Permuted tRNA Genes Expressed via a Circular RNA Intermediate in Cyanidioschyzon merolae

Akiko Soma, *et al.*

Science **318**, 450 (2007);
DOI: 10.1126/science.1145718

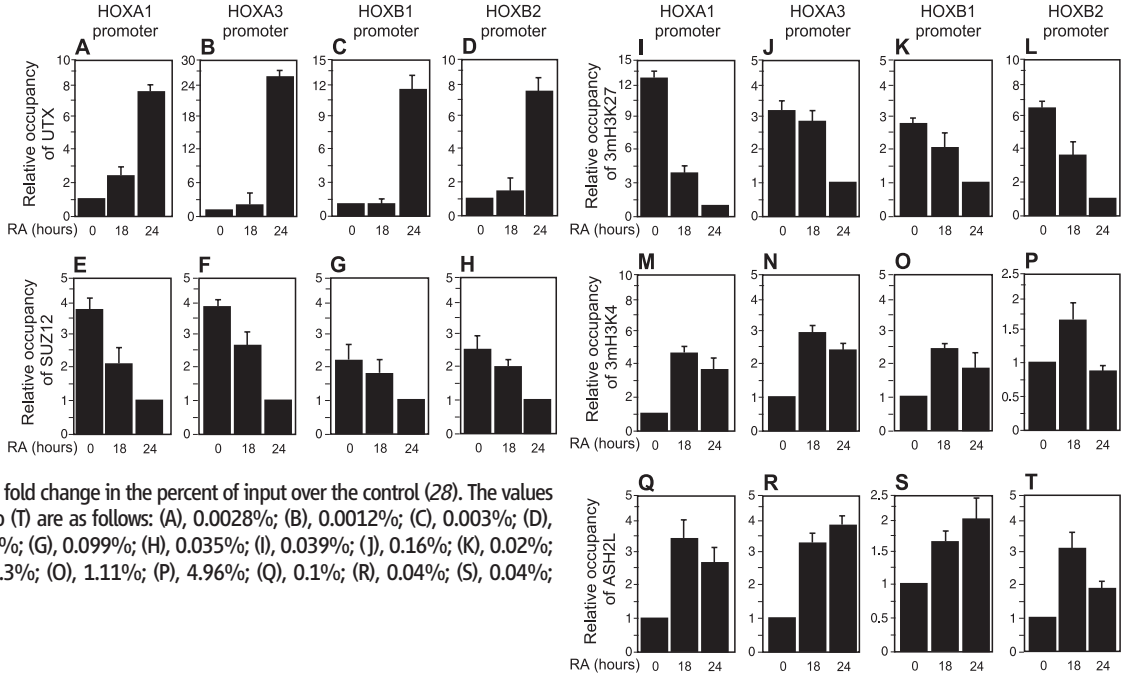

Fig. 4. RA treatment results in recruitment of the UTX/MLL complex, concomitant with decreased levels of trimethyl H3K27 and increased levels of trimethyl H3K4. (**A** to **T**) UTX occupancy (A to D), SUZ12 occupancy (E to H), trimethyl H3K27 levels (I to L), trimethyl H3K4 levels (M to P), and ASH2L occupancy (Q to T) at *HOXA1*, *HOXA3*, *HOXB1*, and *HOXB2* genes were analyzed by a qChIP assay. Data are presented as the mean ± SEM (error bars) ($n \geq 3$ except in the case of ASH2L, where $n = 2$). The relative occupancy represents the fold change in the percent of input over the control (*28*). The values of percent input set as 1 in (A) to (T) are as follows: (A), 0.0028%; (B), 0.0012%; (C), 0.003%; (D), 0.004%; (E), 0.046%; (F), 0.031%; (G), 0.099%; (H), 0.035%; (I), 0.039%; (J), 0.16%; (K), 0.02%; (L), 0.022%; (M), 2.47%; (N), 6.3%; (O), 1.11%; (P), 4.96%; (Q), 0.1%; (R), 0.04%; (S), 0.04%; and (T), 0.07%.

16. P. A. Cloos *et al.*, *Nature* **442**, 307 (2006).
17. R. J. Klose *et al.*, *Nature* **442**, 312 (2006).
18. B. D. Fodor *et al.*, *Genes Dev.* **20**, 1557 (2006).
19. M. G. Lee, J. Norman, A. Shilatifard, R. Shiekhattar, *Cell* **128**, 877 (2007).
20. R. J. Klose *et al.*, *Cell* **128**, 889 (2007).
21. J. Christensen *et al.*, *Cell* **128**, 1063 (2007).
22. J. C. Eissenberg *et al.*, *Nat. Struct. Mol. Biol.* **14**, 344 (2007).
23. A. P. Bracken, N. Dietrich, D. Pasini, K. H. Hansen, K. Helin, *Genes Dev.* **20**, 1123 (2006).
24. R. Cao, Y. Tsukada, Y. Zhang, *Mol. Cell* **20**, 845 (2005).
25. H. Wang *et al.*, *Nature* **431**, 873 (2004).
26. Y. W. Cho *et al.*, *J. Biol. Chem.* **282**, 20395 (2007).
27. I. Issaeva *et al.*, *Mol. Cell. Biol.* **27**, 1889 (2007).
28. Materials and methods are available as supporting material on *Science* Online.
29. We thank A. Shilatifard and T. Jenuwein for their antibodies; Kazusa DNA Research Institute Foundation for UTX cDNA; M. Hart, T. Beer, K. Speicher, and D. Speicher for help with mass spectrometry; G. Harris for figure preparation; and H. Hoff and D. Schultz for protein expression. L.D.C. was supported by a grant from the Spanish–Ministerio de Educación y Cultura and R.S. was supported by a grant from NIH (RO1CA090758) and an intramural fund from Centre de Regulació Genòmica.

Supporting Online Material
www.sciencemag.org/cgi/content/full/1149042/DC1
Materials and Methods
Figs. S1 to S9
Table S1
References

5 June 2007; accepted 20 August 2007
Published online 30 August 2007;
10.1126/science.1149042
Include this information when citing this paper.

Permuted tRNA Genes Expressed via a Circular RNA Intermediate in *Cyanidioschyzon merolae*

Akiko Soma,[1] Akinori Onodera,[1] Junichi Sugahara,[2,3] Akio Kanai,[2,3,4] Nozomu Yachie,[2,3] Masaru Tomita,[2,3,4] Fujio Kawamura,[1,5] Yasuhiko Sekine[1,5]*

A computational analysis of the nuclear genome of a red alga, *Cyanidioschyzon merolae*, identified 11 transfer RNA (tRNA) genes in which the 3′ half of the tRNA lies upstream of the 5′ half in the genome. We verified that these genes are expressed and produce mature tRNAs that are aminoacylated. Analysis of tRNA-processing intermediates for these genes indicates an unusual processing pathway in which the termini of the tRNA precursor are ligated, resulting in formation of a characteristic circular RNA intermediate that is then processed at the acceptor stem to generate the correct termini.

Cyanidioschyzon merolae is an ultrasmall unicellular red alga that inhabits an extreme environment (*1*). The complete sequence of the nuclear genome of *C. merolae* recently became available (*2*). Genome-wide analyses (*1*, *2*) and a molecular phylogenetic analysis (*3*) have demonstrated that this organism is likely to represent one of the most ancestral forms of eukaryote. A search for tRNA genes from the *C. merolae* nuclear genome, using the tRNAscan-SE program (*4*), predicted only 30 tRNA genes encoding 30 species of anticodon, a number that is insufficient to decode all 61 codons (*2*). This prominent paucity of tRNA genes prompted us to search for undiscovered tRNA genes that may elucidate the evolution of tRNAs in early eukaryotes.

To search for *C. merolae* nuclear tRNA genes, we used SPLITS and SPLITSX, new programs that can detect cis-spliced tRNAs containing introns in various positions and trans-spliced tRNAs that are joined at several positions (*5*–*7*). In addition to this analysis, we performed a BLAST search of tRNA genes with conserved sequences in the TΨC arm or the anticodon arm, followed by manual inspection of the results.

[1]Department of Life Science, College of Science, Rikkyo (St. Paul's) University, Toshima, Tokyo 171-8501, Japan. [2]Institute for Advanced Biosciences, Keio University, Tsuruoka, 997-0017, Japan. [3]Systems Biology Program, Graduate School of Media and Governance, Keio University, Fujisawa, 252-8520, Japan. [4]Faculty of Environment and Information Studies, Keio University, Fujisawa, 252-8520, Japan. [5]Research Information Center for Extremophile, Rikkyo (St. Paul's) University, Toshima, Tokyo 171-8501, Japan.

*To whom correspondence should be addressed. E-mail: ysekine@rikkyo.ac.jp

The most important finding was that 11 genes have a novel gene organization in which the 3′ half of the tRNA sequence lies upstream of the 5′ half in the genome (Fig. 1A and fig. S1). Such a gene arrangement is accomplished by circular gene permutation (*8*), and we therefore termed these genes permuted tRNA genes. As shown in fig. S1, a TATA-like sequence is found upstream of the 3′ half in most of the genes. This sequence is also conserved in nonpermuted tRNA genes of the *C. merolae* nuclear genome. Thus, instead of the intragenic bipartite promoter consisting of an A box and a B box, which are conserved sequences in the D arm and TΨC arm (*9*), the upstream TATA-like sequence may play a central role in initiation of transcription in *C. merolae*. The genomic sequence encoding the intervening sequence between the 3′ and 5′ halves varies from 7 to 74 base pairs (bp). Downstream of the 5′ half, a T stretch that corresponds to a termination signal for RNA polymerase III (pol III) is found (*10*). These observations suggest that the pair of putative tRNA halves is transcribed as a linear RNA. As shown in Fig. 1B and fig. S2, permuted tRNA genes can be classified into four types on the basis of the location of the junction between the 3′ end of the 5′ half and the 5′ end of the 3′ half in the inferred secondary structures of the tRNAs. The junctions are located at 20/21 (between position 20 and 21) in the D loop (I), 37/38 in the anticodon loop (II), 50/51 in the TΨC stem (III), and 59/60 in the TΨC loop (IV). We identified one, six, one, and three candidates

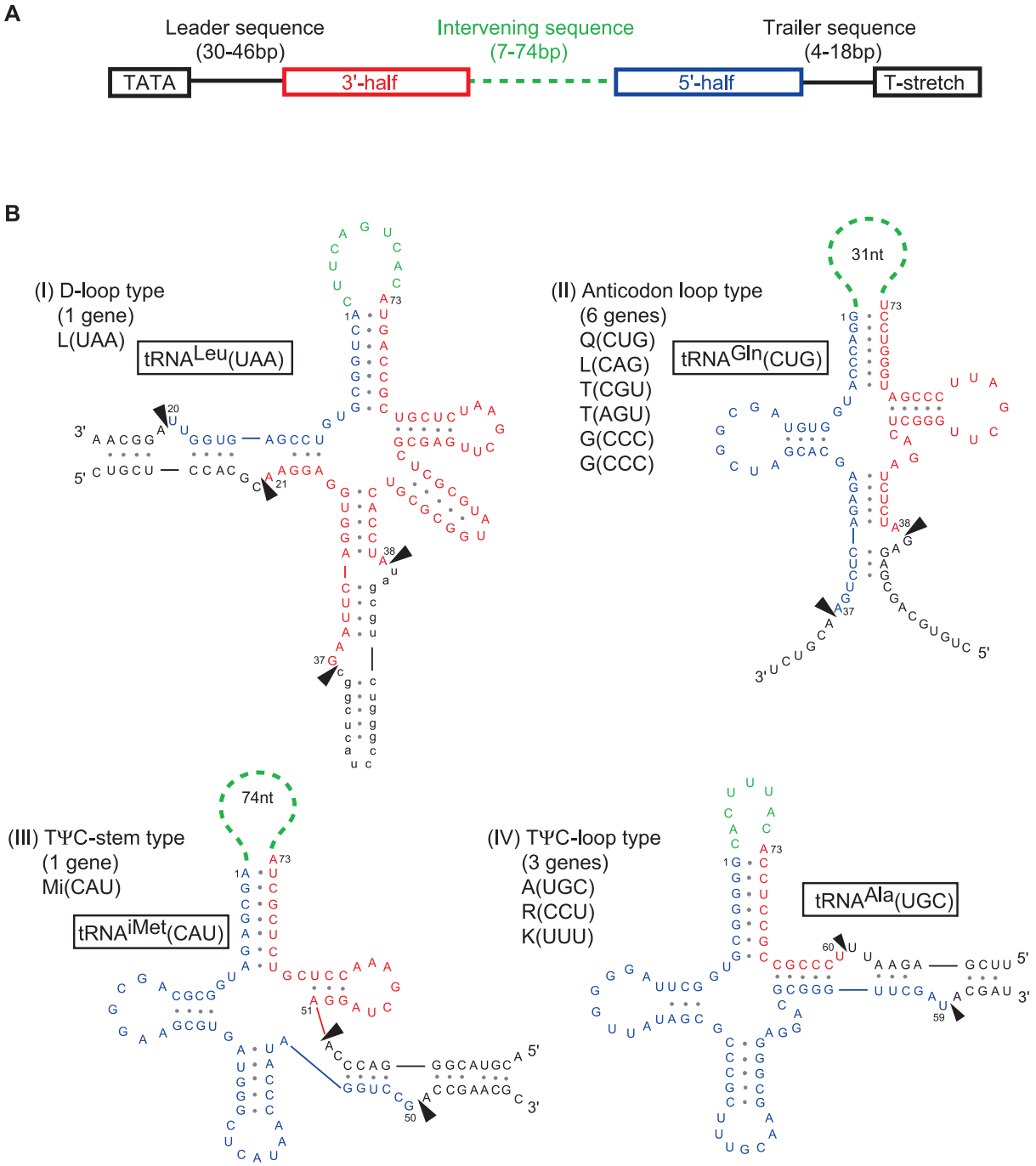

Fig. 1. Permuted tRNA genes in *C. merolae*. (**A**) Schematic representation of a permuted tRNA gene. (**B**) Inferred secondary structures for pre-tRNAs of four types of permuted tRNA genes. Arrowheads indicate the positions to be processed. The intron sequence is shown in lower case. The numbering of the tRNA positions is according to (*19*). Single-letter abbreviations for the amino acid residues are as follows: A, Ala; G, Gly; K, Lys; L, Leu; M, Met; Q, Gln; R, Arg; and T, Thr.

for the type I to IV tRNAs, respectively. Notably, the sequences adjacent to those junctions in tRNA precursors (pre-tRNAs) potentially form bulge-helix-bulge (BHB) motifs, which were originally found around the intron-exon junctions of nuclear and archaeal tRNAs (*11*).

Northern blot analysis using *C. merolae* total RNA verified that permuted tRNA genes are indeed transcribed in the cell (fig. S3). Next, to examine whether these tRNAs are aminoacylated, we performed Northern blot analysis of acid-urea gels with use of total RNA prepared from *C. merolae* cells under acidic conditions. Putative aminoacylated forms, which migrate more slowly than the deacylated forms, were detected, showing that these tRNAs could be aminoacylated in vivo (Fig. 2, lanes 1 and 2, and fig. S4). An in vitro aminoacylation analysis showed that recombinant *C. merolae* methionyl-tRNA synthetase (MetRS) methionylates $tRNA^{iMet}$ in total RNA preparations (Fig. 2, lanes 3 to 5). An in vitro transcribed $tRNA^{iMet}$ with the 3′ terminal CCA sequence was methionylated, although with less efficiency (Fig. 2, lanes 6 to 9). Thus, tRNA molecules expressed from permuted tRNA genes are aminoacylated and are likely to participate in protein synthesis.

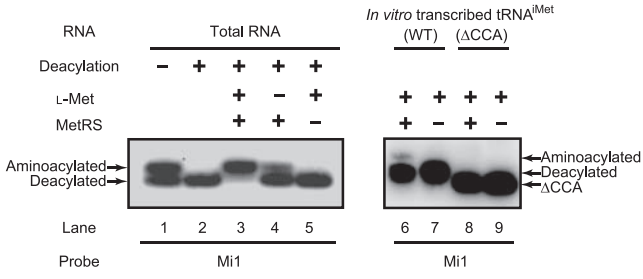

Fig. 2. Aminoacylation analysis of $tRNA^{iMet}$(CAU). Total *C. merolae* RNA prepared under acidic conditions was separated on an acid-urea gel directly (lane 1) or after deacylation (lane 2). In vitro methionylation reaction mixtures were loaded onto the gel (lanes 3 to 9). Deacylated total RNA (lanes 3 to 5) or in vitro transcribed $tRNA^{iMet}$ with [wild type (WT), lanes 6 and 7] or without (ΔCCA, lanes 8 and 9) the CCA sequence was used.

What is the processing mechanism of the pre-tRNA for these unusual tRNA genes? To clarify the processing pathway, we detected processing intermediates by reverse transcription polymerase chain reaction (RT-PCR) with two different sets of primers (Fig. 3A), followed by sequencing analysis of those RT-PCR products (Fig. 3B and fig. S5). Analysis of the $tRNA^{Gln}$(CUG) verified the sequences of the pre-$tRNA^{Gln}$ with a circularly permuted structure in which the leader sequence, the 3′ half, the intervening sequence, the 5′ half, and the trailer sequence were aligned in this order (Fig. 3B, lane 1, and fig. S5A). Interestingly, we also detected a circular RNA intermediate in which the leader and trailer sequences were removed and the resulting ends were ligated, while the intervening sequence was retained (Fig. 3B, lane 2, and fig. S5B). The existence of the circular RNA intermediate was confirmed by the generation of a PCR product representing two rounds of reverse transcription around the circular RNA (Fig. 3B, lane 2). The 3′-terminal CCA sequence is added posttranscriptionally in eukaryotes (*12*). To determine the terminal sequence of the mature $tRNA^{Gln}$, we performed RT-PCR with total RNA circularized by T4 RNA ligase. The sequence of the mature $tRNA^{Gln}$, in which the extra sequences are removed and the CCA sequence is added to the 3′ terminus of the acceptor stem, was verified (Fig. 3B, lane 3, and fig. S5C). As summarized in the model presented in Fig. 3C, maturation of the pre-tRNA probably starts with processing of the leader and trailer sequences, resulting in formation of the circular RNA intermediate. This processing step is most likely carried out by the tRNA-splicing machinery because the sequences adjoining the processing sites potentially form a BHB motif, which is the dominant recognition element for nuclear and archaeal tRNA-splicing endonucleases (*11*). The intervening sequence is then removed, possibly by ribonuclease (RNase) P and tRNase Z (*13*, *14*), followed by the CCA addition, to generate the correct termini. This model would be common to the permuted tRNA genes, because the circular RNA intermediate was detected for all 11 genes.

How could permuted tRNA genes have arisen? Permuted noncoding RNA (ncRNA) genes have been reported for *Trypanosoma*

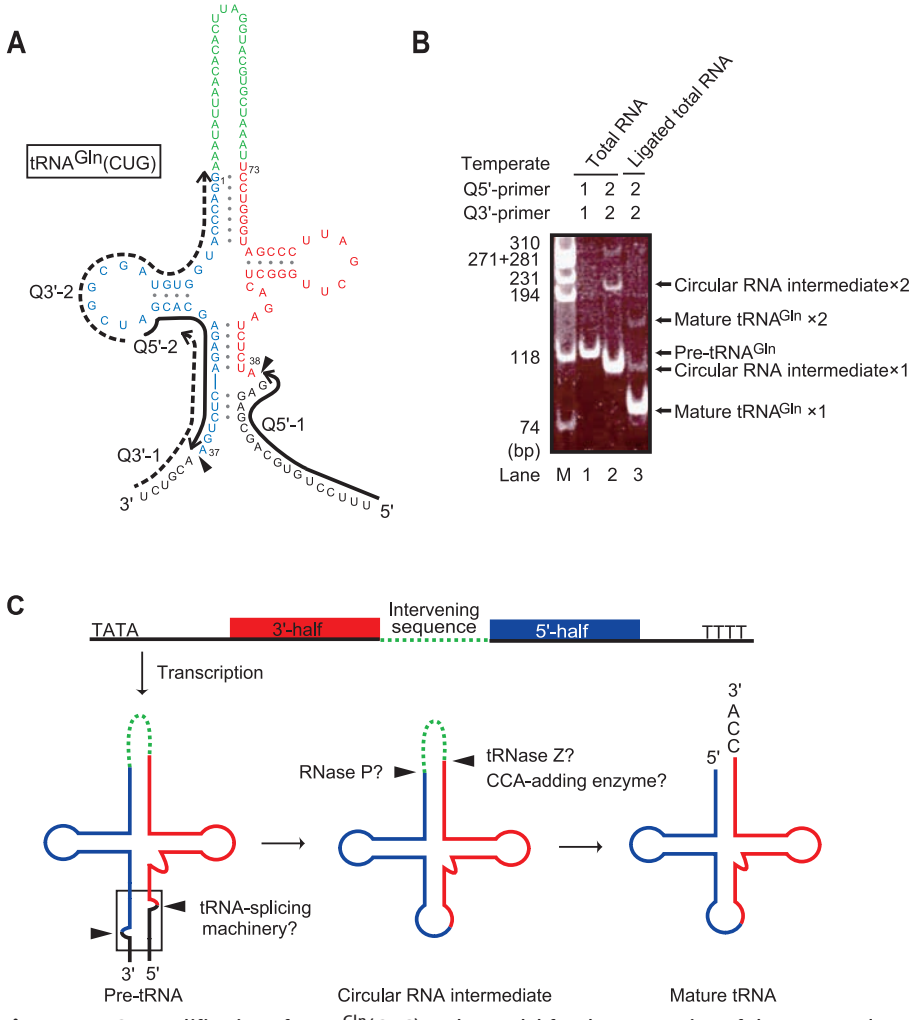

Fig. 3. RT-PCR amplification of $tRNA^{Gln}$(CUG) and a model for the maturation of the permuted pre-tRNAs. (**A**) 5′ and 3′ primers used for RT-PCR are indicated as solid and broken arrows, respectively. (**B**) PCR products amplified from cDNA of pre-$tRNA^{Gln}$ and from cDNA generated from one (×1) or two (×2) rounds of reverse transcription around a circular RNA intermediate or a circularized mature $tRNA^{Gln}$ are indicated. Lane M, DNA molecular weight markers (Φ×174 DNA-Hae III digest). (**C**) Maturation of the pre-tRNA starts with processing of a BHB motif (boxed) by the tRNA-splicing machinery, resulting in formation of a circular RNA intermediate. The intervening sequence is removed by RNase P and tRNase Z, then followed by the CCA addition.

mitochondrial small subunit (SSU) ribosomal RNA (rRNA) (*15*) and a bacterial transfer messenger RNA (tmRNA) (*16*) that function in a two-piece form, in contrast to the *C. merolae* tRNAs reported here that function in a one-piece form. The permuted rRNA and tmRNA genes are hypothesized to have arisen by a gene duplication that formed a tandem repeat, followed by the loss of the outer segment of each copy (*15*, *17*). Even if a circular permutation occurred in tRNA genes, most of the resulting permuted genes would not be retained in the genome because of the failure of expression or the loss of functional structure of the RNA. In *C. merolae*, however, permuted tRNA genes might have persisted in the genome because of the use of the upstream promoter by the transcription system and processing of the circularly permuted pre-tRNA into a canonical tRNA molecule by the tRNA-splicing machinery. Considering that *C. merolae* is an early rooted eukaryote and that the BHB motifs would play a pivotal role in the tRNA processing, it is possible that the permuted tRNA genes might have developed via a common process with the split-tRNA genes of *Nanoarchaeum equitans* (*18*). Further investigation should provide a hint about how to evaluate the evolution of tRNA genes in the early eukaryote.

References and Notes

1. T. Kuroiwa, *Bioessays* **20**, 344 (1998).
2. M. Matsuzaki *et al.*, *Nature* **428**, 653 (2004).
3. H. Nozaki *et al.*, *J. Mol. Evol.* **56**, 485 (2003).
4. T. M. Lowe, S. R. Eddy, *Nucleic Acids Res.* **25**, 955 (1997).
5. Materials and methods are available on *Science* Online.
6. J. Sugahara *et al.*, *In Silico Biol.* **6**, 411 (2006).
7. J. Sugahara, N. Yachie, K. Arakawa, M. Tomita, *RNA* **13**, 671 (2007).
8. T. Pan, O. C. Uhlenbeck, *Gene* **125**, 111 (1993).
9. G. Galli, H. Hofstetter, M. L. Birnstiel, *Nature* **294**, 626 (1981).
10. M. Hamada, A. L. Sakulich, S. B. Koduru, R. J. Maraia, *J. Biol. Chem.* **275**, 29076 (2000).
11. J. Abelson, C. R. Trotta, H. Li, *J. Biol. Chem.* **273**, 12685 (1998).
12. A. M. Weiner, *Curr. Biol.* **14**, R883 (2004).
13. S. Altman, L. Kirsebom, S. Talbot, *FASEB J.* **7**, 7 (1993).
14. H. Schurer, S. Schiffer, A. Marchfelder, M. Morl, *Biol. Chem.* **382**, 1147 (2001).
15. T. Y. Heinonen, M. N. Schnare, P. G. Young, M. W. Gray, *J. Biol. Chem.* **262**, 2879 (1987).
16. K. C. Keiler, L. Shapiro, K. P. Williams, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 7778 (2000).
17. K. P. Williams, *Nucleic Acids Res.* **30**, 2025 (2002).
18. L. Randau *et al.*, *Nature* **433**, 537 (2005).
19. M. Sprinzl, K. S. Vassilenko, *Nucleic Acids Res.* **33**, D139 (2005).

20. Supported by Japan Society of the Promotion of Science (JSPS) Fellowships (7473 to A.S. and 1924 to N.Y.), by Grants-in-Aid for Creative Scientific Research (17GS0314 to Y.S. and 16GS0304 to F.K.) from JSPS and Ministry of Education, Culture, Sports, Science and Technology, respectively, by grants from the Yamagata Prefectural Government and Tsuruoka City in Japan (to M.T., A.K., J.S., and N.Y.), and by the Frontier project Adaptation and Evolution of Extremophile (to Y.S.).

Supporting Online Material

www.sciencemag.org/cgi/content/full/318/5849/450/DC1
Materials and Methods
Figs. S1 to S5
References and Notes

29 May 2007; accepted 31 August 2007
10.1126/science.1145718

Trojan Horse Strategy in *Agrobacterium* Transformation: Abusing MAPK Defense Signaling

Armin Djamei,[1]*† Andrea Pitzschke,[1]† Hirofumi Nakagami,[1]†‡ Iva Rajh,[1] Heribert Hirt[1,2]

Nuclear import of transfer DNA (T-DNA) is a central event in *Agrobacterium* transformation of plant cells and is thought to occur by the hijacking of certain host cell proteins. The T-DNA–associated virulence protein VirE2 mediates this process by binding to the nuclear import machinery via the host cell factor VIP1, whose role in plants has been so far unknown. Here we show that VIP1 is a transcription factor that is a direct target of the *Agrobacterium*-induced mitogen-activated protein kinase (MAPK) MPK3. Upon phosphorylation by MPK3, VIP1 relocalizes from the cytoplasm to the nucleus and regulates the expression of the *PR1* pathogenesis-related gene. MAPK-dependent phosphorylation of VIP1 is necessary for VIP1-mediated *Agrobacterium* T-DNA transfer, indicating that *Agrobacterium* abuses the MAPK-targeted VIP1 defense signaling pathway for nuclear delivery of the T-DNA complex as a Trojan horse.

Higher eukaryotes recognize microbes through pathogen-associated molecular patterns (PAMPs) and activate the innate immune response in animals and plants (*1*). The perception of PAMPs leads to rapid activation of host defense mechanisms, including the activation of mitogen-activated protein kinases (MAPKs), production of reactive oxygen species, and subsequent induction of defense-related genes. However, virulence factors of successful pathogens can inhibit PAMP-elicited basal defenses (*2*). In specific cases, plants have evolved resistance proteins specialized to detect these pathogen-derived virulence factors or their effects on host targets. As a consequence, a hypersensitive response (HR) occurs that includes localized cell death and the arrest of pathogen spread (*3*, *4*).

Agrobacterium transforms plants by transporting a single-stranded copy of the transfer DNA (T-DNA) from its tumor-inducing Ti plasmid into the host cell and integrating it into the host cell genome. The agrobacterial transformation process is mediated by Vir (virulence) proteins. The T-DNA strand that is exported into plant cells has the VirD2 protein covalently attached to its 5′ end. The VirE2 protein, which is translocated into plant cells independently of the T-DNA strand, associates with the T strand in the plant cell (*5*) before nuclear import mediated by cellular karyopherin α, which binds to VirD2. Nuclear import is further facilitated by the host protein VIP1, which functions as an adaptor (*6*), but whose cellular function is as yet unknown.

PAMPs activate a variety of plant MAPK cascades (*7*), which make a major contribution to the host defense responses. In *Arabidopsis thaliana*, PAMPs such as flagellin activate at least three MAPKs—MPK3, MPK4, and MPK6—resulting in altered expression of various stress-responsive genes (*8*–*10*). So far, the direct downstream targets of these plant MAPKs are largely unknown. In this study, we show that *Agrobacterium* triggers the activation of several MAPKs, including MPK3, and we identify VIP1 as a target of MPK3.

A yeast two-hybrid screen was performed in order to find interactors with MPK3. Clones carrying full-length cDNAs of VIP1 (VirE2-interacting protein At1g43700) (*11*) were repeatedly isolated. The VIP1-MPK3 interaction in yeast was confirmed by its ability to induce another reporter gene, *lacZ*, encoding for β-galactosidase (Fig. 1A). In order to test whether VIP1 also interacts with other MAPKs, targeted yeast two-hybrid interaction experiments were performed with VIP1 and representatives of all the *A. thaliana* MAPK subfamilies (MPK2, -3, -4, -5, -6, -7, -16, and -17). VIP1-MAPK interaction was specific for MPK3, because neither MPK6, the closest homolog of MPK3, nor any of the other MAPKs tested interacted with VIP1 (Fig. 1A).

To substantiate these results, we transiently expressed MPK3 and VIP1 in *Arabidopsis*

[1]Department of Plant Molecular Biology, Max F. Perutz Laboratories, University of Vienna, Dr.-Bohr-Gasse 9, 1030 Vienna, Austria. [2]URGV Plant Genomics Laboratory, 2 Rue Gaston Crémieux, 91057 Evry, France.

*Present address: Department of Organismic Interactions, Max-Planck-Institut für terrestrische Mikrobiologie, Karl-von-Frisch-Strasse, 35043 Marburg, Germany.
†These authors contributed equally to this work.
‡Present address: Plant Immunity Research Team, RIKEN Plant Science Center, 1-7-22 Suehiro-cho, Tsurumi, Yokohama 230-0045, Japan.

ERRATUM

Post date 30 November 2007

Reports: "Permuted tRNA genes expressed via a circular RNA intermediate in *Cyanidioschyzon merolae*" by A. Soma *et al*. (19 October, p. 450). The last sentence on p. 452 referred to an incorrect subunit. The sentence should begin "Permuted noncoding RNA (ncRNA) genes have been reported for *Tetrahymena* mitochondrial large subunit (LSU) ribosomal RNA (rRNA) (*15*)."

ERRATUM

Post date 21 December 2007

Reports: "Permuted tRNA genes expressed via a circular RNA intermediate in *Cyanidioschyzon merolae*" by A. Soma *et al*. (19 October 2007, p. 450). The last sentence on p. 452 referred to an incorrect subunit. The sentence should begin "Permuted noncoding RNA (ncRNA) genes have been reported for *Tetrahymena* mitochondrial large subunit (LSU) ribosomal RNA (rRNA) (*15*)…"