Hall, 2010). The 500–bp downstream from gene windows was defined using gtf annotation from the TAIR10 database. We normalized raw read counts by the total of mapped reads (rpm). Fold change (FC) was determined as the ratio between the normalized read counts between mutant and wild type. The list was further filtered to remove readthrough cases where the expression of the upstream genes changes in the ner-d-1 mutant background.

Expression analysis

Total RNA was isolated from 50 mg of seedlings using the TRI reagent (Cat. No. TR-118; Euromedex) according to the manufacturer’s instructions. Genomic DNA was then digested out using the RNase-Free DNase (Cat. No. M6101; Promega): 4 μg of RNA was treated with 4 units of the enzyme in the 1× reaction buffer in a 20-μl total volume at 37°C for 30 min and the reaction stopped by adding 2 μl of RNase-Free DNase Stop Solution and incubating 10 min at 65°C. cDNAs were obtained from 400 ng of RNase-treated RNA using 1 μl of GoScript Reverse Transcriptase (Cat. No. A5003; Promega) in a 20-μl final volume reaction using random primers (Cat. No. C1181; Promega) in the presence of 20 units of RNasin (Recombinant Ribonuclease Inhibitor, Cat. No. C2511; Promega) and dNTP mix at a final concentration of 0.5 mM of each dNTP (Cat. No. U1511; Promega). Semi-quantitative RT–PCR amplifications were performed on 1 μl of cDNA in a 12.5-μl reaction volume to start with. The amplification of EF1-a transcripts (TL786-TL787, Table S7) was used to equilibrate. qRT–PCR was performed on a Light Cycler 480 II machine (Roche Diagnostics) by using the Takyon No ROX SYBR MasterMix blue dTTP kit (Cat. No. UF–NSMT–B0701; Eurogentec). Each amplification reaction was set up in a 10-μl reaction containing each primer at 300 nM and 1 μl of RT template in the case of total RNA with a thermal profile of 95°C for 10 min and 40 amplification cycles of 95°C, 15 s; 60°C, 60 s. Relative transcript accumulation was calculated using the ΔΔCT methodology, using ACTIN2 as internal control (Livak & Schmittgen, 2001). Average ΔΔCT represents three experimental replicates with standard errors. Primers used are listed in Table S1.

PolyA+ RNAs were purified from RNA obtained by the TRI Reagent (Cat. No. TR-118; Euromedex) extraction from 100 μg of tissue, using the PolyATtract mRNA Isolation System III (Cat. No. Z5310; Promega) according to the manufacturer’s instructions. Northern blots were performed using 2 μg of the obtained polyA+ RNA resuspended in 100% formamide. After a 5-min denaturation at 70°C, the RNAs were separated onto a 1% agarose, 0.5% formaldehyde gel in 0.03 M tricine 0.03 M trithethanolamine buffer. The RNAs were transferred onto an NX-Hybrid membrane (GE Healthcare) and cross-linked with EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, Cat. No. E7750; Sigma-Aldrich). For the At4g30570-80 locus, the probe was PCR-amplified on genomic DNA using TL2980 and TL3205 primers (Table S7), purified using the GeneClean Turbo Kit (Cat. No. 111102400; MP Biomedicals), and labeled with the Prime-a-Gen Labeling System (Cat. No. U1100; Promega) according to the manufacturer’s instructions. The hybridization was performed in the PerfectHyb Plus Hybridization Buffer (Cat. No. H7033; Sigma-Aldrich) at 68°C overnight. For At3g17340, the TL3417 primer (Table S7) was labeled using the T4 polynucleotide kinase (Cat. No. M4101; Promega) and used as in a probe in the PerfectHyb Plus Hybridization Buffer (Cat. No. H7033; Sigma-Aldrich) at 42°C overnight. In both cases, the membranes were washed once in a 2× SSC, 0.1% SDS solution for 5 min at room temperature and twice in 0.5× SSC, 0.1% SDS solution for 15 min at 42°C or 68°C before exposure.

RNA m6A quantification by targeted MS (LC–MRM)

Total RNAs from WT, frip37l, and ner-d-1 plants were isolated using TRIzol reagent (Euromedex) according to the manufacturer’s instructions. Polyadenylated RNAs were extracted twice by oligo(dT) magnetic beads (Cat. No. Z5310; Promega), followed by removal of remaining RNA with Ribominus Plant Kit (A10838–08; In vitrogen). mRNA concentration was measured using NanoDrop and Qubit assays. 210 ng of mRNA were diluted in a total volume of 20 μl milliQ water. 3 μl of 0.1 M ammonium acetate, pH 5.3, and 0.001 U of Nuclease P1 (N8630; Sigma-Aldrich) were added. Incubation at 42°C for 2 h was performed. Then, 3 μl of 1 M ammonium acetate and 0.001 U of alkaline phosphatase (P4252; Sigma-Aldrich) were added. The mixture was incubated at 37°C for 2 h. Next, the nucleosides solution was diluted two times and was filtered with 0.22 μm filters (Milllex-GV, SLGVR04NL; Millipore). 1 μl of each sample was injected, and all samples were analyzed in triplicate using LC-MRM.

The nucleosides were then separated by 1290 LC systems (Agilent Technologies) using an Synergi Fusion-RP column (4 μm particle size, 250 mm × 2 mm, 80 Å) (00G–4424–B0; Phenomenex) at flow rates...
of 0.4 ml/min. The mobile phase consisted of 5 mM ammonium acetate, adjusted to pH 5.3 with acetic acid (solvent A) and pure acetonitrile (solvent B). The 30-min elution gradient started with 100% phase A followed by a linear gradient to 8% solvent B at 13 min. Solvent B was increased further to 40% over 10 min. After 2 min, solvent B was decreased back to 0% at 25.5 min. Initial conditions were regenerated by rinsing with 100% solvent A for additional 4.5 min.

The detection was performed using Agilent TripleQuad 6490 in positive ion mode. The multiple reaction monitoring (MRM) transitions used for detection were m/z 268 to 136 for Adenosine (fragmentor voltage: 380 V, collision energy: 21 V, cell accelerator voltage: 1 V, and retention time: 13.1 min) and m/z 282.1 to 150.1 for m/z 268 to 136 for Adenosine positive ion mode. The multiple reaction monitoring (MRM) transition was set up as follows: 4 μl of template using 0.3 μl GoTag Q2 DNA Polymerase (Cat. No. M7848; Promega), 0.2 mM each dNTP, 8 μl 10 μM PCR anchor primer (reverse primer, TL3577, Table S7), and 1 μl 10 μM specific primer (primer 1, Table S7). PCR was performed with a thermal profile of 94°C for 2 min; 35 cycles (94°C, 30 s, 57°C, 30 s, and 72°C, 2 to 4 min) and then 72°C, 10 min. The reaction was checked onto an agarose gel. The second amplification was performed on a 1-μl aliquot from the first PCR with the same conditions as for the first PCR except that 1 μl 10 μM PCR anchor primer (reverse primer, TL3577, Table S7) and 1 μl of a 10 μM nested specific primer (primer 2, Table S7) were used. The final products were separated on an UltraPure Agarose (Cat. No. 16500-500; Invitrogen) gel. Lower bands corresponding to Gene1 polyA sites in WT were chosen to be sequenced as well as upper bands that appeared in fip37 samples that corresponded to readthroughs. To get better sequences for the readthroughs, the second PCR was performed using a primer closer to Gene2 polyA sites (primer 3, Table S7). The DNA corresponding to bands to be sequenced was extracted using the GeneClean Turbo Kit (Cat. No. 111102400; MP Biomedicals) and subcloned into pGEM-T Easy Vector (Cat. No. A1360; Promega). The DNA from 48 to 96 independent clones was sequenced with the SupremRun Service from GATC Biotech. The sequences were aligned and the position of the last nucleotide upstream the polyA tail was identified, the starting point being the first base downstream the ITN GENE1 stop codon. The number of clones and their precise position are reported in Table S7.

**m^6^A-IP-qPCR**

m^6^A-IP-qPCR was performed as previously described (Dominissini et al, 2012; Shen et al, 2016). Total RNA was extracted and fragmented into ~200-nucleotide-long fragments by RNA fragmentation buffer (Cat. No. AM8740; Ambion). Fragmented RNA was incubated with 0.5 mg/ml anti-m^6^A antibody (Cat. No. 202-003; Synaptic Systems) in IP buffer (150 mM NaCl, 0.1% (gepal CA-630, and 10 mM Tris–HCl, pH 7.4) supplemented with RNasin Plus RNase inhibitor (Cat. No. C2511; Promega) for 2 h at 4°C, and subsequently, protein A/G Plus-Agarose (Cat. No. sc-2003; Santa Cruz) that was pre-bound with BSA was added and incubated at 4°C for an additional 2 h. After extensive washing with IP buffer, bound RNA was eluted from the beads by incubation with 6.7 mM N^6^-methyladenosine (Cat. No. M2780; Sigma-Aldrich) in IP buffer and precipitated by ethanol. The input and immunoprecipitated RNAs were reverse-transcribed with random hexamers (Cat. No. N8080127; Invitrogen) using M-MLV Reverse Transcriptase (Cat. No. M1701; Promega). Relative enrichment of each gene was determined by quantitative real-time PCR and calculated first by normalizing the amount of a target cDNA fragment against that of TUB2 as an internal control, and then by normalizing the value for immunoprecipitated sample against that for the input. The primers used for real-time PCR are listed in Table S7.

**Protein extraction and immunodetection**

Total plant protein extracts (up to 100 mg) were ground in liquid nitrogen with 200 μl SDS–PAGE loading buffer. Coomassie staining was used to calibrate loadings. Proteins were separated on SDS/PAGE gels and blotted onto Immobilon-P PVDF membrane (Cat. No. IPVH00010; MerckMillipore). Protein blot analysis was performed using the Immobilon Western Chemiluminescent HRP Substrate (Cat. No. WBKLS0500; MerckMillipore). The antibodies used in this study were HRP–coupled FLAG-specific antibody (Cat. No. A8592; Sigma-Aldrich) and anti-UGPase antibody as a loading control (Cat. No. AS05 086; Agrisera) at 1/5,000 dilution and HRP–coupled Myc-specific antibody (Cat. No. A5598; Sigma-Aldrich) at 1/10,000.

**Chromatin Immunoprecipitation**

2 g of inflorescences were homogenized in 25 ml of Honda buffer (0.44 M sucrose, 1.25% Ficoll, 2.5% dextran T40, 20 mM Hepes KOH, pH 7.4, 10 mM MgCl₂, 0.5% Triton, and 5 mM DTT) supplemented with Complete EDTA-free Protease Inhibitor Cocktail (Cat. No. 11873580001; Roche) was filtered onto two layers of Miracloth (Cat.
Duplicated genes were classified into four categories: 1) whole genome duplication/segmental paralogs are located in corresponding collinear blocks within species. 2) Tandem duplicates correspond to paralogs that are immediately adjacent to each other. 3) Translocated duplicates refer to non-collinear paralogs that have at least one collinear ancestral paralog (Wang et al., 2013b). It should be noted that we prefer here the use of “translocated duplicates” rather than “transposed duplicates” used by the author of MCScanX-transposed because the latest term may be confused with transposition of TEs whereas gene movement and translocation may result from multiple mechanisms. 4) Other duplicates include duplicated genes that did not belong to any of the first three categories. Statistical tests were performed using R and Figs 6A, S2B, and S6A were drawn using EasyFig software (Sullivan et al., 2011).

GENE1/GENE2 evolutionary conservation and expression

To identify homologs of GENE1 and GENE2 in angiosperm and their genomic positions, the reference genome of 325 sequenced angiosperms was first downloaded from NCBI (ftp://ftp.ncbi.nlm.nih.gov/genomes/). The predicted protein sequences of each pair of genes were then blasted against the entire reference genome using the blastn program. BLAST hits with a minimum of $1 \times 10^{-10}$ e-value corresponding to potential homologs sequences were all kept even if multiple alignments were found for each genes. Genes were considered as absent if no hit was found in the target genomes. When hit corresponding to both GENE1 and GENE2 were found, we consider genes as adjacent when any of the unique or multiple hits of GENE1 and GENE2 were located in the same chromosome/scaffold and their relative distance not greater than 5 kbp. Otherwise, GENE1 and GENE2 were classified as distant. For each GENE1/GENE2 pairs, the number of the four different scenarios shown in Fig 1C was calculated and expressed in percentage. The expression score in transcripts per million (TPM) was obtained from AraPoi11 (https://apps.araport.org/thalemine) and heat map generated using heatmap2 function in R.

CS analysis of GENE1/GENE2 pairs

The genomic coordinates of CSs and of GENE1/GENE2 pairs were retrieved from published data (Sequeira-Mendes et al., 2014; Vergara & Gutierrez, 2017), and from the TAIR10 release of the A. thaliana genome. For each gene, the overlap with the nine CSs corresponding to proximal promoters (CS2), transcription start site (CS1), 5’ end of genes (CS3), long coding sequences (CS7), 3’ end of genes (CS6), polycomb chromatin (CS5), distal regulatory intergenic regions (CS4), AT-rich heterochromatin (CS8), and GC-rich heterochromatin (CS9) was determined. A Pearson’s chi-square test was used to statistically assess the similarity between the distribution of GENE2s among the nine CSs for NERD- or FPA-dependent chimeric loci with that of all the genes immediately downstream of a gene associated with CS1 across the genome. Because of small sample size, P-values were computed by Monte Carlo simulation.

Data Availability

Sequencing data have been deposited in NCBI’ GEO under accession number GSE123912.

Supplementary Information

Supplementary information is available at https://doi.org/10.26508/lsa.201900393.
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T Lagrange: conceptualization, supervision, funding acquisition, and writing—original draft.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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