A stable tRNA-like molecule is generated from the long noncoding RNA GUT15 in Arabidopsis

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ABSTRACT

The Arabidopsis GUT15 RNA belongs to a class of noncoding RNAs that are expressed from the intergenic regions of protein-coding genes. We show that the RNA polymerase II transcribed GUT15 transcript serves as a precursor for two stable RNA species, a tRNA-like molecule and GUT15-TF-F5, which are both encoded by the final intron in the GUT15 gene. The GUT15-encoded tRNA-like molecule cannot be autonomously transcribed by RNA polymerase III. However, this molecule contains a CCA motif, suggesting that it may enter the tRNA maturation pathway. The GUT15-encoded tRNA-like sequence has an inhibiting effect on the splicing of its host intron. Moreover, we demonstrate that the canonical tRNA genes nested within introns do not affect the splicing patterns of their host protein-coding transcripts.

Introduction

Recent advances in RNA sequencing technology and computational analyses have resulted in the identification of a notable number of long transcripts known as long noncoding RNAs (lncRNAs) that lack protein-coding capacity [1]. The heterogeneity in the biogenesis, expression levels, stability and evolution of this diverse class of molecules reflects the broad array of functions performed by lncRNAs in the cell. Based on animal studies, lncRNAs have emerged as novel factors regulating different aspects of gene expression, and these factors have been shown to participate in transcriptional silencing by recruiting chromatin remodeling complexes [2]. Several lines of evidence suggest that lncRNAs play a role in establishing and maintaining the architecture of nuclear compartments [2].

Numerous lncRNAs have been predicted and identified in several plant species [3]; however, our knowledge regarding their biogenesis and molecular functions remains limited to a few examples. Both AtCOOLAIR and AtCOLDAIR noncoding RNAs play roles in the epigenetic silencing of the FLC locus during vernalization [4,5]. The epigenetic landscape is also affected by the dual noncoding transcription of the APOLO locus (performed by both RNA polymerase II and V) that controls the auxin-driven chromatin loop dynamics to regulate the expression of the neighboring PID gene, which encodes an essential regulator of polar auxin transport [6]. The APOLO lncRNA-dependent oscillating chromatin topology further reflects an additional function ascribed to plant lncRNAs, i.e., the production of small interfering RNAs [7]. Generally, the biogenesis of 24 nt siRNAs requires noncoding transcription, which is driven by plant RNA polymerases IV and V, and leads to transcriptional gene silencing (TGS) by directing the DNA methylation of their locus of origin and adjacent genes [8]. In addition, plant lncRNAs have been associated with other biological processes. Phosphate homeostasis is regulated by the IPS1 lncRNA, which acts as an endogenous target mimic of miR399 [9]. Photomorphogenesis in Arabidopsis seedlings is regulated by the HID3 lncRNA, which is known to associate with PIF3 locus chromatin and repress its transcription [10]. In rice, the LDMAR noncoding transcript is required for normal male fertility under long-day conditions [11]. Plant lncRNAs are also involved in root nodule organogenesis; for example, the ENOD40 lncRNA plays a role in the cytoplasmic re-location of the nuclear protein RBP1 during nodulation in Medicago truncatula [12]. Bardou and coworkers [13] identified that the ASCO lncRNA is a novel player (competitor) in the regulation of alternative splicing. Recently, the ELENA1 noncoding transcript has been shown to be involved in the transcriptional regulation of plant innate immunity by interacting with Mediator and enhancing the expression of PRI (PATHOGENESIS-RELATED 1) [14].

Long noncoding RNAs can be classified into the following five different classes according to their genomic location: natural antisense transcripts (NATs), intronic noncoding RNAs, enhancer RNAs, promoter-associated transcripts and large intergenic noncoding RNAs (lincRNAs) [1]. The lincRNA group is represented in A. thaliana by the GUT15 long
noncoding transcript, which was first reported in tobacco as a short-lived RNA [15].

In this paper, we prove the existence of a tRNA-like structure nested within the gene encoding the Arabidopsis IncRNA GUT15. Interestingly, the biogenesis of this tRNA-like molecule relies on GUT15 transcript processing rather than on RNA polymerase III-driven transcription. Furthermore, the CCA trinucleotide, which is indicative of functional tRNAs, is added to the 3′-end of the tRNA-like molecule encoded by the final intron of the GUT15 primary transcript, and this molecule is most likely aminoacylated. Additionally, we show that the GUT15-encoded tRNA-like structure inhibits the splicing of its hosting intron.

**Results**

**A tRNA-like molecule is encoded within the Arabidopsis IncRNA GUT15 locus**

The re-annotation of the tRNA genes in the Arabidopsis genome performed in our laboratory revealed the presence of a tRNA-like sequence within the locus encoding the IncRNA GUT15 (Gene with unstable transcript 15; At2g18440) [16]. This tRNA-like sequence exhibits the conserved structural features present in canonical tRNAs. In particular, the length of the acceptor, the D- and T-stems, as well as the size of the D- and T-loops are consistent with the standard tRNA structure cloverleaf model (Fig. 1A, left panel) [17]. Interestingly, compared with bona fide tRNA sequences, the sequence of the T-loop (positions 3–9 of the B-box in the sequence logo) in the identified tRNA-like structure is very well conserved. This feature plays a role in the formation of the distinct L-shaped tertiary structure that is essential for tRNA function. In contrast, the A-box of the GUT15 tRNA-like structure differs from the consensus sequence due to two additional nucleotides (Fig. 1A, middle panel). Moreover, the region corresponding to the anticodon stem-loop in the tRNA-like structure cannot form the canonical 5-bp stem and 7-nucleotide loop. Another feature distinguishing the GUT15 tRNA-like structure from standard tRNA molecules is the presence of three rather than two unpaired bases at the junction between the acceptor and

![Figure 1](image-url)
sRNA-seq results (homemade libraries and public samples) for in transcript GUT15

Nicotiana benthamiana GUT15 transcripts are capped, polyadenylated and gene variant 0 GUT15 Arabidopsis GUT15, right gene transcripts. Additionally, using the tRNA-like molecule contains a leaves using the gene (both under the control of the CaMV 35S pro-

transcripts (Fig. 1A locus, we performed 5 lncRNA from cDNA revealed the presence of a 0 GUT15 was detected in the seedlings was 100 bp longer than the GUT15 gene contains 4 introns. The seedlings was 100 bp longer than the GUT15 transcript described in the TAIR10 database [18]. However, this discrepancy may be due to differences in the regulation of GUT15 gene transcription in specific tissues or the different developmental stages during which the RNA samples were collected. The Arabidopsis GUT15 gene contains 4 introns. The discovered tRNA-like sequence is encoded by the final intron in the GUT15 transcripts (Fig. 1B). Interestingly, the RT-PCR amplification of the GUT15 cDNA revealed the presence of a series of splicing isoforms, and only three isoforms contained the tRNA-like-bearing intron (Fig. 1C).

The GUT15 transcripts are capped, polyadenylated and undergo alternative splicing

To determine the structure of the transcripts originating from the Arabidopsis GUT15 locus, we performed 5' and 3' RACE experiments using primers that hybridize to the tRNA-like sequence, followed by RT-PCR amplification of the products obtained with primers designed to bind to the 5'- and 3'-ends of the longest RACE fragments. Using this approach, we identified polyadenylated GUT15 transcripts. Additionally, using the 5'-RLM-RACE procedure to selectively amplify only full-length capped RNAs, we showed that all analyzed GUT15 transcripts contained the cap structure, which, along with the poly(A) tail, constitutes the characteristics of transcription driven by RNA polymerase II (RNAPII). Based on the longest 3'- and 5'- RACE products, we calculated the length of the GUT15 IncRNA as 2399 bp (Fig. 1B). The presence of this full-length transcript was confirmed by RT-PCR. The experimentally established length of the GUT15 IncRNA from Arabidopsis seedlings was 100 bp longer than the GUT15 transcript described in the TAIR10 database [18]. However, this discrepancy may be due to differences in the regulation of GUT15 gene transcription in specific tissues or the different developmental stages during which the RNA samples were collected. The Arabidopsis GUT15 gene contains 4 introns. The discovered tRNA-like sequence is encoded by the final intron in the GUT15 transcripts (Fig. 1B). Interestingly, the RT-PCR amplification of the GUT15 cDNA revealed the presence of a series of splicing isoforms, and only three isoforms contained the tRNA-like-bearing intron (Fig. 1C).

The GUT15-encoded tRNA-like sequence affects the splicing of its host intron

The mapping of the small RNA-seq data to the GUT15 gene revealed a short sequence originating from the 5'-flanking region of the tRNA-like coding region in the final GUT15 intron (Fig. 1B). We called this new small RNA molecule GUT15-tRF-F5 according to our previously proposed nomenclature [19]. The abundance of this molecule in different small RNA libraries is summarized in Table 1.

The close localization of the tRNA-like and GUT15-tRF-F5 sequences within the GUT15 IncRNA intron raised the following two possible scenarios regarding the biogenesis of the tRNA-like molecule and GUT15-tRF-F5: 1) GUT15-tRF-F5 and the tRNA-like molecule are generated from a tRNA-like precursor that is transcribed by RNA polymerase III (RNAPIII) or 2) alternatively, the GUT15-tRF-F5 and tRNA-like sequences are processed from GUT15 transcripts or already spliced introns carrying both the tRNA-like molecule and GUT15-tRF-F5. To examine the relationship between the proximity of the tRNA-like structure and biogenesis of GUT15-tRF-F5, we created genetic constructs encoding the following two versions of the GUT15 gene (both under the control of the CaMV 35S promoter): the full-length (35S-GUT15wt) version or a version lacking the tRNA-like sequence (35S-GUT15Δ); we introduced these constructs into Nicotiana benthamiana leaves using the agroinfiltration method (Fig. 2A). Using RT-PCR, we determined that the deletion of the tRNA-like sequence increases the splicing efficiency of its hosting intron (Fig. 2B). Moreover, the Northern blotting results showed an accumulation of processed GUT15-tRF-F5 from the mutated GUT15 gene variant

Table 1. tRNA-seq results (homemade libraries and public samples) for GUT15-tRF-F5 AAAAGAGAGTCAAAGAG.

| sample       | count |
|--------------|-------|
| cbc          | 251   |
| cep1-3       | 140   |
| serrate      | 226   |
| hyv1-2       | 178   |
| db2          | 83    |
| db3          | 136   |
| db4          | 112   |
| db5          | 57    |
| dd2-5_14     | 164   |
| dd3-1_14     | 138   |
| dd4-2_14     | 203   |
| rdr6-15      | 85    |
| sgs3-13      | 556   |
| xrn2-3       | 605   |
| xrn4-3       | 208   |
| rdr2-2       | 353   |
| rdr3b        | 126   |
| rp601-2      | 721   |
| rp602-1      | 224   |
| rp63-1       | 155   |
| ns2-2        | 12    |
| trz4         | 182   |
| wt31         | 83    |
| wt14         | 589   |
| heat_c1      | 1     |
| heat_c2      | 1     |
| heat_c3      | 1     |
| heat_30min_s1| 1     |
| heat_30min_s2| 1     |
| heat_6h_s3   | 1     |
| drought_30pct_c3| 1   |
| cu_d_s1      | 1     |
| cu_d_s2      | 1     |
| cu_d_s3      | 1     |
| nacl_e_s1    | 5     |
| nacl_e_s3    | 1     |
| sulfur_d_s1  | 1     |
| cadmium_e_s1 | 1     |
| cadmium_e_s2 | 4     |
| SRR037653    | 20    |
| SRR037656    | 6     |
| SRR037657    | 17    |
| SRR037658    | 13    |
| SRR037659    | 6     |
| SRR037660    | 26    |
| SRR037663    | 18    |
| SRR037664    | 27    |
| SRR037665    | 20    |
| SRR037666    | 8     |
| SRR037667    | 24    |
| SRR037669    | 17    |
| SRR037670    | 4     |
| SRR037675    | 24    |
lacking the tRNA-like sequence, suggesting that the proximity of the tRNA-like molecule negatively affects the expression of GUT15-tRF-F5 (Fig. 2C, middle panel). Importantly, this result excluded the possibility that GUT15-tRF-F5 is produced from the tRNA-like precursor because the deletion of the tRNA-like molecule resulted in GUT15-tRF-F5 overexpression. Overall, the intronic GUT15 tRNA-like sequence affects both the splicing of the final intron in the GUT15 lncRNA and the accumulation of GUT15-tRF-F5.

**The GUT15-encoded tRNA-like molecule is generated from the GUT15 lncRNA**

Thus far, we have shown that the tRNA-like sequence is transcribed as a part of the lncRNA GUT15, which is synthesized by RNAPII. However, this observation does not exclude that the tRNA-like locus can be independently transcribed also by RNAPII. To address this issue, we transiently expressed N. benthamiana protoplasts with a GUT15 native construct lacking any upstream regulatory sequences (Δp-GUT15wt) to investigate the activity of the internal A-box and B-box tRNA promoter elements within the GUT15 tRNA-like sequence (Fig. 3A, upper panel, and Fig. 1A). The internal promoter elements within the tRNA-like coding sequence were not sufficient to drive its autonomous transcription by RNAPIII (Fig. 3B-C). In contrast, the GUT15 tRNA-like molecule was detected in tobacco protoplasts transfected with the UBQ promoter-driven GUT15 construct (Fig. 3B-C). Thus, the GUT15-encoded tRNA-like molecule can be processed from RNAPII-produced GUT15 transcripts independently of the promoter used, i.e., the native or UBQ promoter. Moreover, no differences were observed in the tRNA-like sequence expression levels when the 35S-GUT15Δ5′/3′ss construct with mutated 5′- and 3′-splice sites in the tRNA-like hosting intron was transiently expressed in the tobacco leaves (Fig. 3A, lower panel, and 3D-E). Thus, the biogenesis of the tRNA-like molecule encoded within the GUT15 intron is reliant on RNAPII GUT15 transcription but independent of the GUT15 final intron splicing.

**Canonical tRNA genes nested within introns of protein-coding genes do not affect the splicing patterns of their host transcripts**

Because the tRNA-like structure in the GUT15’s final intron affects its splicing, we globally examined the occurrence of tRNAs in genes across the A. thaliana genome. The computational analyses identified 19 protein-coding genes containing tRNA sequences in a sense context (in introns and untranslated regions (UTRs)) (Table 2). RT-PCR performed for 3 of these genes revealed that introns containing tRNAs are efficiently spliced in Arabidopsis seedlings (Fig. S3). Subsequently, we selected a gene encoding oligosaccaryltransferase (At3g12587) as an example to study the influence of tRNA sequences on the splicing of their host introns using a transient expression assay in N. benthamiana leaves (Fig. 4A-B). In contrast to the GUT15 transcript, no notable differences were detected in the intron splicing efficiency when the naturally occurring tRNA-Arg^CCG was removed from its host intron (Fig. 4C). Thus, the standard tRNA molecules embedded in the introns of protein-coding genes do not have a negative effect on the splicing of their host introns. To further validate this observation, we prepared the following two variants of the original GUT15 intron 4 (pGUT15): in one variant, the tRNA-like sequence was removed (pGUT15Δ), and in the other variant, the GUT15 tRNA-like sequence was replaced with the tRNA-Ala^AGC gene (At1g06610; pGUT15(Ala)) (Fig. 5A-B, left panel). These constructs were introduced into N. tabacum protoplasts, and their intron excision efficiency was tested using RT-qPCR. All analyzed versions of the GUT15 intron were efficiently spliced in the tobacco protoplast transient expression system. However,
according to the investigation of the specific copy numbers of the isoforms using an qPCR-based absolute quantification method, the GUT15 intron lacking the tRNA-like sequence (pGUT15D construct) was excised more efficiently than the other two intron variants (pGUT5 and pGUT5(Ala)) (Fig. 5C, left panel). These observed changes are consistent with our previous tobacco agroinfiltration experimental results.

To determine whether the tRNA-like molecule and tRNA-AlaAGC affect the splicing of intronic sequences, additional constructs were prepared and tested in transfected tobacco protoplasts. We selected CBP80 (CAP-BINDING PROTEIN 80; At2g13540) intron number 3 (similar in size to the GUT15 tRNA-like hosting intron) [20], which does not naturally possess any tRNA-resembling elements. The original intron construct was modified to contain either the GUT15 tRNA-like sequence or tRNA-AlaAGC (called pCBP80(tRNA-like) and pCBP80(Ala), respectively) (Fig. 5B, right panel). RT-qPCR analysis confirmed the inhibitory effect of the GUT15 tRNA-like molecule but not that of tRNA-AlaAGC on CBP80 intron 3 splicing (Fig. 5C, right panel). Therefore, the presence of tRNA
loci within introns of RNAPII-dependent genes does not generally interfere with the splicing of their primary transcripts. However, the tRNA-like sequence from the \textit{GUT15} gene inhibits the splicing of the introns in which it is embedded.

Discussion

The \textit{GUT15} IncRNA was first identified in the tobacco BY-2 cell line as a transcript with a relatively short half-life [15]. In contrast, \textit{GUT15} RNA was found among highly stable transcripts in pollen from \textit{N. tabacum} plants [21]. This differential stability suggests that the regulation of the \textit{GUT15} RNA lifetime is tissue specific [21]. Interestingly, two \textit{Arabidopsis} IncRNAs, the \textit{GUT15} and \textit{CR20-1}, are hormonally regulated members of a family conserved among monocots and dicots [22,23]. Using a 3’-RACE approach, we confirmed that the \textit{AtGUT15} IncRNA is polyadenylated [23], which is similar to \textit{AtCR20-1} [22]. Furthermore, we demonstrated that \textit{AtGUT15} is capped and undergoes alternative splicing. The same mRNA-like features have also been observed in mammalian and other plant IncRNAs [4,6,24].

\textit{AtGUT15} and \textit{AtCR20-1} are known to share a highly similar segment that forms a stable secondary structure [25], and a part of this conserved region is present in another location of the \textit{Arabidopsis} genome (chromosome 1), suggesting that this region could be potentially targeted by the sequence-specific activity of the \textit{GUT15/CR20} gene family [23]. Our computational analysis revealed the presence of a second conserved region within the \textit{Arabidopsis GUT15} locus. We identified a tRNA-resembling sequence in its final intron (intron 4). Notably, a highly similar sequence, i.e., lacking twelve nucleotides and differing by

| Gene symbol / annotation | tRNA | Context |
|--------------------------|------|---------|
| AT1G64130 – polyketide cyclase/dehydrase and lipid transport superfamily | LeuCCA | CDS/intron |
| AT1G68760 – nudix hydrolase 1 hydrolyzes 8-oxo-(d)GTP to 8-oxo-(d)GMP | ProAGG | 3’-UTR |
| AT1G71697 – choline kinase 1 increased in response to wounding | GlyGCC | 3’-UTR |
| AT2G07771 – cytochrome C assembly protein | IleAAU+ | CDS/3’-UTR |
| AT2G4030 – unknown protein | GlyCC | CDS/3’-UTR |
| AT2G4390 – Alg2-like (avirulence induced gene) family protein | ArgAGC | 5’-UTR (longest mRNA isoform) |
| AT4G30410 – peroxisomal membrane 22 kDa (Mpv17/PMP22) family protein | GinUG | 5’-UTR |
| AT4G25640 – detoxifying efflux carrier 35, multidrug and toxin efflux family transporter | LeuCAA | CDS (mRNA isoform 2) |
| AT5G07630 – lipid transporter | LeuCAA | CDS/intron |
| AT5G45720 – AAA-type ATPase family protein DNA polymerase III complex | GluUUC | 3’-UTR |
| AT2G0781 – cytochrome C biogenesis | IleAAU+ | intron |
| AT2G07760 – unknown protein | MetCAU+ | intron |
| AT2G07815 – cytochrome C biogenesis | GlyGCC- | intron |
| AT3G11402 – Cys/His-rich C1 domain family protein | ValAC | intron |
| AT3G12587 – oligosaccarytransferase | ArgCCG | intron |
| AT4G2060 – minichromosome maintenance (MCM2/3/5) family protein | HisUG | intron (longest isoform, alternative transcription initiation) |
| AT2G6145 – unknown protein | AsnGUU | intron |
| AT5G29530 – unknown function (DUF1997) | ProAGG | intron |
| AT5G57880 – multipolar spindle 1 involved in meiotic spindle organization | MetCAU | intron |

‘potential pseudogenes

Figure 4. Standard tRNAs do not affect the splicing of their hosting genes. (A) Schematic structure of the At3g12587 hosting tRNA-ArgCCG within its intron. The white box represents an exon, the gray boxes represent UTRs, the lines depict introns, and the arrow corresponds to the tRNA (adapted from TAIR10). (B) Schematic representation of the oligosaccarytransferase gene variants used. NOS, transcription terminator. (C) Splicing isoforms of the oligosaccarytransferase transcript recorded by RT-PCR in infiltrated \textit{N. benthamiana} leaves expressing the oligosaccarytransferase gene variants shown in B. Schematic structures of the identified isoforms are presented on the left. MOCK is a negative control for the agroinfiltration experiment (leaves infiltrated only with MES buffer). ctrl, amplification of genomic DNA; NTC, non-template control; and marker, 100 bp plus (Thermo Fisher Scientific).
only a few nucleotides, is also located within the second exon of the *Arabidopsis CR20-1* gene (Fig. S1A). Similar to the *GUT15*-encoded tRNA-like sequence, this sequence does not fold into a secondary structure with canonical antico-di stem-loop characteristics (Fig. S1B); however, this sequence was not identified among actively transcribed tRNAs or tRNA-resembling genes according to our tRNA-seq data. Importantly, the tRNA-like molecule from *AtGUT15* is recognized by the tRNA maturation machinery, as evidenced by its 3′-end CCA motif that is not encoded in the genome. This posttranscriptional modification specific to mature tRNA molecules has recently been shown to be present (a complete or partial CCA-terminus) in certain non-tRNA substrates, such as the *rps12*, *cox2* and *atp9* mitochondrial mRNA transcripts in maize, human spliceosomal U2 snRNA and the 3′-end of the tobacco mosaic virus RNA [26]. The biological consequences of these unusual nucleotide incorporations remain unexplored and may reflect unspecific reactions of CCA-adding enzymes [26] because most of these transcripts contain a stem-loop structure at the 3′-end that resembles the tRNA minihelix, which is known to be an efficient substrate for CCA-attachment in vitro [27,28].

In addition to having the CCA sequence, the *GUT15*-encoded tRNA-like molecule was detected only in the tRNA-seq library prepared from the deacylated tRNA fraction, suggesting that this molecule may be charged by an amino acid. The biological role of this newly identified tRNA-like sequence, notably its potential involvement in protein translation, remains to be elucidated. However, certain plant-specific RNA viral genomes contain tRNA-like structures that can be specifically aminoacylated by valine, histidine and tyrosine, and this tRNA mimicry is important for diverse aspects of viral infectivity [29]. In bacteria, it plays a role in tagging abnormal proteins for proteolysis [30].

Furthermore, the tRNA-like molecule from *AtGUT15* is not expressed by RNAPIII and instead is processed from the *GUT15* transcripts synthesized by RNAPII. A similar mechanism has been reported for two tRNA-like molecules generated from the mammalian lncRNAs *MALAT1* and *MenB*. In both...
cases, RNase P recognizes the tRNA-like structure in a nascent RNAPII transcript and cleaves it to simultaneously generate the 3′-end of the mature nuclear-retained lncRNA and the 5′-end of the tRNA-like small RNA [31, 32]. Recently, 132 genomic loci resembling the MALAT1 3′-end processing module have been identified among several vertebrate genomes [33]. Interestingly, both mascRNAs (MALAT1-associated small cytoplasmic RNAs) and the GUT15-tRNA-like molecule are marked with a CCA [31]. Furthermore, the RNAPII-mediated transcription of conventional tRNAs has already been reported for tRNAsec in Trypanosoma brucei [34].

The AtGUT15 transcript serves as a precursor for another small noncoding RNA, the GUT15-tRF-F5. This small RNA is also generated from the final intron of the AtGUT15 lncRNA. Interestingly, the GUT15 tRNA-like sequence exerts an inhibitory effect on both the splicing of its host intron and GUT15-tRF-F5 biogenesis. Therefore, the GUT15-tRF-F5 fragment may be produced from the spliced intron. In contrast, the accumulation of GUT15 tRNA-like molecules is not influenced by GUT15 lncRNA splicing, suggesting that it is not produced from the intron excised from the AtGUT15 primary transcripts.

The GUT15 lncRNA has been previously characterized as a peptide-coding transcript, because both N. tabacum and A. thaliana gene contains putative short open reading frames (sORFs) of 78 and 75 amino acids, respectively [16]. In 2016, an sORF encoding a 37-aa peptide was identified in GUT15 in Arabidopsis roots using the Ribo-Seq method [35]. However, this peptide does not correspond to the 75-aa peptide predicted by van Hoof and coworkers [40]. This peptide has homologs in multiple species within the Brassicaceae family [35], but the function of this conserved short peptide is unknown. In the same study, additional 26 small ORFs were identified in annotated lncRNAs. These are not the only cases of transcripts believed to solely function as RNA molecules that do in fact code for small peptides [12,36]. In our studies we also detected the AtGUT15 transcripts to be attached to translating ribosomes. Interestingly, only the isoform lacking the tRNA-like sequence was associated with polyribosomal fractions (Fig. S2).

Thus, we speculate that the spliced version of the GUT15 transcript can physically interact with ribosomes to produce a small peptide from its first exon, whereas primary GUT15 transcripts serve as a source of tRNA-like molecules. In contrast, Carlevaro-Pita et al. [37] have recently shown that ribosomes are the default destination of most cytoplasmic lncRNAs and may play a role in their degradation because blocking ribosomal elongation results in the stabilization of many associated lncRNAs, which may be also the case for the GUT15 lncRNA. In 1995, Taylor and coworkers found that the level of the shorter GUT15 transcript was significantly increased in an actinomycin D (inhibitor of RNAPII transcription)- and cycloheximide (translational inhibitor)-treated tobacco cell line [15]. Therefore, a complex regulatory mechanism underlies the biogenesis and functions of the noncoding RNAs and small peptides originating from the GUT15 lncRNA.

Our experiments shed light on the phenomena of tRNA and tRNA-like sequences embedded within introns of their host genes. By screening the TAIR10 database, we identified 19 host protein-coding genes containing annotated tRNAs in their introns and UTRs in a sense orientation. Additionally, our global approach revealed two Arabidopsis lncRNAs, GUT15 and CR20-1, that contain similar tRNA-like sequences in their intron and exon, respectively. Our transient expression experiments in tobacco protoplasts confirmed the negative effects of the GUT15 tRNA-like sequence on the splicing of both its original host and the CBP80 intron. Similarly, this influence was also observed when the tRNA-Ala^{XGC} gene was inserted into the AtGUT15 intron, suggesting that this effect may be due to this specific intron. Moreover, the tRNA-Arg^{COG} embedded in the oligosaccaryltransferase gene does not affect the splicing pattern of its transcript. Accordingly, we conclude that the presence of a tRNA coding sequence in the host protein-coding genes does not interfere with gene splicing. Interestingly, Zhang and coworkers [38] revealed an additional role of tRNA sequences in systemic mRNA transport in plants. These authors found that mobile mRNAs in Arabidopsis are enriched in tRNA-like motifs or are transcribed from genes located in close proximity to annotated tRNA loci, which form di-cis-tronic mRNA-tRNA transcripts at high frequency (according to paired-end RNA-seq data). However, the GUT15 lncRNA was not identified among the genes producing mobile transcripts [39], suggesting that the tRNA-related sequence derived from this lncRNA plays other roles.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana Columbia-0 wild-type plants were grown for 10 or 14 days as previously described [40].

N. benthamiana and N. tabacum var. Xanthi plants were grown for 4 and 8 weeks, respectively, at 22°C (16/8-hour light/dark cycle, 50% humidity, and 150–200 μmol m⁻² s⁻¹ photon flux density).

Construct preparation

For the agroinfiltration of the tobacco leaves, the genomic sequences of At2g18440 (GUT15) and At3g12587 (gene encoding oligosaccaryltransferase) were amplified (from A. thaliana genomic DNA isolated using the DNeasy Plant Mini Kit, Qiagen) and cloned into the pCR8 plasmid (Thermo Fisher Scientific) using the NotI and Ascl restriction sites. The GUT15 and oligosaccaryltransferase constructs lacking the tRNA-like or tRNA sequences were created using a three-step PCR approach as previously described [40]. The mutagenesis of the GUT15 final intron splice sites was performed using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies). To detect the expression in plants, the At2g18440 and At3g12587 gene versions were cloned into the pMDC32 Gateway binary vector [41] using the Gateway LR Clonase II Enzyme Mix (Thermo Fisher Scientific).

For transient expression in the tobacco protoplasts, Gateway LR Clonase II Enzyme Mix was used to generate expression clones carrying the genomic sequence of At2g18440 without or under the control of the UBCQ10 promoter.

The intron mini-constructs were based on the following two Arabidopsis thaliana introns: the 4th intron from
GUT15 (674 bp) and the 3rd intron from the CBP80 gene (485 bp). The intron sequences together with the fragments (>55 bp) from the original 5’- and 3’-exons were isolated from Arabidopsis genomic DNA by PCR and introduced to the plant expression vector pDH515 within an intronless zein gene using the unique BamHI restriction site [42,43]. For the mutant intron construct generation (with different tRNA types), a two-step PCR-based approach was used. First, the desired tRNA sequence was amplified from genomic DNA using primers containing intron-specific (CBP80 or GUT15) overhangs. Second, the PCR products were used as primers in the mutagenesis reaction using the appropriate original intron construct as a template and Pfu Ultra High Fidelity polymerase (Agilent Technologies). The sequences of all constructs were verified by Sanger sequencing. All oligonucleotide sequences are listed in Table 3.

### Transient expression in N. benthamiana leaves

The tobacco leaf agroinfiltration was performed as previously described [40,44].

### Mesophyll protoplast transfection

The constructs were transfected into protoplasts of Nicotiana tabacum var Xanthi isolated from 8-week-old leaves using a previously described protocol for Arabidopsis protoplasts with the following modifications [40]: (1) 10 µg of the vector were used for the transfection, and (2) the transfected protoplasts were incubated overnight in the dark at 22°C before RNA isolation using a modified TRIzol method [45].

### RNA isolation and cDNA preparation

For the Northern blot, RACE, qPCR and RT-PCR analyses, total RNA from 100 mg of tissue or protoplast suspensions was isolated using the TRIzol reagent (Thermo Fisher Scientific). Total RNA from 100 mg of tissue or protoplast suspensions was isolated using the TRIzol method [42,43].

RNA isolation from small RNAs was performed as described [44]. The integrity and quality of the NGS-dedicated RNA were verified using an Agilent RNA 6000 Nano Kit (Agilent Technologies).

For the cDNA preparation, total RNA was first treated with Turbo DNase I (Thermo Fisher Scientific); double treatment was required for RNA isolated from transfected protoplasts. The reverse transcription reactions were prepared from 0.5–3 µg of total DNase-treated RNA using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) and either the oligodT(18) primer (Thermo Fisher Scientific) or the Zein-specific primer. The stem-loop pulsed reverse transcription was performed using 200 ng of total RNA enriched for small RNAs [47].

### sRNA-seq, tRNA-seq and bioinformatics analyses

A. thaliana total RNA enriched for the small RNA species was used for the small RNA library generation (TruSeq Small RNA Library Prep Kit), and sequencing was performed using an Illumina HiSeq 2000 system. A bioinformatics analysis of the sRNA-seq data was performed as previously described [19].

For the tRNA sequencing, total RNA was isolated and decylated as previously described [48]. After size fractionation in a PAA gel, the RNA molecules (65–100 nucleotides) were used for the library generation, followed by sequencing using an Illumina HiSeq Platform (Fastersis SA, Switzerland). The GUT15-encoded tRNA-like molecule was identified from the NGS data by mapping the obtained reads to the A. thaliana genome.

The sequence profiles of the A-box and B-box promoter elements were derived by analyzing all A. thaliana tRNA genes. The sequence logos in the profiles were produced using the WebLogo application using multiple sequence alignments of corresponding regions from tRNA genes [49].

### Northern blot analyses

For the Northern blot analyses, 5–30 µg of total RNA were used to detect sRNA and tRNA-like sequences. The RNA electrophoresis, blot transfer and hybridization were performed as previously described [46,50]. All hybridizations were performed with three biological replicates.

### RT-PCR analyses and quantitative real-time PCR (qPCR)

The RT-PCR amplifications were performed as previously described [51] using the gene-specific oligonucleotide primer pairs listed in Table 3. The amplification of the GUT15-encoded tRNA-like molecule was carried out as previously described [47]. The identity of the PCR product was verified by sequencing.

RT-qPCR was performed as previously described [40]. The amplification efficiency of each primer pair was calculated by making a 10-fold dilution series of the plasmid templates, calculating a linear regression based on the data points and estimating the efficiency from the line slope. The primer pairs with the highest almost equal amplification efficiency (max. difference of 10% was approved) and only one visible peak on the dissociation curve were used for the analysis (Table 3).

The expression levels of particular splicing isoforms were calculated by performing the absolute quantification method using standard curves obtained for a 10-fold dilution series of the plasmid bearing the appropriate isoform. To estimate the splicing efficiency, events identified for the analyzed intron (expressed in copy numbers) were summed and treated as 100%, and the contribution of the fully spliced and unspliced isoforms was then calculated.

All results were analyzed using the SDS 2.3 software (Thermo Fisher Scientific). Error bars were calculated using the SD Function in the Microsoft Excel software. The statistical significance of the presented results was estimated using Student’s t-test at a significance level of *p < 0.05.

### 3′-5′-RACE and 5′ RLM-RACE experiments

The 5′- and 3′-RACE cDNA template synthesis and two-step RACE-PCR experiments were conducted using the
### Primers used for constructs preparation

| Name | Sequence | gene/cDNA fragment amplified using the primer pair | Construct prepared using the amplified fragment |
|------|----------|-------------------------------------------------|-----------------------------------------------|
| A01  | ATTTGCGGCCGCATCACCGCCTCCATATTCTTTC | GUT15 (At2g18440) | pUBQ10-GUT15wt, Δp-GUT15wt, 35S-GUT15wt, 35S-GUT15Δ, 35S-GUT15ΔS3’s |
| A02  | TTTGGCGCGCCAATTAATCGTTCAACTATTATTCTATATTCTATATAACATG | 1-1680 bp fragment of GUT15 | 35S-GUT15Δ |
| A03  | CCTCAAAGATCTTCTTCTACAGATTTGGAATTCATTGCTGCTATGTCATGTTG | mutagenesis of 5’ss in 35S-GUT15wt | 35S-GUT15ΔS3’s |
| A04  | TTTGGCGCGCCAAAGAAAAAAATTCAAAGTTTCATCAAACAT | mutagenesis of 3’ss in 35S-GUT15wt | 35S-GUT15Δ |
| A05  | TCCACAACAGCTTTTGTCAAGATGGCTTCTTCAAAACAAATCTTACAGA | Oligosaccharyltransferase (At3g12587) | 35S-oligosac, 35S-oligosacΔ |
| A06  | TTTGGCGCGCCAAAGAAAAAAATTCAAAGTTTCATCAAACAT | 1-369 bp fragment of Oligosaccharyltransferase | 35S-oligosacΔ |
| A07  | CGGGATCCCTGGAAATTAGCGTGGACACTTTC | GUT15 intron with fragments of flanking exons | pGUT15, pGUT15(Ala), pGUT15Δ |
| A08  | CGGGATCCCTGGAAATTAGCGTGGACACTTTC | GUT15 intron with fragments of flanking exons | pGUT15, pGUT15(Ala), pGUT15Δ |
| A09  | CGGGATCCCTGGAAATTAGCGTGGACACTTTC | GUT15 intron with fragments of flanking exons | pGUT15, pGUT15(Ala), pGUT15Δ |
| A10  | CGGGATCCCTGGAAATTAGCGTGGACACTTTC | GUT15 intron with fragments of flanking exons | pGUT15, pGUT15(Ala), pGUT15Δ |
| A11  | CGGGATCCCTGGAAATTAGCGTGGACACTTTC | GUT15 intron with fragments of flanking exons | pGUT15, pGUT15(Ala), pGUT15Δ |
| A12  | CGGGATCCCTGGAAATTAGCGTGGACACTTTC | GUT15 intron with fragments of flanking exons | pGUT15, pGUT15(Ala), pGUT15Δ |
| A13  | CGGGATCCCTGGAAATTAGCGTGGACACTTTC | GUT15 intron with fragments of flanking exons | pGUT15, pGUT15(Ala), pGUT15Δ |
| A14  | CGGGATCCCTGGAAATTAGCGTGGACACTTTC | GUT15 intron with fragments of flanking exons | pGUT15, pGUT15(Ala), pGUT15Δ |
| A15  | CGGGATCCCTGGAAATTAGCGTGGACACTTTC | GUT15 intron with fragments of flanking exons | pGUT15, pGUT15(Ala), pGUT15Δ |
| A16  | CGGGATCCCTGGAAATTAGCGTGGACACTTTC | GUT15 intron with fragments of flanking exons | pGUT15, pGUT15(Ala), pGUT15Δ |
| A17  | CGGGATCCCTGGAAATTAGCGTGGACACTTTC | GUT15 intron with fragments of flanking exons | pGUT15, pGUT15(Ala), pGUT15Δ |
| A18  | CGGGATCCCTGGAAATTAGCGTGGACACTTTC | GUT15 intron with fragments of flanking exons | pGUT15, pGUT15(Ala), pGUT15Δ |
| A19  | CGGGATCCCTGGAAATTAGCGTGGACACTTTC | GUT15 intron with fragments of flanking exons | pGUT15, pGUT15(Ala), pGUT15Δ |
| A20  | CGGGATCCCTGGAAATTAGCGTGGACACTTTC | GUT15 intron with fragments of flanking exons | pGUT15, pGUT15(Ala), pGUT15Δ |
| A21  | CGGGATCCCTGGAAATTAGCGTGGACACTTTC | GUT15 intron with fragments of flanking exons | pGUT15, pGUT15(Ala), pGUT15Δ |
| A22  | CGGGATCCCTGGAAATTAGCGTGGACACTTTC | GUT15 intron with fragments of flanking exons | pGUT15, pGUT15(Ala), pGUT15Δ |
| A23  | CGGGATCCCTGGAAATTAGCGTGGACACTTTC | GUT15 intron with fragments of flanking exons | pGUT15, pGUT15(Ala), pGUT15Δ |
| A24  | CGGGATCCCTGGAAATTAGCGTGGACACTTTC | GUT15 intron with fragments of flanking exons | pGUT15, pGUT15(Ala), pGUT15Δ |
| A25  | CGGGATCCCTGGAAATTAGCGTGGACACTTTC | GUT15 intron with fragments of flanking exons | pGUT15, pGUT15(Ala), pGUT15Δ |
| A26  | CGGGATCCCTGGAAATTAGCGTGGACACTTTC | GUT15 intron with fragments of flanking exons | pGUT15, pGUT15(Ala), pGUT15Δ |

### Primers used in RACE, RT-PCR and qPCR analyses

| Name | Sequence | gene/cDNA fragment amplified using the primer pair | Experiments in which the primer pair was used |
|------|----------|-------------------------------------------------|-----------------------------------------------|
| B01  | TTCGAAACCAAACTCTCTGACACACTTGGTCA | GUT15 (At2g18440) | 5’ RLM-RACE |
| B02  | TTCGAAACCAAACTCTCTGACACACTTGGTCA | GUT15 (At2g18440) | 3’ RACE |
| B03  | TTCGAAACCAAACTCTCTGACACACTTGGTCA | GUT15 (At2g18440) | RT-PCR (RACE confirmation) |
| B04  | TTCGAAACCAAACTCTCTGACACACTTGGTCA | GUT15 (At2g18440) | RT-PCR (splicing isoforms analysis) |
| B05  | TTCGAAACCAAACTCTCTGACACACTTGGTCA | GUT15 (At2g18440) | RT-PCR (splicing isoforms analysis) |
| B06  | TTCGAAACCAAACTCTCTGACACACTTGGTCA | GUT15 (At2g18440) | RT-PCR (splicing isoforms analysis) |
| B07  | TTCGAAACCAAACTCTCTGACACACTTGGTCA | GUT15 (At2g18440) | RT-PCR (splicing isoforms analysis) |
| B08  | TTCGAAACCAAACTCTCTGACACACTTGGTCA | GUT15 (At2g18440) | RT-PCR (splicing isoforms analysis) |
| B09  | TTCGAAACCAAACTCTCTGACACACTTGGTCA | GUT15 (At2g18440) | RT-PCR (splicing isoforms analysis) |
| B10  | TTCGAAACCAAACTCTCTGACACACTTGGTCA | GUT15 (At2g18440) | RT-PCR (splicing isoforms analysis) |
| B11  | TTCGAAACCAAACTCTCTGACACACTTGGTCA | GUT15 (At2g18440) | RT-PCR (splicing isoforms analysis) |
| B12  | TTCGAAACCAAACTCTCTGACACACTTGGTCA | GUT15 (At2g18440) | RT-PCR (splicing isoforms analysis) |
| B13  | TTCGAAACCAAACTCTCTGACACACTTGGTCA | GUT15 (At2g18440) | RT-PCR (splicing isoforms analysis) |
| B14  | TTCGAAACCAAACTCTCTGACACACTTGGTCA | GUT15 (At2g18440) | RT-PCR (splicing isoforms analysis) |
| B15  | TTCGAAACCAAACTCTCTGACACACTTGGTCA | GUT15 (At2g18440) | RT-PCR (splicing isoforms analysis) |
| B16  | TTCGAAACCAAACTCTCTGACACACTTGGTCA | GUT15 (At2g18440) | RT-PCR (splicing isoforms analysis) |
| B17  | TTCGAAACCAAACTCTCTGACACACTTGGTCA | GUT15 (At2g18440) | RT-PCR (splicing isoforms analysis) |
| B18  | TTCGAAACCAAACTCTCTGACACACTTGGTCA | GUT15 (At2g18440) | RT-PCR (splicing isoforms analysis) |
| B19  | TTCGAAACCAAACTCTCTGACACACTTGGTCA | GUT15 (At2g18440) | RT-PCR (splicing isoforms analysis) |

(Continue on next page)
| Name | Sequence | Detection |
|------|----------|-----------|
| C01  | GACTCTTTAACTCTATT | GUT15 tRF-F5 |
| C02  | ACCAACCTCTCGCTCCTAGAAGA | GUT15 tRNA-like |
| C03  | TCAATCTGCGAGGGGCA | U6 snRNA |

| Name | Sequence | Experiments in which the primers were used |
|------|----------|------------------------------------------|
| D01  | GTCGATCCAGGTTCCACGAGGATCCGATTCCGACTCG | stem-loop reverse transcription primer |
| D02  | TGGTAGAGCCGCTCCTTTT | end-point RT-PCR of tRNA-like from GUT15 |
| D03  | GTGCAGGGTCCAGG | |
SMARTer RACE cDNA Amplification Kit (Clontech) according to the manufacturer’s protocol. The cDNA template used in the 5′-RLM-RACE analysis was created using the GeneRacer™ Kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. PCR reactions were performed using the Advantage 2 PCR Enzyme System (Clontech) on a Veriti thermal cycler (Applied Biosystems). PCR products were cloned into the pGEM T-Easy vector (Promega) and sequenced. The primer sequences are listed in Table 3.

Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

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