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Riboregulation in plant-associated α-proteobacteria

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Abbreviations: AS, acetosyringone; asRNA, *cis*-acting antisense RNA; CM, covariance model; dRNA-seq, differential RNA-seq; GABA, γ-amino butyric acid; IGR, intergenic region; sRNA, small non-coding RNA; ORF, open reading frame; RFM, RNA family model; RNase, ribonuclease; TDM, thermodynamic matcher; *trans*-sRNA, trans-encoded sRNA; TSS, transcriptional start site

The symbiotic α-rhizobia *Sinorhizobium meliloti*, *Bradyrhizobium japonicum*, *Rhizobium etli* and the related plant pathogen *Agrobacterium tumefaciens* are important model organisms for studying plant-microbe interactions. These metabolically versatile soil bacteria are characterized by complex lifestyles and large genomes. Here we summarize the recent knowledge on their small non-coding RNAs (sRNAs) including conservation, function, and interaction of the sRNAs with the RNA chaperone Hfq. In each of these organisms, an inventory of hundreds of *cis*- and *trans*-encoded sRNAs with regulatory potential was uncovered by high-throughput approaches and used for the construction of 39 sRNA family models. Genome-wide analyses of *hfq* mutants and co-immunoprecipitation with tagged Hfq revealed a major impact of the RNA chaperone on the physiology of plant-associated α-proteobacteria including symbiosis and virulence. Highly conserved members of the SmelC411 family are the AbcR sRNAs, which predominantly regulate ABC transport systems. AbcR1 of *A. tumefaciens* controls the uptake of the plant-generated signaling molecule GABA and is a central regulator of nutrient uptake systems. It has similar functions in *S. meliloti* and the human pathogen *Brucella abortus*. As RNA degradation is an important process in RNA-based gene regulation, a short overview on ribonucleases in plant-associated α-proteobacteria concludes this review.

Introduction

Soil bacteria interacting with plants are of high agricultural importance. They include several research model organisms which belong to the Rhizobiales order within the much larger α-proteobacterial phylum. Among them are the so-called α-rhizobia which are capable of entering a symbiosis with leguminous plants, such as *Bradyrhizobium japonicum, Rhizobium etli* and *Sinorhizobium meliloti*, the nitrogen fixing endosymbionts of soybean, common bean and alfalfa, respectively.[1-3] This order also includes plant pathogens belonging to the *Agrobacterium/Rhizobium* lineage. A well-studied representative is *A. tumefaciens*, also used for genetic engineering of plants.[4] *A. tumefaciens*, *R. etli* and *S. meliloti* belong to the family of *Rhizobiaceae* while *B. japonicum* is a member of the distinct family of *Bradyrhizobiaceae* in the *Rhizobiales*. Recent high-throughput analyses revealed that these α-proteobacterial species harbor hundreds of RNAs with regulatory potential.[5-12] It is the challenge now to understand their physiological roles and the underlying molecular mechanisms. A systematic comparison of the sRNA repertoires of selected model organisms may help to understand the commonalities and differences between these phylogenetically and ecologically related bacteria.

The α-rhizobia and *A. tumefaciens* are metabolically versatile soil bacteria specifically interacting with host plants. *B. japonicum, R. etli* and *S. meliloti* infect a quite limited spectrum of leguminous plants leading to the formation of specialized root organs named nodules, in which the differentiated bacteria (bacteroids) live as endosymbionts, obtain nutrients from the plant and convert atmospheric nitrogen into ammonia to the benefit of the host.[2,3,13] In contrast, *A. tumefaciens* is a broad-host-range phytopathogen that transfers bacterial T-DNA into plant cells and leads to the formation of tumors (crown galls) on most dicotyledonous and some monocotyledonous plants.[4] Interestingly, there are fundamental similarities in the mechanisms of microbe-plant interactions and the interactions between pathogenic bacteria and mammalian cells.[14] Beside their agricultural importance, this is one of the reasons for the intense investigation of the three α-rhizobial species and *A. tumefaciens* as model organisms.

All four plant-associated species persist in the soil under a variety of environmental conditions, compete with other bacteria in

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the rhizosphere, interact with the plant and, in the case of rhizobia, differentiate into bacteroids. These complex lifestyles are reflected by large genomes (5.7 Mb in *A. tumefaciens*, 6.5 Mb in *R. etli*, 6.7 Mb in *S. meliloti* and 9 kb in *B. japonicum*) coding for a plethora of protein transcription factors.[15-19] Comparatively little is known about the regulation of gene expression at the posttranscriptional level in these bacteria. Small non-coding RNAs (sRNAs) are major players in RNA-based gene regulation together with the RNA chaperone Hfq and ribonucleases (RNases) or RNase-containing complexes.[20,21] They participate in the response and adaptation to diverse stress conditions, and are important for the regulation of quorum sensing and virulence.[22-25] The capability of *A. tumefaciens*, *B. japonicum*, *R. etli* and *S. meliloti* to occupy diverse ecological niches is mediated by complex regulatory networks which include riboregulation as well. This view is supported by the large amount of cis- and trans-encoded sRNAs detected by genome-wide analyses in these plant-associated bacteria.[5-12]

Since conservation of sRNAs is mostly limited to closely related bacteria, the phylogenetic relationships between α-rhizobia and *Agrobacterium* species allow the functional comparison of sRNAs with different degrees of conservation in function and regulation. Here, we summarize the current knowledge on the non-coding RNome as inferred from genome-wide surveys and discuss the incipient insights into biological functions and mechanisms of riboregulation in these organisms.

Transcriptome Landscape: Inventory of Non-Coding RNAs in α-Rhizobia and the Plant-Pathogen *A. tumefaciens*

Genome-wide identification of small non-coding RNAs in the Rhizobiales has been pioneered by computational predictions in *S. meliloti* and *R. etli*.[5-7,9] These were based on comparative genomics combining features characteristic of trans-encoded sRNAs (*trans*-sRNAs): transcription from genomic regions separating protein-encoding genes, association with promoter and/or Rho-independent transcriptional terminator sequence motifs, sequence conservation between closely related species, and conservation and thermodynamic stability of the predicted RNA secondary structure. However, complementation of these in silico approaches by microarray- and RNA-seq based surveys of the non-coding transcriptome of the model α-rhizobial species *S. meliloti*, *R. etli* and *B. japonicum* and the phytopathogen *A. tumefaciens* represented the major breakthrough in this field. Here we give an overview of inventories of the non-coding transcriptomes of these α-proteobacteria, which provides a firm foundation for comparative and functional studies of regulatory RNAs.

The non-coding transcriptome of *S. meliloti*

S. meliloti is well-studied for its engagement in a nitrogen fixing symbiosis with its plant hosts *Medicago*, *Melilotus*, and *Trigonella*. Strain Rm1021 possesses a large, tripartite genome composed of a chromosome (3.54 Mb), and the megaplasmids pSymA (1.35 Mb) and pSymB (1.68 Mb).[15] Several RNA-seq based approaches were undertaken in this *S. meliloti* strain and its close relative Rm2011. These screens aimed at profiling the transcriptome landscape and identifying sRNAs. Differential RNA-seq (dRNA-seq) of size-fractionated RNA using the Genome Sequencer *FLX Titanium* System (Roche Diagnostics) was applied to discover short RNAs of a size range from 50 to 350 nucleotides in RNA populations derived from exponential and stationary growing batch cultures, and from six stress conditions.[8] dRNA-seq involves enrichment of RNAs with primary 5′-ends by terminal exonuclease (TEX) treatment of one of the samples and its comparison to a non-treated sample still containing the original amount of processed 5′-monophosphorylated transcripts.[26] This approach revealed a total of 1,125 sRNA candidates of which 173 were grouped to *trans*-sRNAs, 117 to *cis*-encoded antisense sRNAs (asRNAs), 379 to mRNA leader sequences, and 456 to sense sRNAs overlapping coding regions. Short RNAs derived from 5′-UTRs of mRNAs may include metabolite-controlled riboswitches and RNA thermometers. This approach identified a surprisingly low number of antisense transcripts compared with RNA-seq studies of other prokaryotes.

An RNA-seq strategy designed for genome-wide identification of transcriptional start sites (TSS) of *S. meliloti* from 16 different growth and stress conditions revealed 3,720 asRNAs that were assigned to approximately 35% of the protein-coding genes.[27] Thus, the lower number of growth conditions and the size fractionation of RNA populations analyzed in the first approach was likely responsible for the small number of antisense transcripts identified.[8] Genome-wide TSS mapping not only increased the number of known asRNAs, but also identified new members of the classes of *trans*-sRNAs and sense RNAs, and identified TSS of mRNAs.[27] A total of 17,001 TSS falling into six categories were reported in this study: mRNA (4,430 TSS assigned to 2,657 protein-coding genes), leaderless mRNAs (171), putative mRNAs (425), internal sense transcripts (7,650), asRNA (3,720), and *trans*-sRNAs (605 TSS associated with 440 *trans*-sRNAs). This raised the number of *trans*-sRNAs to 550 in *S. meliloti*. *Trans*-sRNAs were found on all three replicons of the multipartite genome of *S. meliloti*. Taking into account the individual sizes of the three replicons these sRNAs were overrepresented on pSymA.[27]

Both RNA-seq approaches that aimed at identifying primary 5′-ends revealed a strikingly high number of sense transcripts overlapping coding regions, but not involving an open reading frame (ORF).[8,27] Such sense RNAs have also been identified in similar studies of other prokaryotic transcriptomes. It is likely that a high proportion of these sense RNAs were degradation products derived from the corresponding mRNAs that survived the enrichment procedure. However, upstream promoter motifs were predicted for 632 sense RNAs.[8,27] Whether these transcripts have a functional role remains to be investigated.

The precise determination of TSS by RNA-seq involving enrichment of primary transcripts allowed for identification of promoter motifs and improved consensus sequences.[8,27] The RNA-seq approaches clearly outperformed previous microarray-based strategies.[7,8] Comparison of RNA-seq based identification to computational predictions of *trans*-sRNAs demonstrated the power of deep sequencing strategies that rediscovered 59 out of

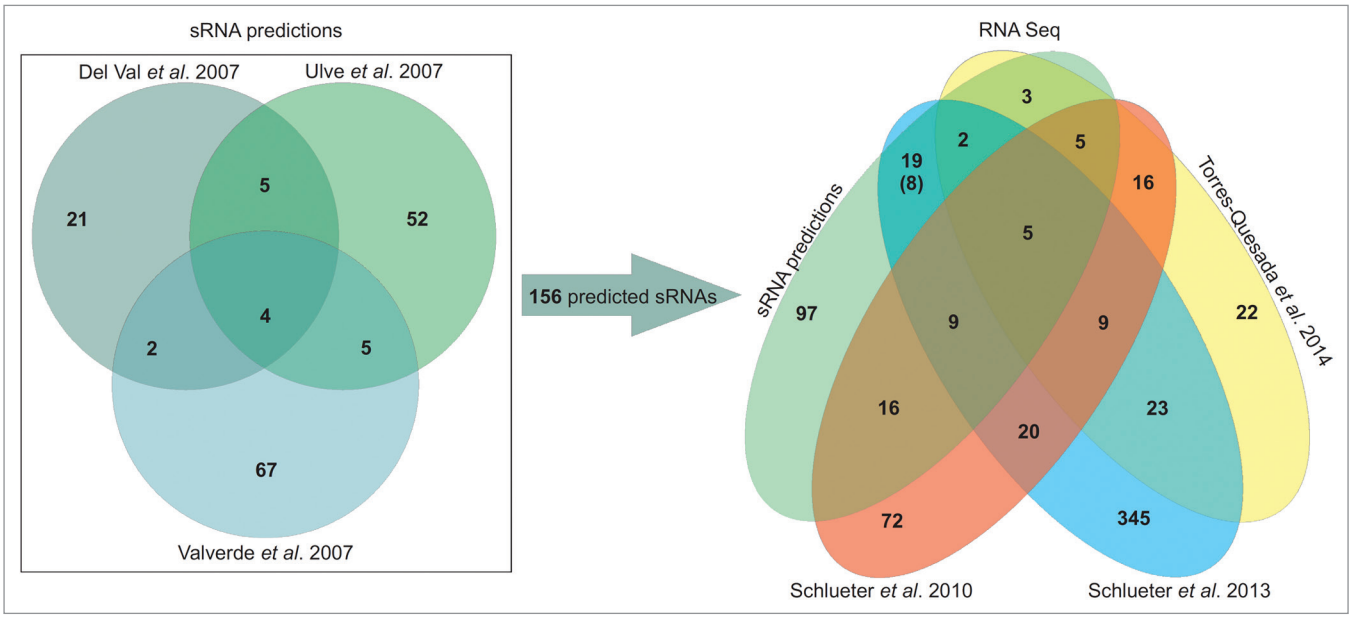

Figure 1. Overview of computational predicted and experimentally identified sRNAs in *S. meliloti*. On the left, Venn-diagram illustrating the number of potential sRNA encoding regions predicted by del Val et al.,[5] Ulve et al.,[6] and Valverde et al.[7] On the right, Venn-diagram representing sRNA transcripts identified via RNA-seq studies by Schlüter et al.,[8] Schlüter et al.,[27] and Torres-Quesada et al.[28] and a comparison to the 156 predicted candidates. Number in brackets: clustered sRNA genes (at least two experimentally identified neighboring sRNA genes) which were spuriously predicted and summarized as a single sRNA gene region were counted as individual transcription units.

156 predicted candidates and identified numerous novel trans-sRNAs (**Fig. 1**).[8,27,28]

The non-coding transcriptome of *R. etli*

In silico searches were combined with transcriptome profiles of *R. etli* CFN42 at various time points during free-living growth and during symbiosis with *Phaseolus vulgaris*.[9] This study aimed at the identification of transcriptionally active intergenic regions (IGR) applying a whole-genome-high-resolution tiling oligonucleotide array. The expression profiles suggested 89 non-coding RNAs mapping to all replicons of the *R. etli* CFN42 genome, the 4.38 Mb chromosome and the six megaplasmids encompassing additional 2.15 Mb.[19] 66 of these candidates were classified as novel non-coding RNAs since they did not group to well-characterized house-keeping RNAs and had not been reported before in *R. etli* or related α-proteobacteria. These candidates, comprising 17 *trans*-sRNAs and 49 putative asRNAs, overlapped with the group of computationally predicted non-coding RNAs.[9] Several of these non-coding RNAs were differentially expressed in free-living conditions and during symbiosis. Clustering identified three classes characterized by dominant expression during exponential growth, in the stationary phase, or *in planta* implying a role in adaptation to different environmental factors.[9]

The non-coding transcriptome of *A. tumefaciens*

Two deep sequencing strategies uncovered the transcriptome of the plant pathogen *A. tumefaciens* under non-virulent and virulence induced conditions.[11,12] Wilms et al.[11] used the Roche 454 platform and libraries from cells grown in minimal medium with or without the phenolic compound and virulence gene inducer Acetosyringone (AS). A total number of 348,998 cDNAs reads were mapped to the genome. The 228 identified sRNA candidates (152 *trans*-sRNAs, 76 *cis*-asRNAs) are distributed on all replicons: 129 on the circular chromosome (*trans*-sRNA: 96, asRNA: 33), 59 on the linear chromosome (*trans*-sRNA: 41, asRNA: 18), 20 on the At plasmid (*trans*-sRNA: 8, asRNA: 12) and 20 on the Ti plasmid (*trans*-sRNA: 7, asRNA: 13). Among them were the known sRNAs RepE, AbcR1, and AbcR2.[29] 22 of these identified sRNAs were independently confirmed by northern blot analysis. The observation that several sRNAs were differentially expressed in response to growth media, growth phase, temperature, or pH, and that one sRNA from the Ti-plasmid was massively induced in the presence of AS suggests that sRNAs play a role in various cellular processes including virulence.

The virulence-induced sRNA Ti2 is encoded downstream of a promoter strictly dependent on the VirA/G two-component system.[11] Its location in the 5′-UTR of the *atu6155virK* operon suggests that it may be a processed or prematurely terminated product of the UTR as proposed by Lee et al.[12] The latter study made use of an Illumina GAII platform to sequence cDNA libraries from cells grown in nutrient-rich medium at mid-log phase, nutrient-rich medium at late stationary phase, and minimal medium with or without AS. Mapping of a total number of 490,552 million reads identified 475 highly expressed non-coding RNAs distributed across all four replicons (101 *trans*-RNAs, 354 antisense RNAs and 20 5′-UTRs), including RepE, AbcR1 and AbcR2. Disregarding the 5′-UTRs, 210 were on the circular chromosome (*trans*-sRNA: 56, asRNA: 154), 158 on the linear chromosome (*trans*-sRNA: 33, asRNA: 125), 41 on the At plasmid (*trans*-sRNA: 8, asRNA: 33) and 44 on the Ti plasmid (*trans*-sRNA: 4, asRNA: 42). Fifteen sRNAs were induced and 7

were suppressed by the virulence inducer AS. A total number of 374 novel sRNA were found in this study (**Fig. 2**). Interestingly, this study found fewer *trans*-sRNAs but many more antisense RNAs than Wilms et al.[11] demonstrating the value of several independent approaches in sRNA discovery (see also **Figure 1**).

Both studies reported the presence of antisense sRNAs transcribed from the reverse strand of virulence genes, for example of *virC2* (required for excision of the T-DNA) and *virB9* (component of the type IV secretion system) suggesting that they might play regulatory roles in the plant infection process. Two candidates antisense to *atsD* (might be important for bacterial attachment on plants) and antisense to *virB10* (Atu6176, essential component of type IV secretion system) were tested for their role in *A. tumefaciens* virulence. However, their depletion by anti-antisense technology did not significantly affect tumor genesis in three different virulence assays suggesting that the real targets of these *cis*-antisense sRNAs might not be the sense-strand genes.[12] Likewise, deletion of the massively AS-induced sRNA Ti2 did not significantly affect tumor genesis of *Kalanchoe daigremontiana* leaves and potatoe tumor discs (unpublished data).

The non-coding transcriptome of *B. japonicum* USDA 110

The initial genome-wide search for sRNAs in *B. japonicum* USDA 110 relied on computational predictions based on comparison of genomes of *Bradyrhizobium* sp.BTAi1 and *B. japonicum* USDA 110 to the genome of the free-living purple bacterium *Rhodopseudomonas palustris* BisB5a.[10] Approximately 100 so-called "clusters" representing potential *trans*-sRNAs were predicted, and seven candidate sRNAs (BjrC2a, BjrC2b, BjrC2c, BjrC68, BjrC80, BjrC174 and BjrC1505) were verified by northern blot hybridization in addition to conserved sRNAs with housekeeping function like SRP RNA, RNase P RNA, tmRNA and 6S rRNA. The detected sRNA candidates have a different degree of conservation. Three of these sRNAs (BjrC2a, b and c) belong to the previously described RNA family RF00519.[30] Multiple imperfect copies of RF00519 sRNAs are present in many species in the Rhizobiales including *S. meliloti* and *A. tumefaciens*. Another well conserved sRNA is BjrC1505, homologs of which are present in *Bradyrhizobiaceae* and some genera of *Rhizobiaceae* like *Brucella* and *Mesorhizobium* but not in *Sinorhizobium* and *Agrobacterium/Rhizobium*. The other sRNAs are limited to *Bradyrhizobiaceae*. The least conserved BjrC68 corresponds to the 3′-UTR of blr0613 and its homologs are found only in the closely related genera *Bradyrhizobium* and *Rhodopseudomonas*.[10]

As in most similar studies, the expression of the verified sRNAs was monitored by northern blot hybridization under diverse stresses and at different growth phases. The results were directly compared with data from microarray and dRNA-seq analyses and displayed similar tendencies - increases or decreases in the amounts of the particular sRNA in bacteroids were supported by at least two of the methods.[10] Specific accumulation of full-length forms or processing/degradation products of sRNAs in nodules suggested functional relevance in symbiosis for BjrC174, BjrC68 and BjrC80. Interestingly, although the amount of 6S RNA was not increased in the stationary phase in liquid cultures, it was increased in nodules suggesting a similar role for 6S RNA

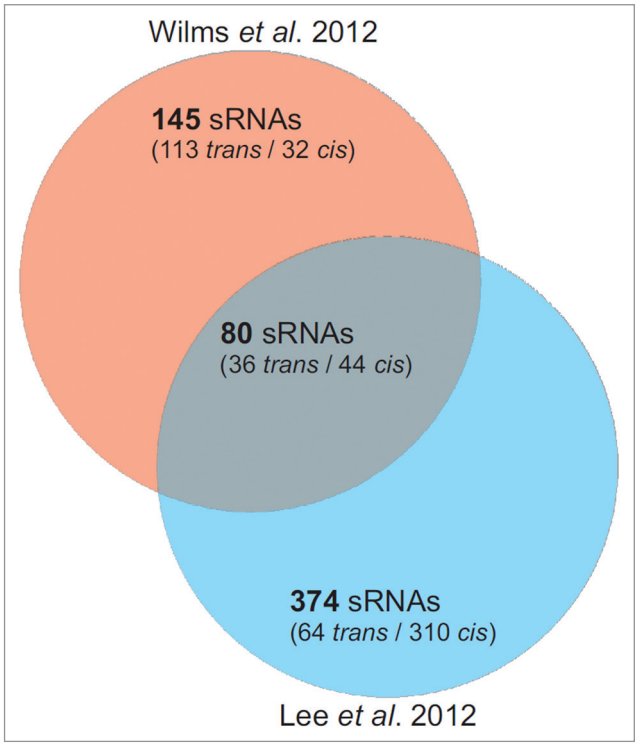

Figure 2. Comparison of two *A. tumefaciens* dRNA-seq studies. Venn diagram comparing the sRNAs identified by Wilms et al.[11] and Lee et al.[12]

in the non-dividing bacteroids of *B. japonicum* USDA 110 as in stationary cultures of *E. coli*.[10,31,32] Using dRNA-seq, comparable levels of "product RNAs" (pRNAs) of 10 to 17 nt in length were detected in free-living bacteria and in bacteroids.[10] The pRNAs are short primary transcripts synthesized by the RNA polymerase using 6S RNA as template during the process of release, and their detection suggests turnover of 6S RNA of *B. japonicum* USDA 110 under both free-living and symbiotic conditions.[33]

α-Proteobacterial sRNA Families

Constructing sRNA family models (RFMs) from transcripts observed in an RNA-seq study serves two purposes. For a transcript with a known or suspected regulatory function, this information can be transferred to related bacteria that host a family member in their genome, and experimental evidence available from different organisms may be interpreted in combination. For an sRNA transcript without associated functional evidence, successful construction of an RFM provides evidence for conservation in sequence and/or structure, and indirectly hints at the existence of a conserved function. Furthermore, the specific pattern of conservation observed may point to functions related to the lifestyle of a closely related group.

Starting from the sRNAs identified by Schlüter et al.,[8] a set of 52 *trans*-sRNAs was chosen, some of which apparently were multiple homologs of the same sRNA on different replicons of *S. meliloti*. From these, 39 family models were constructed employing two different bioinformatics approaches.[34]

• Covariance models (CMs) are stochastic models, capturing sequence and structure conservation in an alignment of family members. CMs can be automatically constructed by INFERNAL, given such an alignment.[35]

• Thermodynamic matchers (TDMs) are RNA folding programs, based on the established thermodynamic model, but tailored to a specific structural motif. Production of such matchers is supported by the graphical editor LOCOMOTIF and by the Bellman's GAP system.[36,37]

CMs are the more commonly used approach, which is employed e.g., with the Rfam database. The method is applicable when sequence conservation is high enough to obtain a reliable multiple alignment from candidate sequences. 37 out of the 39 family models were constructed in this fashion. In two cases where structure was conserved, but sequence was highly diverged (except for short loop motifs), the TDM approach was applied. TDMs focus on structure and folding energy; they can ignore sequence conservation in some parts, e.g., in helices, and yet insist on conserved sequence motifs elsewhere, e.g., in loops. Building such a matcher requires human design decisions and some experimentation, and hence, it is more laborious than the standard route to family model construction. The precise workflows employed were described by Reinkensmeier et al.[34]

del Val et al.[38] applied a similar approach to analyze the distribution of six sRNA candidates (SmrC7/SmelC023, SmrC9/SmelC289, SmrC45/SmelC706, SmrC15/SmelC411, SmrC14/SmelC397, SmrB35/SmelB053) identified in *S. meliloti* 1021.[5] Conservation and occurrence of sRNAs in the Rhizobiales derived from these studies is summarized in **Figure 3**. Three of these sRNAs, SmelB053, SmelC023, and SmelC289, were investigated in both comparative studies that suggested consistent phylogenetic distributions. Microsynteny of the genomic regions containing sRNA genes further supported 27 of the 39 RFMs.[34]

RNA families founded by *S. meliloti* sRNA candidates encoded by the megaplasmids pSymA or pSymB were restricted to *Sinorhizobium* species (**Fig. 3**). The only exception to this observation was SmelA033 that was also found in *R. etli* strains. This is in agreement with the assumption that the large rhizobial plasmids mostly contain accessory genetic information.[39] Most sRNA families were restricted to the *Rhizobiaceae* and only eight families were present in all species of the *Rhizobiaceae* investigated in these comparative studies, including five *Rhizobium*, five *Sinorhizobium*, and four *Agrobacterium* strains (**Fig. 3**).[34,38] Among these was the family SmelC411 (SmrC15) including the AbcR sRNAs that have been first described and functionally studied in *A. tumefaciens* and *S. meliloti* (**Fig. 4A**).[29,40] The AbcR family contains two highly conserved modules resembling the anti-Shine–Dalgarno sequence, one with a 5′-CCUCCC-3′ sequence (M1) within loop 1 and a second 5′-GUUCCCCUC-3′ sequence (M2) between the first and the second loop (**Fig. 4A**). Homologs of AbcR1 and AbcR2 (SmrC15/SmrC16, Smr15c1/Smr15c2, SmelC411/SmelC412, Sra41, and Sm3/Sm3′, ReD01, ReC58/ReC59) have been reported in the closely related plant symbionts *S. meliloti, S. fredii, R. etli, Rhizobium gallicum, Rhizobium giardinii, Rhizobium leguminosarum*, and in the human pathogen *Brucella abortus*.[5-7,31,41] In several *Sinorhizobium* species, comparative sequence analyses predicted a third member of this family on megaplasmid pSymA in addition to the chromosomal members AbcR1 and AbcR2.

Only ten sRNA families showed broad conservation in the *Rhizobiales*, but neither occurred in all subtaxa of this order. These RFMs mostly showed only conservation of short sequence motifs combined with structural similarities. Among these are the RFMs founded by SmelA075 and SmelA099 (**Fig. 4B**). Like the AbcR1 family, both contain highly conserved 5′-CCUCCUCCC-3′ loop motifs resembling the anti-Shine–Dalgarno (aSD) sequence. sRNAs composed of two to four stem loops containing this conserved loop motif were also found in closely related as well as more distant α-proteobacteria.[34,42] The *Rhodobacter spaeroides* sRNA family RSs0680 showing similar features has been implicated in the oxidative stress response.[42] Comprehensive sequence and structural conservation facilitates identification of sRNA homologs in close relatives and also suggests functional conservation. Though, prediction of homologs in more distantly related bacteria is complicated by poor conservation. This may result in spurious and misleading assignments of RNAs to RFMs. Nevertheless, the conservation patterns of *trans*-sRNAs and the frequent occurrence of paralogs in the Rhizobiales point to the evolutionary history of this class of riboregulators and imply that duplication events followed by divergent evolution probably provide an adaptive advantage.

RNA-Binding Proteins in α-Rhizobia and *A. tumefaciens*

sRNA activity in many bacterial species involves RNA-binding proteins. One of the best studied examples are CsrA/RsmA family RNA-binding proteins acting as regulators of translation.[43] Csr/Rsm mimic sRNAs compete with the target mRNA for binding of these proteins thereby releasing the translational block. With exception of a CsrA/RsmA-like protein encoded by the cryptic plasmid pMBA19a in *S. meliloti* MBA19, α-proteobacterial genomes lack genes homologous to *csrA*/*rsmA*.[44]

In contrast, *hfq* encoding an RNA-binding protein predicted to be present in at least 50% of all bacterial species was identified in all α-proteobacterial genomes sequenced so far.[21] Hfq proteins found in this group of bacteria share strong sequence similarities with enterobacterial Hfq proteins suggesting a similar quaternary organization into a hexameric toroid with at least two different RNA binding surfaces.[45] This is further supported by successful complementation of an *E. coli hfq* mutant by the *S. meliloti hfq* gene and vice versa.[46,47] Hfq assists RNA-mediated gene regulation.[21,48] Its functional role exceeds the interaction with *trans*-sRNAs and their mRNA targets to binding of asRNAs and tRNAs.[49-53] Translational control or regulation of mRNA decay mediated by binding of Hfq to mRNAs in a trans-sRNA independent manner has been reported.[54,55] Like in other groups of bacteria, α-proteobacterial *hfq* genes appear to be strongly expressed and autoregulated at posttranscriptional level.[46,47,56]

In most α-proteobacteria, *hfq* mutants were affected in a broad range of phenotypes, including growth behavior, adaptation to

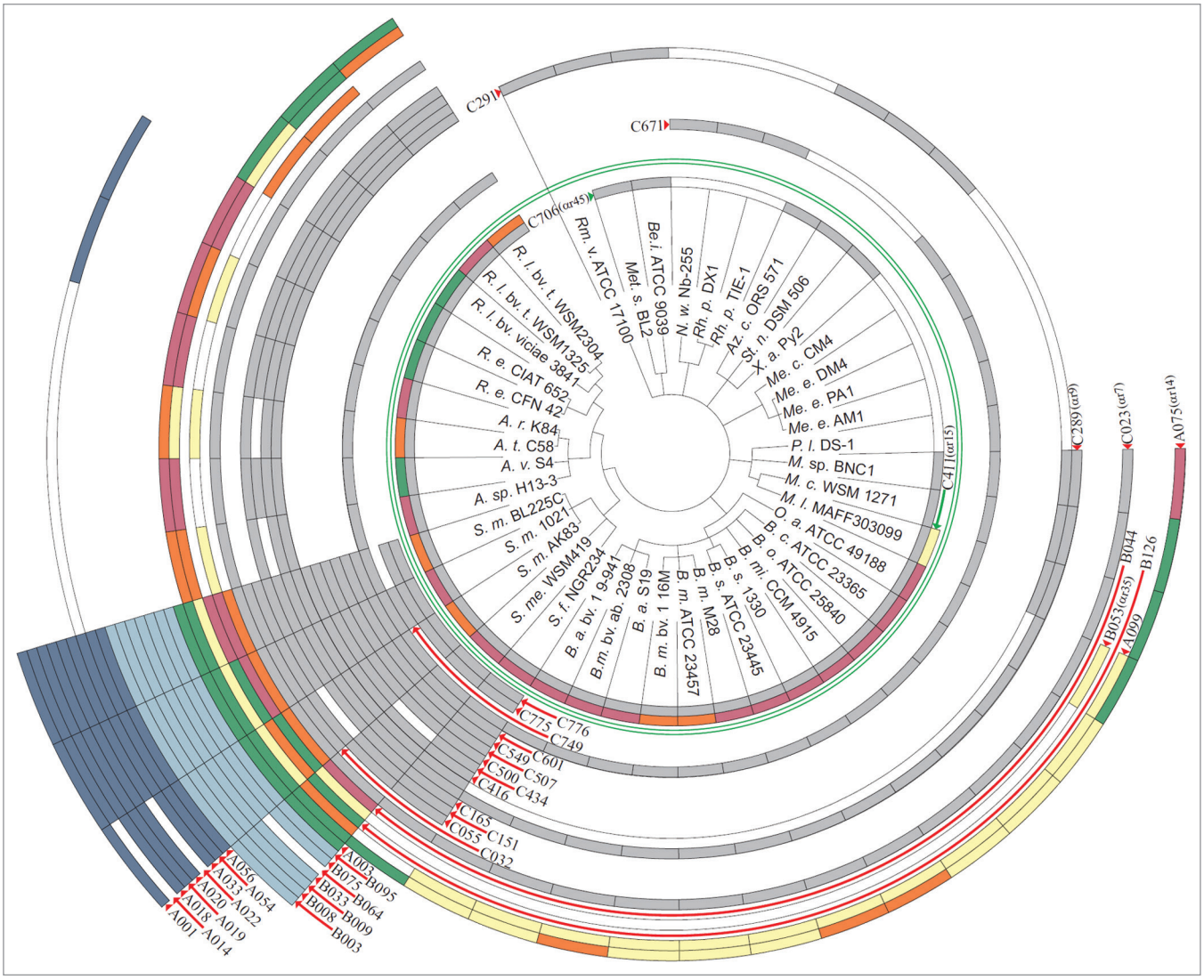

Figure 3. Distribution of *trans*-sRNAs in the Rhizobiales. The circular plot is based on a simplified phylogenetic tree adopted from Reinkensmeier et al. and del Val et al.[34,38] Sequenced strains which harbor at least one sRNA of a defined RNA family model (RFM) are illustrated. Each circular row incorporates all sRNA members of an RFM and shows their assignment to members of the Rhizobiales. The two RFMs separated by the green circle were defined exclusively by del Val et al.[38] The RFM nomenclature in brackets indicates the availability of alternative RFMs calculated by del Val et al.[38] (not shown here). Grey, chromosomal sRNAs; light blue, sRNAs encoded on pSymB-like replicons; dark-blue, sRNAs encoded on pSymA-like replicons. Multi-colored rows indicate RFMs with several RNA gene homologs per strain/genome: Yellow, single gene; orange, two homologs; pale violet red, three homologs; green, four or more homologs. Species glossary: R. l., *Rhizobium leguminosarum*; R. e., *Rhizobium etli*; A. r., *Agrobacterium radiobacter*; A. t., *Agrobacterium tumefaciens*; A. v., *Agrobacterium vitis*; A. sp., *Agrobacterium species*, S. m., *Sinorhizobium meliloti*; S. e., *Sinorhizobium meliloti*; S. f., *Sinorhizobium fredii*; B. a., *Brucella abortus*; B. m., *Brucella melitensis*; B. s., *Brucella suis*; B. mi., *Brucella microti*; B. o., *Brucella ovis*; B. c., *Brucella canis*; O. a., *Ochrobactrum antrophi*; M. l., *Mesorhizobium loti*; M. c., *Mesorhizobium cicero*; M. sp., *Mesorhizobium species*; P. l., *Parvibaculum lavamentivorans*; M. e., *Methylobacterium extorquens*; M. c., *Methylobacterium chloromethanicum*; X. a., *Xanthobacter autotrophicus*; St. n., Starkeya novella; Az. c., *Azorhizobium caulinodans*; Rh. p., *Rhodopseudomonas palustris*; N. w., *Nitrobacter winogradskyi*; Be. i., *Beijerinckia indica*; Met. s., *Methylocella silvestris*; Rm. v., *Rhodomicrobium vannielii*.

stress factors, motility, cell morphology, host colonization, or virulence. The *A. tumefaciens hfq* mutant was delayed in growth, impaired in motility, showed altered cell morphology and was attenuated in tumor formation.[57] *A. caulinodans* and *Rhizobium leguminosarum* bv. *viciae hfq* mutants were deficient in symbiotic nitrogen fixation.[46,58,59] A number of different studies characterized phenotypes caused by *hfq* knockouts and their impact on the *S. meliloti* transcriptome and proteome. *S. meliloti hfq* mutants were delayed in growth, impaired in motility and more sensitive to heat, oxidative, or pH stresses. They showed alterations in the utilization of nitrogen sources, in profiles of quorum sensing signals, and symbiotic traits.[45,56,60-63] Competitiveness for infection, nodule development, intracellular survival of bacteroids and nitrogen fixation was negatively affected in *S. meliloti hfq* mutants. These pleiotropic phenotypes can largely be traced back to global changes in gene expression patterns at RNA and protein levels. Although the various profiling studies involved different *S. meliloti* strains, growth conditions, and host plants

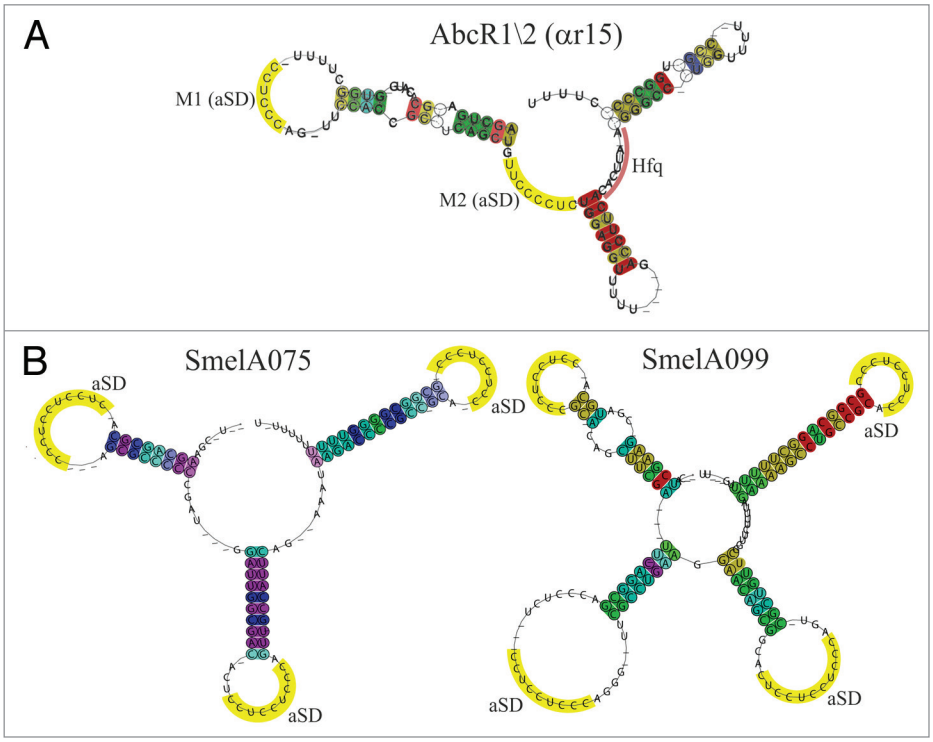

Figure 4. Structural Alignment of AbcR1/2 (SmelC411), SmelA075, and SmelA099. Consensus secondary structures are colored according to the Vienna RNA conservation coloring scheme.[110] A. Structural alignment of RFM αr15 (AbcR1/2) defined by del Val et al.[38] In yellow, anti-Shine-Dalgarno (aSD) sequences (modules M1 and M2) in single-stranded regions and loop structures. Pale red, Hfq binding site. B. Structural alignment of RFM SmelA075 and SmelA099 defined by Reinkensmeier et al.[34]

some common observations were made. Massive differential expression of genes mediating tolerance to a variety of stress factors and to genes involved in transport of small molecules relevant to carbon and nitrogen metabolism suggests a major role of Hfq in physiology of *S. meliloti*.[56,60-62] These findings were further supported by an *A. tumefaciens hfq* mutant showing overproduction of several ABC transporter components when compared with the wild type.[57]

A positive contribution of Hfq to the post-transcriptional regulation of *nifA*, encoding the major transcriptional regulator of nitrogen fixation, has been observed in several free-living and symbiotic α-proteobacterial diazotrophs.[46,59,64] Furthermore, *R. leguminosarum* Hfq negatively regulates the broad range amino acid transport systems Aap and Bra which have a key role in nitrogen exchange between the bacteroid and the infected plant cell and suppressor mutations in *hfq* rescued *R. leguminosarum* GOGAT mutants that were deficient in nitrogen fixation.[65] α-proteobacterial *hfq* mutants impaired in virulence and symbiosis, as observed for several symbiotic α-rhizobia, phytopathogenic *A. tumefaciens*, and animal-pathogenic *Brucella* species, imply that Hfq is not only important for free-living states but also for establishment and maintenance of chronic microbial infections of eukaryotic hosts.[66,67]

Co-immunoprecipitation (CoIP) of an epitope-tagged Hfq and its associated RNA in *S. meliloti* followed by PCR identified a few selected *trans*-sRNAs.[40,62] Recently, Hfq-CoIP combined with deep sequencing of the recovered RNA generated a genome-wide atlas of *S. meliloti* Hfq-bound transcripts in five growth/stress conditions.[28] This study suggested 1,315 Hfq-bound transcripts derived from non-coding RNAs, both *trans*-encoded (6.4%) and antisense (asRNAs) (6.3%), and mRNAs (86%) with the largest proportion of RNAs enriched from stress conditions. However, only 14% of *trans*-sRNAs and 2% of asRNAs were identified in previous studies. Hfq was associated with 18% of the predicted *S. meliloti* mRNAs encoding proteins related to transport and metabolism, σ^{E2}-dependent stress responses, quorum sensing, flagella biosynthesis, ribosome and membrane assembly, or symbiotic nitrogen fixation. This suggests that in *S. meliloti* stress responses and symbiotic gene expression are substantially affected by Hfq-mediated post-transcriptional control.

Another candidate for an RNA binding protein that may be related to riboregulation is the *S. meliloti* SMc01113 protein, a homolog to *E. coli* YbeY. This protein is broadly conserved in bacteria and comprises an RNA-binding region reminiscent of the MID domain of eukaryotic Argonaute proteins involved in RNA silencing.[68] Phenotypes of a SMc01113 mutant are pleiotropic and resemble those of *hfq* mutants.[68] In *E. coli*, YbeY modulates Hfq-dependent and Hfq-independent sRNA-mRNA interactions.[69] Furthermore, YbeY is related to rRNA processing and transcription termination and recently, it was found that YbeY is an RNase playing a role in rRNA maturation and quality control.[70-72] The functional role of YbeY-like proteins in α-proteobacteria remains to be investigated.

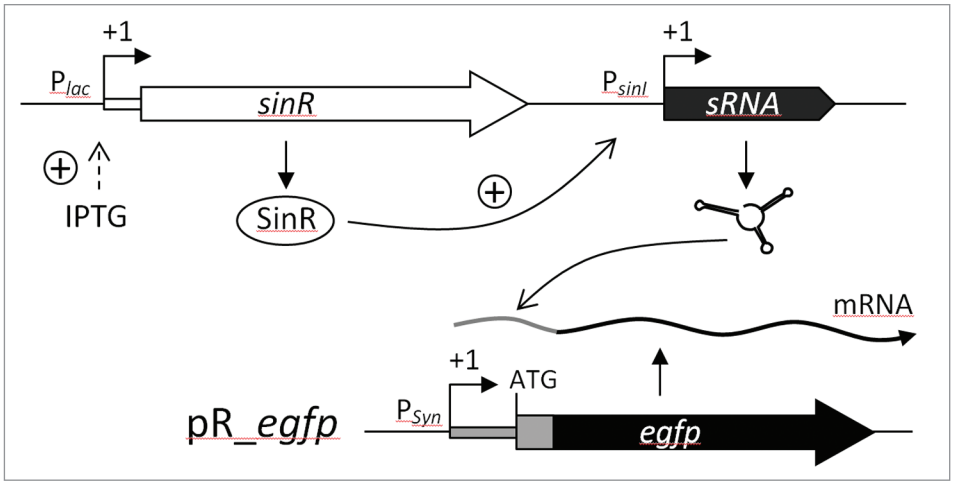

Figure 5. sRNA pulse overexpression construct based on IPTG inducible expression of *sinR* which activates P_{sinI}-driven sRNA gene expression. The effect of sRNA overproduction is monitored applying a reporter construct based on plasmid pR_egfp.[40] P_{Syn} is a constitutive promoter derived from P_{lac}.

Regulatory Functions of Non-Coding RNAs: AbcR1 and AbcR2

The function of most sRNAs in the Rhizobiales is unexplored. Apart from housekeeping rRNAs and tRNAs, the regulatory 6S RNA, and the housekeeping 4.5S and RNase P RNAs are ubiquitous in bacteria and their genes have also been identified in α-proteobacterial genomes.[73-76] Random mutagenesis led to the identification of the *B. japonicum* tmRNA encoded by *sra* (for symbiotic ribonucleic acid). A *B. japonicum sra* mutant was deficient in nodulation and nitrogen fixation.[77] In silico searches confirmed the prevalence of the tmRNA also in α-proteobacteria.[76, 78, 79] In *S. meliloti* this RNA was identified in genome-wide profiling and Northern analysis. It accumulated under stress conditions.[6] Two stable 214 nt and 82 nt RNAs derived from an unstable precursor were reported.[8] These likely correspond to the mRNA and tRNA domains. The plasmid-encoded *incA* antisense RNA mediates incompatibility within the large *repABC* family of α-proteobacterial plasmids.[80] A vast number of α-rhizobia carries symbiotic plasmids of this family.

Apart from RepE, which regulates Ti plasmid replication, AbcR1 (ABC transporter regulator 1) was the first functionally characterized sRNA in *A. tumefaciens*.[29,81] At least three target mRNAs of AbcR1, all encoding proteins related to ABC transport systems, were identified by one dimensional protein gels and computer predictions.[29] Expression of these three targets is downregulated by AbcR1. Among them is *atu2422* encoding the binding protein for GABA (γ-amino butyric acid), a plant-derived defense molecule that interferes with quorum sensing in *Agrobacterium*.[82,83] AbcR1 inhibits initiation of *atu2422* translation through direct RNA-RNA interaction masking the ribosome binding site and is the first described bacterial sRNA that controls uptake of a plant-generated signaling molecule.[29] Most recent data suggests that AbcR1 is a central regulator of nutrient uptake systems in *A. tumefaciens*.[84] The sRNA has numerous targets, most of them coding for ABC transporter components. Two single-stranded regions of AbcR1 expose conserved single-stranded anti-SD sequences (module M1 and M2, **Figure 4A**) and are able to basepair with target mRNAs either in the translation initiation region or up to 500 nucleotides downstream into the coding region.[84]

AbcR1 is encoded in an intergenic region in tandem with the related sRNA AbcR2.[29] Their transcripts are maximally expressed in the late exponential phase. To date, there is no evidence for AbcR2-mediated gene regulation in *A. tumefaciens*. Homologs of AbcR1 and AbcR2 have been also reported in the closely related plant symbiont *S. meliloti*, in other α-rhizobia and in more distantly related α-proteobacteria.[5-7,31,41] In contrast to *A. tumefaciens*, AbcR1 and AbcR2 are differently expressed in *S. meliloti*, suggesting that they operate at diverse conditions.[5,31] One protein of an ABC transporter, the amino acid binding protein LivK, was overrepresented in the periplasmic proteome of an *S. meliloti* AbcR1 mutant. As in *A. tumefaciens*, AbcR2 does not play a role in *livK* regulation and the role of this sRNA remains to be elucidated.[40] This is remarkably different in *B. abortus*, where both AbcR1 and AbcR2 seem to have a regulatory and somewhat redundant function.[41] Quantitative proteomics and microarray analysis revealed elevated levels of multiple proteins and transcripts related to ABC transport systems in the double mutant. At least three of these transcripts were shown to be controlled by AbcR1 or AbcR2 alone.

The rhizobial AbcR1 was shown to belong to the large group of Hfq-associated sRNAs and a putative binding sequence for Hfq was predicted (**Fig. 4A**).[28,31,41,57,62] Both AbcR1 and AbcR2 were enriched in Hfq-CoIP-RNA libraries.[28] Moreover, the *livK* mRNA (*SMc01946*) was also among the Hfq-bound transcripts identified in this study. A double-plasmid reporter system assay showed downregulation of *SMa0495* and *prbA* (*SMc01642*) by both AbcR1 and AbcR2.[28,40] *SMa0495* and *pbrA* encode periplasmic solute binding proteins, the latter probably mediating uptake of proline betaine both at high and low osmolarities. Putative involvement of Hfq in this regulation was further supported by enrichment of the *SMa0495* and *prbA* mRNAs in the Hfq-CoIP-RNA libraries derived from exponentially growing and salt-shocked cultures.[28]

Quantitative proteomics of the Hfq-regulon in *A. tumefaciens* and *S. meliloti* or Hfq-coimmunoprecipitations in *R. sphaeroides* supported the notion that transport processes (like ABC transport) in α-proteobacteria are regulated by AbcR1 and other sRNAs as has been reported for many other bacteria.[42,57,63]

Tools for In Vivo Analysis of sRNA-mRNA Interactions in *Rhizobiaceae*

Computational and experimental screens have uncovered a staggering number of *trans*-sRNAs in plant-associated α-proteobacteria that demand functional characterization. Identification of the mRNA targets is crucial to fully understand the biological function of a given sRNA. Biocomputational algorithms can help to in silico predict conserved sRNA-target recognition at the genomic scale, but sRNA-mRNAs interactions require in vivo validation.[85] Similar to the reporter system developed for enterobacteria, a double-plasmid reporter assay has been implemented in *S. meliloti*.[40,86] Basically, the sRNA and the translational fusion of a candidate target mRNA to a reporter gene are co-expressed from constitutive promoters to primarily assay post-transcriptional regulation. This system has confirmed the regulation of several targets genes encoding ABC transporters by the AbcR1/2 sRNAs.[28,40] *S. meliloti* cells were co-transformed with a low-copy reporter plasmid (pR_egfp) and a mid-copy plasmid constitutively expressing the sRNA from a modified P_{lac} promoter (pSRK_C). The translational fusion of the mRNA region of interest to *egfp* was expressed under the control of a constitutive promoter to specifically detect posttranscriptional effects rather than transcriptional regulation.

As shown by Torres-Quesada et al.[40] sRNA expression from the modified P_{lac} promoter allowed for target identification. However, constitutive overexpression of some sRNAs may have indirect pleiotropic, toxic or lethal effects. IPTG-inducible sRNA overexpression driven by the native P_{lac} promoter would partially overcome these limitations. However, it does not ensure sRNA yields that are high enough to test weak sRNA-mRNA regulatory pairs in most α-proteobacteria since regulatory effects can be masked by the contribution of native transcription from the target gene. Therefore, we have modified an IPTG-inducible system that relies on the *S. meliloti sinR-sinI* quorum sensing genes (M. McIntosh, personal communication) to drive strong pulse overexpression of a desired sRNA. The *sinI* gene encoding an N-acyl homoserine lactone synthase is the target gene of the LuxR-type transcriptional regulators SinR and ExpR.[87] This inducible expression construct for sRNA genes takes advantage of the strong activation of the P_{sinI} promoter upon moderate induction of *sinR* expression even in the absence of ExpR (**Fig. 5**).[86,88] *S. meliloti sinR*$^-$ *sinI*$^-$ *expR*$^-$ cells harboring the target reporter fusion of interest were transformed with plasmids carrying this construct for pulse overexpression of *S. meliloti* sRNA genes. GFP fluorescence of the resulting transformants confirmed the expected effects of the sRNAs on the expression of their predicted mRNA targets in *S. meliloti*. Furthermore, this system was also proven to be suitable for induced sRNA overexpression in other members of the *Rhizobiaceae*, such as *S. medicae*, *S. fredii*, *R. tropicii, R. radiobacter, R. etli*, and *A. tumefaciens* (M. McIntosh, M. Robledo, personal commmunication).

RNA Degradation

The understanding of the mechanisms for processing and degradation of RNA (ribolysis) is a prerequisite for the understanding of RNA-based gene regulation for the following reasons: 1) Abundance and availability of an sRNA is important for its impact in the cell.[89,90] 2) The abundance (steady-state amount) of an sRNA is determined by the rate of decay in addition to the rate of transcription, and the rate of decay is a subject of regulation.[31,91,92] 3) Some sRNAs are generated by processing of primary transcripts.[93,94] 4) Often the interaction between sRNAs and their mRNA targets regulates the stability of the target and in many cases both molecules are degraded simultaneously.[20,95,96] Generally two different mechanisms participate in this degradation. On the one hand, binding of an sRNA to the target RNA may result in a cleavage site specifically recognized by the double-strand specific ribonuclease (RNase) III.[97,98] On the other hand, the imperfect basepairing of sRNAs and mRNAs is often mediated by Hfq, which directly binds to RNase E, a single strand specific RNase with major importance for decay of RNA in many bacteria. In this way RNase E is guided by an sRNA and Hfq to its cleavage site on an mRNA.[20] The guiding sRNA carries a monophosphate at the 5′-end and can be rapidly degraded by RNase E and other RNases which prefer processed RNAs.[99-101] On the other hand, Hfq stabilizes the sRNAs which do not interact with the target protecting them from RNase E cleavage.[31,102]

The impact of RNA processing and degradation on sRNA-mediated gene regulation is best understood in *Enterobacteriaceae*.[20,95,99,103] For most bacteria with genome-wide mapped sRNAs this information is still missing. In the case of plant-associated α-proteobacteria, some progress was made for the sRNA AbcR1 (originally named SmrC16), the levels of which are decreased in the stationary phase and in the absence of Hfq in *S. meliloti*.[5,31,40] In both cases increased degradation contributes to the decrease in the amount of the sRNA. In the wild type *S. meliloti*, the half-life of AbcR1 was 2.5-fold shorter during transition to the stationary phase when compared with the exponential growth phase, showing regulation of the AbcR1 expression at the posttranscriptional level.[31] In a Δ*hfq* mutant, the half-life was decreased 2-fold during logarithmic growth and 6.5-fold during transition to the stationary phase when compared with the wild type.[31] This suggests a similar role for *S. meliloti* Hfq in riboregulation like in *E. coli*.[21] Most probably RNase E is responsible for the posttranscriptional regulation of AbcR1 expression, since the amounts and the stability of AbcR1 are strongly increased in an RNase E mutant of *S. meliloti* (E. Evguenieva-Hackenberg and A. Becker, unpublished). Probably RNase E and Hfq physically interact in α-proteobacteria like in *E. coli*. Their association was documented in *Rhizobium leguminosarum,* where Hfq is necessary for the RNase E-dependent activation of the translation of NifA.[59]

The set of predicted degradative RNases in plant-associated α-proteobacteria includes the above mentioned endoribonucleases RNase E and RNase III, the 3′-5′ exoribonucleases RNase R and polynucleotide phosphorylase (PNPase), and RNase J, which is not present in *E. coli* but is an endo- and 5′-3′ exoribonuclease in *B. subtilis*.[14-19,104,105] The analysis of mini-Tn*5* mutants with insertions in the genes encoding RNase E and RNase J enabled first insights into mechanisms of RNA processing and degradation in *S. meliloti*. Both mutants showed specific and overlapping effects in a microarray analysis which suggested a role for both RNases in quorum sensing (E. Evguenieva-Hackenberg and A. Becker, unpublished).[106] RNase E was found to be necessary for the 5′-degradation of *sinI* mRNA encoding an autoinducer synthase. It specifically cleaves in the 5′-UTR of this mRNA between the Shine-Dalgarno sequence and the start codon in an Hfq-independent manner.[107] The role of RNase J in riboregulation is still not clear but it is responsible for the maturation of 5′-ends of rRNA in *S. meliloti*.[108]

Conclusions

It is now well established that sRNAs are very abundant in plant-associated bacteria. Antisense transcripts and *trans*-sRNAs constitute the largest class of riboregulators in these bacteria. The functional role of only a small number of these riboregulators has been deciphered. Nonetheless, it is already emerging that some of them are global regulators with an important impact on bacterial physiology. Identifying the target genes of other sRNAs by bioinformatic and experimental methods and revealing the mechanistic principles of RNA-mediated regulation in these bacteria is going to be an interesting task for future research. Experimental tools tailored to plant-associated α-proteobacteria are being developed and will significantly promote functional analysis of sRNAs in these bacteria. Studies of the role of the ubiquitous RNA chaperone Hfq in several *Rhizobiaceae* suggested a broad impact of this protein on riboregulation, but also provided evidence for trans-RNAs that may not require Hfq for their function. This implies Hfq-independent regulatory mechanisms or the presence of additional rhizobial RNA chaperones involved in riboregulation that still await to be studied. A particularly fascinating endeavor will be to study the role of sRNAs in plant-microbe interaction, not only from the bacterial site but also from the plant perspective. It is conceivable that the plant response to contact with a bacterial symbiont or pathogen involves microRNAs or other regulatory RNAs in the plant. A promising technique to look into this on a global scale would be dual RNA-seq of microbe and host.[109]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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