Small Noncoding RNAs in *Agrobacterium tumefaciens*

Keunsub Lee and Kan Wang

Contents

Abstract During the last decade, small noncoding RNAs (ncRNAs) have emerged as essential post-transcriptional regulators in bacteria. Nearly all important physiological and stress responses are modulated by ncRNA regulators, such as riboswitches, *trans*-acting small RNAs (sRNAs), and *cis*-antisense RNAs. Recently, three RNA-seq studies identified a total of 1534 candidate ncRNAs from *Agrobacterium tumefaciens*, a pathogen and biotechnology tool for plants. Only a few ncRNAs have been functionally characterized in *A. tumefaciens*, and some of them appear to be involved in virulence. AbcR1 regulates multiple ABC transporters and modulates uptake of a quorum-sensing inhibitor produced by plants. RNA1111, a Ti plasmid-encoded sRNA, might regulate the dispersal of the Ti

K. Lee · K. Wang (✉)
Crop Bioengineering Center, Iowa State University,
Ames, IA, USA
e-mail: kanwang@iastate.edu

K. Lee · K. Wang
Department of Agronomy, Iowa State University, Ames, IA, USA

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plasmid and virulence. In addition, a chromosomally encoded sRNA Atr35C is induced by the vir gene regulator VirG and its expression is affected by iron, manganese, and hydrogen peroxide, suggesting a possible role in oxidative stress responses and *Agrobacterium*–plant interactions. Progress in ncRNA functional analysis is slow, likely resulting from innate challenges, such as poor sequence conservation and imperfect base-pairing between sRNAs and mRNAs, which make computational target predictions inefficient. Advances in single-cell-based RNA-seq and proteomics approaches would provide valuable tools to reveal regulatory networks involving ncRNA regulators.

1 Introduction

1.1 Discovery of ncRNAs

Regulatory noncoding RNAs (ncRNAs) have emerged as important regulators of physiological responses in bacteria to survive in ever-changing environments. RNA-mediated response regulation is more advantageous for bacteria than is regulation by proteins (e.g., transcription factors) because it requires less time and energy for synthesis (transcription only vs. transcription and translation) and the responses can be rapidly reversed when needed thanks to a short ncRNA turnover time. Numerous RNA molecules have been discovered that modulate most biological processes and stress responses via various mechanisms. The first studied bacterial small ncRNAs were exosome-encoded antisense RNAs that block plasmid replication (Stougaard et al. 1981; Tomizawa et al. 1981) and inhibit transposon movement (Simons and Kleckner 1983). Although these findings precede the discovery of microRNAs (miRNAs) and small interfering RNAs (siRNAs), the importance of bacterial ncRNAs as regulators had not been much appreciated until the early 2000s when genome-wide identification of chromosomally encoded ncRNAs from *E. coli* and other bacteria were reported (reviewed in Livny and Waldor 2007). Since then, tens to hundreds of candidate ncRNAs have been identified from diverse bacterial species including plant-associated bacteria (reviewed in Becker et al. 2014; Harfouche et al. 2015).

1.2 Classification and Mode of Action

Ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) are well-characterized ncRNAs regulating protein translation but they are not discussed in this review. We will focus on riboswitches, *trans*-acting small RNAs (sRNAs), and *cis*-antisense RNAs (asRNAs) in this chapter (Waters and Storz 2009). Each group of RNAs uses a variety of mechanisms to modulate physiological and stress responses. Below, we review how these ncRNAs exert regulatory effects in the model bacterial species.

1.2.1 Riboswitches

Riboswitches are part of untranslated regions (UTRs) of mRNAs and affect cognate gene expression at the transcriptional or post-transcriptional levels. Riboswitches are evolutionarily conserved among distantly related bacteria and their functional counterparts are also found in archaea, plants, and fungi. Bacterial riboswitches were first discovered in 2002 as sensors of intracellular small molecules (Mironov et al. 2002; Nahvi et al. 2002; Winkler et al. 2002). Three independent studies reported that part of the mRNA binds to vitamin B derivatives and affects downstream gene expression via transcription attenuation or translation inhibition. Since then, many different riboswitches have been identified and functionally characterized in various bacteria (Winkler and Breaker 2005; Serganov and Nudler 2013). Most riboswitches have two distinct parts: the ligand-binding aptamer domain and the expression platform domain. The aptamer is the sensor region which binds to a specific ligand or metabolite, and the expression platform domain is the response region which adopts alternative structures to affect gene expression. Most well-characterized riboswitches are metabolite sensors and are located in the 5′ UTR of mRNAs encoding enzymes responsible for the biosynthesis the metabolites. Under normal conditions, the ribosome binding site (RBS) is open and accessible to the translation machinery, resulting in the production of functional proteins. Under high metabolite conditions, however, a metabolite binds to the aptamer domain, leading to a conformational change. This change can result in transcriptional attenuation by forming a terminator or translational inhibition by masking the RBS. This type of negative feedback loop prevents overproduction of a specific metabolite, ensuring balanced resource utilization.

Riboswitches are highly selective and many different types of riboswitches have been discovered (reviewed in Serganov and Nudler 2013). A wide range of ligands can be sensed by riboswitches: fluoride anions, metals, purines and their derivatives, cofactors, and amino acids. Recent studies showed that these *cis*-acting regulatory elements can also affect ncRNA expression and modulate RNA–protein interactions (reviewed in Mellin and Cossart 2015). In addition, some riboswitches can act as catalytic enzymes (Tinsley et al. 2007) or as *trans*-acting sRNAs (Loh et al. 2009), suggesting that bacteria have unexpectedly complex regulatory networks involving different classes of ncRNAs.

1.2.2 Trans-encoded sRNAs

sRNAs are 50–500 nt in size and are often encoded in intergenic regions. This group of ncRNAs represents the most well-known RNA regulators and are involved in many physiological and stress responses (Waters and Storz 2009; Gottesman and Storz 2011). sRNAs appear to evolve rapidly, because primary sequence conservation is very limited among closely related bacterial species (Gottesman and Storz 2011). Many sRNAs modulate gene expression via imperfect base-pairing with their target mRNAs, which are transcribed from distinct genomic locations (Waters

and Storz 2009). Another feature of sRNAs is their requirement for the global RNA chaperone Hfq for optimal sRNA–target interactions (De Lay et al. 2013). Hfq-binding affects RNA secondary structure and might facilitate sRNA–mRNA interactions, presumably by binding to both molecules (Gottesman and Storz 2011). Interestingly, however, Hfq is not required for sRNA functions in some bacteria, such as *Staphylococcus aureus* and *Bacillus subtilis*. A recent study by Smirnov et al. (2016) discovered another major sRNA-binding protein, ProQ, which forms stable complexes with small RNAs. It is possible that evolutionarily distant bacteria might have adopted different RNA-binding proteins to facilitate sRNA–mRNA interactions. The exact mechanisms of how sRNAs select and interact with their target mRNAs are still largely unknown.

Another group of sRNAs modulate RNA-binding proteins by sequestering them or directly affecting enzymatic activity (reviewed in Storz et al. 2011). For example, 6S RNA sequesters the house-keeping RNA polymerase (Wassarman and Storz 2000) and the CsrB family sRNAs negatively regulate the activity of CsrA (carbon storage regulator), the regulator of secondary metabolism, by sequestering multiple subunits (Romeo 1998). Many protein-binding sRNAs contain multiple protein-binding sequences, and direct competition by mimicry is the underlying mechanism of these sRNAs (Storz et al. 2011). The presence of multiple protein-binding sRNAs and RNA-binding proteins suggests that bacteria utilize complex sRNA-protein pairs to fine-tune the regulatory networks.

1.2.3 Cis-antisense RNAs

asRNAs are transcribed from the complementary strand of a target gene, and thus exert effects via base-pairing with perfect complementarity (Waters and Storz 2009). The most well-studied asRNAs are encoded on mobile elements, such as plasmids, transposons, and bacteriophages, and maintain proper copy numbers via various mechanisms (Waters and Storz 2009). Common mechanisms are to inhibit plasmid replication by blocking replication primer formation and to inhibit the translation of transposases and protein toxins encoded by these mobile elements (Brantl 2007; Wagner and Simons 1994).

There are an increasing number of asRNAs discovered from diverse bacteria (Georg and Hess 2011; Thomason et al. 2015). However, it is not clear how many of these are actually regulatory asRNAs. Because a low level of pervasive transcription occurs throughout the entire genome (reviewed in Wade and Grainger 2014; Lloréns-Rico et al. 2016), systematic approaches need to be developed to distinguish regulatory asRNAs from these pervasive antisense transcripts. Adding another level of complexity, recent studies discovered that genes and operons encoding proteins performing opposing functions can modulate the expression of genes encoded on the opposite strand (reviewed in Sesto et al. 2013). The total number of asRNAs inventories in bacterial genomes will likely increase in the near future. However, it remains challenging to study how these asRNAs exert regulatory effects, if any, to counter ever-changing environmental stresses.

1.3 Genome-Wide Identification of ncRNAs

Early genome-wide ncRNA identification studies utilized whole genome sequencing data and searched for conserved bacterial promoter and *rho*-independent terminator sequences in the conserved intergenic regions of *E. coli* (reviewed in Livny and Waldor 2007; Livny et al. 2008). The predicted ncRNAs were experimentally validated to prove the effectiveness of the computational predictions. However, the bias toward intergenic regions and low throughput validation procedures limited the thorough discovery of inventories of regulatory RNAs in a genome. High-resolution tiling arrays were successfully used to identify 20–50 sRNAs (Landt et al. 2008; Toledo-Arana et al. 2009) before whole transcriptome sequencing (RNA-seq) became a powerful tool to discover many hundreds of candidate ncRNAs from various bacterial species (Sharma and Vogel 2009). In this chapter, we review recent advances in regulatory ncRNA research in the "natural genetic engineer" *A. tumefaciens* C58 and discuss current challenges and remaining questions.

2 Identification of Small ncRNAs in *Agrobacterium*

Three RNA-seq studies have identified numerous small ncRNAs from *A. tumefaciens* strain C58 thus far (Table 1; Wilms et al. 2012a; Lee et al. 2013; Dequivre et al. 2015). Each study employed some unique approaches, and therefore provided nonredundant identification of novel candidate ncRNAs.

The first genome-wide identification study was done by Wilms et al. (2012a), who used a Roche FLX platform and identified 228 novel ncRNAs. Briefly, total RNA was extracted from *A. tumefaciens* C58 cultures grown under two different conditions: AB minimal medium in the presence (+Vir) or absence (−Vir) of the *vir* gene inducer acetosyringone (AS). To identify transcription start sites (TSS), each total RNA sample was treated or not treated with the Terminator™ 5′-Phosphate-Dependent Exonuclease (TEX), which selectively degrades transcripts containing a 5′-mono-phosphate, to enrich primary transcripts which contain a 5′-tri-phosphate. Four cDNA libraries were constructed and sequenced using a Roche FLX sequencer. The resulting 422,204 cDNA sequences were compared to the C58 reference genome and 348,998 sequences longer than 18 nt were mapped. Sequences mapping to intergenic regions or complementary to protein-coding genes were manually analyzed for ncRNA discovery. Putative ncRNAs were identified if there were a minimum of five cDNA reads in at least one of the four cDNA libraries. A total of 228 candidate ncRNAs were identified from all four replicons: 129 on the circular chromosome, 59 on the linear chromosome, 20 on the pAt plasmid, and 20 on the Ti plasmid. The list also included widely conserved ncRNAs, such as 6S RNA, SRP RNA 4.5S, RNase P, and tmRNA, as well as the previously published *Agrobacterium* ncRNAs RepE, AbcR1, and AbcR2.

Table 1 Comparison of three *Agrobacterium* RNA-seq studies

RNA-seq studies	Wilms et al. (2012a)	Lee et al. (2013)	Dequivre et al. (2015)
Agrobacterium strain	*Agrobacterium tumefaciens* C58	*Agrobacterium tumefaciens* C58	*Agrobacterium tumefaciens* C58
Growth conditions	AB minimal medium with or without AS	YEP complex medium (log and stationary phases); AB minimal medium with or without AS	YPG rich medium (log and stationary phases); AB minimal medium (log and stationary phases)
Total RNA extraction method	Hot acid phenol method (Aiba et al. 1981)	RNeasy Protect Bacteria mini kit	Frozen acid phenol method (Maes and Messens 1992)
Sample treatment	Terminator™ 5′-phosphate-dependent exonuclease	Terminator™ 5′-phosphate-dependent exonuclease; MICROBExpress™ kit	Size fractionation (25–500 nt); First Strand cDNA synthesis kit: RNaseH and DNase I treatment
NGS platform	Roche FLX	Illumina GAII (PE: 2x 50 bp)	Illumina GAII (1x 36 bp)
Total UMR (×1000)	308	48,302	28,386
Identified ncRNAs	228	475	1108
Experimentally validated ncRNAs	22	36	14
Validation methods	RNA gel blot hybridization	RNA gel blot hybridization and RACE	RACE

Twenty-two ncRNAs were validated by RNA-blot hybridization: 10 from the circular chromosome, six from the linear chromosome, two from the pAt plasmid, and four from the pTi plasmid. Among these, 152 were intergenic sRNAs, whereas 76 were antisense to known protein-coding genes (asRNAs). Several independently validated ncRNAs were differentially expressed under varying growth conditions such as medium, temperature, and pH. One ncRNA encoded by the Ti plasmid, Ti2, was highly induced under Vir gene induction conditions, and its expression was diminished in *virA* and *virG* deletion mutants.

Lee et al. (2013) identified 475 highly expressed candidate ncRNAs under four different growth conditions: YEP logarithmic and stationary phases and AB minimal medium in the presence or absence of AS. Because ribosomal RNAs represent the vast majority of the total cellular RNA (He et al. 2010), two commercial kits were used to deplete rRNAs and tRNAs, the TEX and MICROBExpress™ kits. All four total RNA samples were treated with reagents in the MICROBExpress™ kit, which removed ∼55% of the 16S and 23S rRNAs, followed by TEX treatment

(±TEX). RNA samples were fragmented to ~200–300 nt before cDNA library construction. A total of eight cDNA libraries (four growth conditions; ±TEX) were sequenced using the Illumina GAII platform. A total of 842 million 50-bp reads were obtained and 48.3 million reads were mapped exactly once to the reference genome (Uniquely Mapped Reads, UMRs). These UMRs were used for data analysis. The use of TEX treatment substantially improved the UMR ratio from 7.5 (−TEX) to 12.5% (+TEX), indicating RNA-seq sensitivity was considerably enhanced. The highly expressed ncRNA identification procedure began with calculating the depth of coverage data for each individual nucleotide position on both forward/reverse strands of all four replicons. Candidate ncRNAs were identified in the intergenic regions or complementary sequences of protein-coding genes, where the average depth of coverage of a candidate ncRNA region was at least 10 times greater than those of immediate upstream and downstream regions. A total of 101 sRNAs and 310 asRNAs were identified, as well as 20 5′ UTR leader sequences. Thirty-six ncRNAs were experimentally validated by RNA-blot hybridization and RACE (Rapid Amplification of cDNA Ends)-PCR (Gerhart et al. 2014). Twenty-two ncRNAs were differentially expressed by Vir gene induction: 15 were up-regulated and seven were down-regulated. Fourteen of the 15 AS-induced ncRNAs contain a putative *vir* box, a conserved motif for VirG binding, in the promoter regions. In addition to the identified ncRNAs, a stranded RNA-seq approach also revealed interesting features of *Agrobacterium* transcripts: (i) highly expressed antisense transcripts from the complementary strands of important *vir* genes and operons and (ii) novel transcripts within the known protein-coding genes (e.g., virD4* internal transcript; Lee et al. 2013). It is likely that many putative asRNAs might have been ignored due to the high stringency of the informatics cutoff, i.e., a minimum of 10 times higher expression level compared to adjacent regions, whereas internal transcripts had not been considered for ncRNA identification.

Most recently, Dequivre et al. (2015) conducted another RNA-seq study using size-fractionated RNA samples (25–500 nt). *A. tumefaciens* strain C58 was grown under four different growth conditions: logarithmic/stationary phases in YPG rich medium and in AB minimal medium. tRNAs were depleted using a First Strand cDNA synthesis kit. tRNA-specific primers were used to synthesize the first strand cDNAs, and RNaseH was used to degrade tRNAs in the RNA–DNA duplex, followed by DNase I treatment. A total of 193.1 million reads were obtained and 28.4 million reads were mapped once to the reference genome (UMRs). Genomic regions whose average depth was at least 10 times greater than the adjacent regions were considered transcribed, and candidate ncRNAs were identified only when a transcript was presented in all four libraries. A total of 1108 candidate ncRNAs were evenly distributed among all four replicons: 602 on the circular chromosome, 291 on the linear chromosome, 140 on the pAt plasmid, and 75 on the Ti plasmid. Four hundred and seven were intergenic sRNAs and 262 were asRNAs. Additionally, 402 and 37 were derived from 5′ and 3′ UTRs, respectively. Seventeen candidate ncRNAs were independently validated by RACE-PCR. An intergenic sRNA encoded by the Ti plasmid, RNA1111, was conserved among

Table 2 Summary of the three RNA-seq identifications of ncRNAs in *A. tumefaciens* C58

Dataset	Number of ncRNAs exclusively identified by the combined dataset		
	Wilms et al. (2012a)	Lee et al. (2013)	Dequivre et al. (2015)
Wilms et al. (2012a)	98	28[a]	48
Lee et al. (2013)	28[a]	300	94
Dequivre et al. (2015)	48	94	912
All three datasets[b]	54	54	54
Total	228	475	1108

[a]A sRNA identified by Lee et al. (2013) corresponds to two tandemly encoded sRNAs identified by Wilms et al. (2012a)

[b]Fifty four ncRNAs (3.5%) were identified by all three studies, while 170 (11.1%) and 1310 (85.4%) were identified by two and one studies, respectively

other Ti plasmids, and the deletion mutant exhibited reduced tumorigenicity in tomato, suggesting the involvement of this sRNA in bacterial virulence.

These three genome-wide RNA-seq studies identified a total of 1534 candidate ncRNAs from *A. tumefaciens* C58. As summarized in Table 2, 54 (3.5%) candidate ncRNAs were identified by all three studies, whereas 170 (11.1%) and 1310 (85.4%) ncRNAs were identified by two and one studies, respectively. The numbers presented in Table 2 are slightly different from those presented in the Venn Diagram of Dequivre et al. (2015) due to two small differences. First, one sRNA identified by Lee et al. (2013) corresponds to two tandemly encoded sRNAs identified by Wilms et al. (2012a); thus the total number of ncRNAs identified by Wilms et al. (2012a) was 228, not 227. The second was a simple calculation error, as the total number of ncRNAs is 1534 (=98 + 300 + 912 + 28 + 48 + 94 + 54; Table 2) not 1560 (Dequivre et al. 2015). As each RNA-seq investigation employed unique approaches, the collective efforts led to a thorough inventory of *Agrobacterium* ncRNAs. Functional analyses of these ncRNAs, however, have not been comprehensive; only several conserved ncRNAs have been characterized in *Agrobacterium* and other closely related species.

3 Functions of *Agrobacterium* ncRNAs

Although over a thousand candidate ncRNAs have been discovered from *A. tumefaciens*, the regulatory functions of all but a few remain unknown. Only a handful of ncRNAs have been functionally characterized thus far: *repE* (Chai and Winans 2005), *AbcR1* (Wilms et al. 2011), a TPP riboswitch (Lee et al. 2013), and *RNA1111* (Dequivre et al. 2015). Here we describe how these ncRNAs have been discovered and how they exert regulatory functions via various mechanisms. We also report how a chromosomally encoded and AS-induced sRNA, Atr35C (Lee et al. 2013), is expressed under iron deficiency and oxidative stress conditions.

3.1 Thi-Box Riboswitch

Thiamine, also known as vitamin B1, is an essential coenzyme for carbohydrate and branched-chain amino acid metabolism in all living cells. Maintaining a proper level of thiamine is critical and a highly conserved RNA structure called the Thi-box riboswitch or TPP (thiamine pyrophosphate) riboswitch regulates the biosynthesis and transport of thiamine in bacteria, archaea, and eukaryotes (Serganov and Nudler 2013; RF00059 in Rfam database). The Thi-box riboswitch binds to TPP to cause RNA structural changes which can lead to transcriptional attenuation or translational inhibition (Serganov and Nudler 2013). Three TPP riboswitches have been identified in *A. tumefaciens* C58, two on the circular and one on the linear chromosome (Rfam database: http://rfam.xfam.org/search?q=Agrobacterium%20fabrum%20AND%20rna_type:%22riboswitch%22%20and%20TPP%20AND%20alignment_type:%22full%22). All three Thi-box riboswitches are located in the 5′ UTR of operons encoding putative thiamine biosynthesis enzymes or transporters. Lee et al. (2013) demonstrated, using Northern Blot analysis, that a Thi-box riboswitch (C1_2541934R) located in the 5′ UTR of the thiamine biosynthesis operon *thiCOGG* indeed regulates gene expression via transcriptional attenuation. The *thiCOGG* mRNA was detected when *A. tumefaciens* was grown in minimal medium lacking thiamine, but only the ∼110 nt riboswitch accumulated when grown in nutrient-rich medium-containing thiamine, suggesting that the *thiCOGG* promoter has constitutive activity and a transcriptional attenuator is formed to block transcription of the full-length mRNA of the thiamine biosynthesis genes (Lee et al. 2013). Thi-box riboswitch-mediated transcriptional attenuation was also observed in the nitrogen-fixing bacterium *R. etli* (Miranda-Ríos et al. 2001).

In addition to the Thi-box riboswitches, the *A. tumefaciens* C58 genome was predicted to encode six Cobalamin (vitamin B12), two SAM (S-Adenosyl Methionine), one Flavin mononucleotide (FMN; vitamin B2), and one glycine riboswitches (http://rfam.xfam.org/search?q=Agrobacterium%20fabrum%20AND%20rna_type:%22riboswitch%22), but their functional roles have not yet been confirmed.

3.2 RepE

The first characterized sRNA in *Agrobacterium* was *RepE*, a sRNA that regulates the replication of an octopine-type tumor-inducing Ti plasmid (Chai and Winans 2005). *RepE* is encoded in the intergenic region of the *repABC* operon, whose products are responsible for the replication of the Ti plasmid (Chai and Winans 2005). RepABC-type replication is widespread among plasmids found in alpha-proteobacteria, especially in Rhizobiales (Palmer et al. 2000). All known

repABC operons include at least three genes: *repA*, *repB*, and *repC* (reviewed in Cevallos et al. 2008). RepA and RepB are involved in plasmid partitioning and segregation, whereas RepC is responsible for initiation of the DNA synthesis. Chai and Winans (2005) demonstrated that *RepE* is ~54 nt in size and suppresses the replication of a mini-Ti plasmid when expressed in *trans* (Chai and Winans 2005). In addition, mutations introduced at the promoter region resulted in downregulation of *RepE*, which subsequently increased plasmid copy number, further suggesting that *RepE* is a negative regulator of Ti plasmid replication. *RepE* likely form duplexes with *repC* mRNAs resulting in transcriptional attenuation (Brantl et al. 2002). Because the *repE*-encoded intergenic region is highly conserved in other repABC-type plasmids, it is likely that *repE*-mediated transcriptional attenuation is an important mechanism to maintain plasmid copy numbers in Rhizobiales.

3.3 *AbcR1*

The *AbcR1* (ABC regulator) was the first studied chromosomally encoded sRNA in α-proteobacteria (Wilms et al. 2011). *AbcR1* was discovered by a computational search (Wilms et al. 2011) in the conserved intergenic region between *atu2186* and *atu2187*, in tandem with a homologous sRNA *AbcR2*. Both *AbcR1* and *AbcR2* are well conserved in α-proteobacteria and belong to the α*r15* sRNA family (del Val et al. 2012). *AbcR1*/*AbcR2* orthologues have been identified in other α-proteobacteria: *Sinorhizobium meliloti* (*SmrC15*/*SmrC16*; del Val et al. 2007), *Rhizobium etli* (*ReC58*/*ReC59*; Vercruysse et al. 2010), and *Brucella abortus* 2308 (*AbcR1*/*AbcR2*; Caswell et al. 2012). Hfq is likely required for *AbcR1*-meditated negative regulation of atleast for some target genes because their expression levels were elevated in both *hfq* and *abcR1* knockout mutants (Wilms et al. 2012b).

AbcR1 regulons have been identified by one- and two-dimensional PAGE analysis (Wilms et al. 2011; Overlöper et al. 2014) or computational predictions using the CopraRNA algorithm (Wright et al. 2013). *AbcR1* regulates at least 16 mRNAs including several periplasmic substrate-binding proteins required for sugar and amino acid ABC transporters (Wilms et al. 2011, 2012b; Overlöper et al. 2014): AtpH, AttC, Atu0857, Atu1879, Atu2422, Atu3114, Atu4046, Atu4259, Atu4431, Atu4577, Atu4678, ChvE, DppA, FrcB, and NocT. Among these, several target genes are involved in *A. tumefaciens* virulence. Atu2422 encodes a periplasmic protein which is responsible for uptake of the plant defense molecule γ-amino butyric acid (GABA) (Chevrot et al. 2006). GABA can suppress the quorum-sensing signal within *A. tumefaciens*, thus attenuating bacterial virulence (Chevrot et al. 2006). ChvE is a sugar-binding protein that senses host-released sugars and directly interacts with the VirA/VirG two-component system to induce *vir* gene expression (He et al. 2009; Hu et al. 2013). AttC and NocT are responsible for the uptake of spermidine/putrescine and nopaline, respectively (Matthysse et al. 1996).

AbcR1 possesses two separate target-binding regions (Overlöper et al. 2014) and each region binds to a set of target mRNAs either near the ribosomal binding site (RBS) to block translation and accelerate target mRNA turnover, or the coding DNA sequence (CDS) to cause transcriptional attenuation (Wilms et al. 2011, 2012b; Overlöper et al. 2014). Further studies may greatly expand the AbcR1 regulon because a large number of proteins differentially expressed by an *avcR1* deletion have yet to be validated (Overlöper et al. 2014).

Recent studies showed that regulation by *AbcR1*/*AbcR2* orthologues has become diversified in α-proteobacteria. In *A. tumefaciens*, *AbcR1*/*AbcR2* have near identical promoter sequences and are highly expressed in late stationary phase, but only *AbcR1* has regulatory functions (Wilms et al. 2011). Conversely, *AbcR1*/*AbcR2* orthologues in the human pathogen *B. abortus* 2308 have some redundant functions, because only the *abcR1*/*abcR2* double knockout mutant exhibited reduced survival in cultured murine macrophages (Caswell et al. 2012). *B. abortus AbcR1*/*AbcR2* have multiple unique and shared target genes (Caswell et al. 2012). In the nitrogen-fixing bacterium *S. meliloti* Rm1021, *AbcR1*/*AbcR2* orthologues (SmrC15/SmrC16) are divergently expressed: *AbcR1* was expressed in actively growing cells but was not detected in stationary phase, whereas *AbcR2* was highly expressed in the stationary phase and under various stress conditions (Torres-Quesada et al. 2014). Together, these data suggest that *AbcR1*/*AbcR2* orthologues may have evolved rapidly in α-proteobacteria, but it is not yet known whether AbcR1 regulons in the plant pathogenic *A. tumefaciens* are also evolutionarily conserved in the human pathogen *B. abortus* or the nitrogen-fixing symbionts *S. meliloti* and *R. etli*.

3.4 RNA1111

RNA1111 is a recently identified sRNA from the intergenic region between *atu6186* (*virE3*) and *Atu6188* (*virE0*) on the complementary strand (Dequivre et al. 2015). *RNA1111* is ∼173 nt in length and highly conserved among the nopaline-type Ti plasmids. Although *RNA1111* was located within the *vir* gene region, its expression level was not affected by *vir* gene induction conditions (Dequivre et al. 2015). Interestingly, however, an *rna1111* deletion mutant exhibited reduced virulence on tomato plants: an *rna1111* mutant strain harboring an empty expression vector produced an average of two tumors per plant, whereas the wild-type and *rna1111* mutant harboring the complementation construct produced 20 and 9.5 tumors per plant, respectively. The complementation construct alone does not restore a full level of virulence, presumably because the deleted *rna1111* gene region contains the *vir* box of *virE0*.

Because *RNA1111* may be involved in *A. tumefaciens* virulence, the next step was to identify the regulatory targets of this sRNA. Three sRNA target search programs (RNApredator, sTarPicker, and IntaRNA) were utilized to identify a total of eight putative target genes, which were predicted by all three programs. Six candidate target genes were encoded on the pTiC58 plasmid, including three

virulence-related genes (*6b*, *virC2,* and *virD3*), two conjugal transfer genes (*traA* and *trbD*), and a gene encoding a hypothetical protein (*atu6072*). Interestingly, Möller et al. (2014) found that *virC2*, *virD3,* and *traA* mRNAs were enriched by Hfq tagged by 3xFlag. Further studies are needed to determine if *RNA1111* interacts with Hfq to regulate its putative target genes.

Quantitative reverse transcription PCR (qRT-PCR) analyses showed that *trbD* RNA was not detectable, and *virC2*/*virD3* did not show altered expression in the *rna1111* mutant compared to the wild-type strain. Three genes, *6b*, *traA*, and *atu6072*, however, exhibited significantly lower expression levels in the *rna1111* mutant. Importantly, *6b*, *traA*, and *atu6072* expression levels were not different in the *rna1111* mutant strain harboring the complementation construct from those in the wild-type strain, further suggesting that *RNA1111* might stabilize these target mRNAs or protect them from degradation. Together, these results suggest that *RNA1111* might regulate genes involved in *A. tumefaciens*–plant interactions as well as in the dispersal of the Ti plasmid.

3.5 Atr35C

In our previous RNA-seq study, we identified 475 candidate ncRNAs from *A. tumefaciens* C58 (Lee et al. 2013). Fifteen of these were up-regulated by the *vir* gene inducer acetosyringone, and among these was a chromosomally encoded sRNA, C2_132595F (=*Atr35C*), which belongs to the *αr35* sRNA family (http://rfam.xfam.org/family/ar35). *Atr35C* is encoded in the intergenic region between *atu3124* (hypothetical protein) and *atu3126* (hypothetical protein) on the linear chromosome. The first *αr35* RNA family member, *Smr35B*, was identified from the symbiotic bacterium *S. meliloti* 1021 by computational prediction and experimental validation (del Val et al. 2007). A comparative genomics approach suggested that this sRNA family is conserved among certain members of the order Rhizobiales, which include both symbiotic (e.g., *R. etli and R. leguminosarum*) and pathogenic species (e.g., *A. tumefaciens* and *Ochrobactrum anthropi*; del Val et al. 2012).

The expression of *αr35* RNA was first reported in *S. meliloti* 1021 (del Val et al. 2007) and interestingly, it was induced by luteolin, the plant flavone that induces nodulation genes, suggesting a possible role during host–bacterial interactions. Similarly, our previous RNA-seq study found that *Atr35C* is induced by the *vir* gene inducer AS (Lee et al. 2013). qRT-PCR analysis confirmed that *Atr35C* is indeed induced by AS (Fig. 1a). To verify further that *Atr35C* is regulated by VirG, a *virG* mutant was generated as previously described (Lee et al. 2013), and *A. tumefaciens* strains were grown in induction medium (IM) containing (+Vir) or lacking AS (−Vir). qRT-PCR analysis showed that *Atr35C* expression was 21-fold lower in the *virG* mutant than in the wild-type strain in the presence of AS, and *Atr35C* expression was 15-fold higher in the presence of AS in the wild-type strain (Fig. 1a). By comparison, in our previous RNA-seq study, the *Atr35C* level was 6.1-fold higher in the presence of AS in the wild-type strain C58 (Lee et al. 2013,

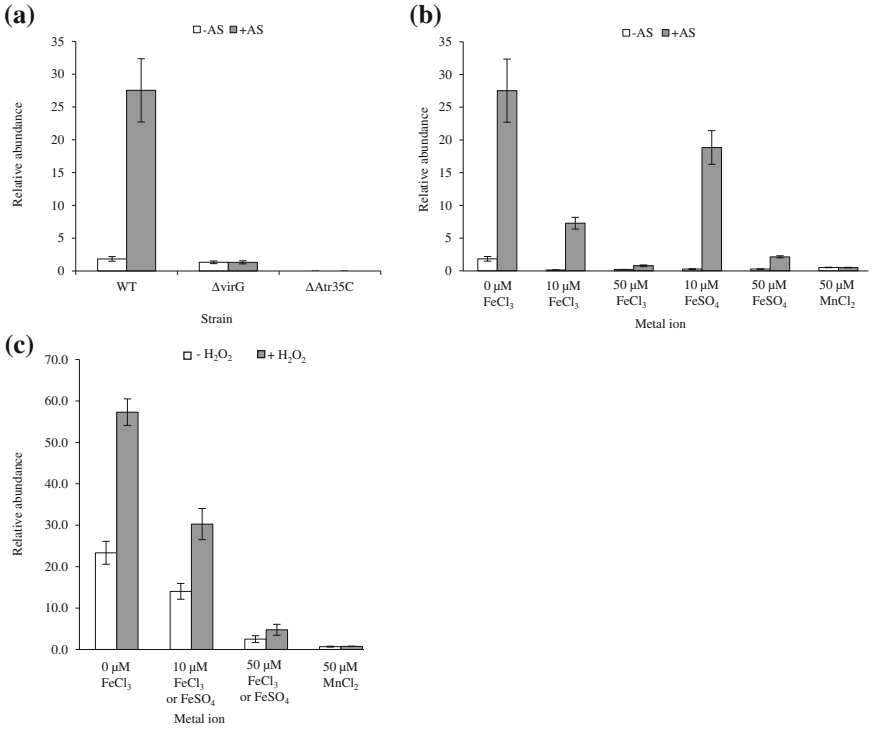

Fig. 1 *Atr35C* transcript levels were estimated by RT-qPCR using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) as described previously (Lee et al. 2013). **a** *Atr35C* transcript level was not induced by AS in the *virG* mutant, suggesting VirG-dependent expression. **b** *Atr35C* expression was negatively correlated with the concentration of iron and manganese ions. **c** *Atr35C* transcript level was further enhanced by 9 mM hydrogen peroxide (H_2O_2). Error bars represent standard errors

Table S4A. C2_132595F). Together, these results strongly suggest that the chromosomally encoded sRNA *Atr35C* is regulated by VirG.

Because *Atr35C* appears to be part of the VirG regulon, we examined if an *atr35C* mutant has altered virulence. However, neither transient GUS expression using an *Arabidopsis* seedling assay (Wu et al. 2014) nor tumorigenicity assay using tobacco leaf disks (Clemente 2006) showed significant differences between the *atr35C* mutant and wild-type C58 strains.

In search of environmental/stress stimuli that trigger *Atr35C* expression, several transition metals were added to the IM, and qRT-PCR assays were used to monitor *Atr35C* transcript levels. When added to a concentration of 100 μM, $FeCl_3$ and $MnCl_2$ greatly reduced *Atr35C* expression levels in the presence of AS (Fig. 1b), whereas $CuSO_4$ and $ZnSO_4$ did not have a significant impact. Because typical IM contains 10 μM $FeSO_4$ (Gelvin 2006), we tested if there were a dosage effect of iron and manganese. Addition of 10 μM ferric ($FeCl_3$) and ferrous ($FeSO_4$) irons to

IM resulted in a mild reduction of *Atr35C* expression levels by about 3.8- (27.5 vs. 7.3) and 1.5-fold (27.5 vs. 18.9), respectively. A higher iron concentration further reduced *Atr35C* transcript levels: addition of 50 μM $FeCl_3$ and $FeSO_4$ reduced *Atr35C* transcript levels by 35.3- (27.5 vs. 0.8) and 12.9-fold (27.5 vs. 2.1), respectively. Thus, *A. tumefaciens* is more responsive to ferric than to ferrous iron (3.8- vs. 1.5-fold changes at 10 μM; 35.3- vs. 12.9-fold change at 50 μM). Addition of 50 μM $MnCl_2$ reduced *Atr35C* transcript levels by 55-fold (27.5 vs. 0.5). These results strongly suggest that *Atr35C* might be involved in iron and manganese homeostasis.

Interestingly, iron and manganese play important roles in oxidative stress responses and virulence in *A. tumefaciens* (Saenkham et al. 2008; Kitphati et al. 2007). We therefore tested if hydrogen peroxide (H_2O_2), a primary defense molecule of plants (Wojtaszek 1997; Dan et al. 2015), affects *Atr35C* expression. Wild-type *A. tumefaciens* C58 was grown in the presence of AS for 24 h and 9 mM H_2O_2 was added to the culture and further incubated for 30 min. *Atr35C* transcript levels increased by ∼twofold in the presence of 9 mM H_2O_2 compared to the control with 0, 10, or 50 μM $FeCl_3$ or $FeSO_4$ (Fig. 1c). However, *Atr35C* transcript levels were not affected by 9 mM H_2O_2 in the presence of 50 μM $MnCl_2$. Taken together, our results suggest that there is cross-talk between the Ti plasmids and the chromosomally encoded sRNA *Atr35C*, which might be involved in oxidative stress responses or iron/manganese homeostasis. Further studies are needed to identify the target genes regulated by *Atr35C* and to elucidate how this sRNA exerts regulatory functions.

4 Challenges

Regulatory ncRNAs are versatile and provide bacteria many adaptive advantages in rapidly changing environments. As mentioned above, however, the biological functions of most ncRNAs remain largely unknown; only a small number of ncRNAs have been functionally characterized in *A. tumefaciens*. This can be attributed to the characteristics of ncRNAs and their interactions with targets: (1) poor sequence conservation in homologous ncRNAs, (2) imperfect complementarity in sRNA–mRNA base-pairing, and (3) quantitative changes in target gene expression.

Bacterial ncRNA homologs have a low level of primary sequence conservation among evolutionarily distantly related species. Consequently, most ncRNA homologs are only found among closely related bacteria. This observation significantly limits data mining, which can provide useful information such as conserved domains, putative functions, and interactions with putative targets and transcription factors. For instance, although 1534 candidate ncRNAs have been discovered in *A. tumefaciens* C58 thus far, only 45 families are found in the Rfam database (http://rfam.xfam.org/search?q=UP000000813%20AND%20entry_type:%22Family%22). Among these are 5S rRNA (RF00001), RNase P RNA (RF00010), SRP RNA

(RF00169), and 6S RNA (RF00013). Other than these broadly conserved ncRNAs, α-proteobacterial ncRNA families, such as αr7 (RF02342), αr9 (RF02343), αr14 (RF02344), αr15 (RF02345), αr35 (RF02346), and αr45 (RF02347) still lack known functions. Extended searches for conserved secondary structures and adjacent protein-coding genes have proven useful to facilitate homologous ncRNA discovery (Barrick et al. 2005), but it still remains challenging to identify functional analogs among distantly related bacteria.

In contrast to eukaryotic miRNAs and siRNAs that base-pair with target mRNAs with near-perfect complementarity (Brodersen et al. 2008), bacterial ncRNAs, especially sRNAs, interact with target mRNAs via base-pairing with a less perfect complementarity and with gaps (Storz et al. 2011). This finding poses a difficult challenge to identify sRNA targets using existing computational algorithms (Pain et al. 2015). Even validated sRNA-mRNA target pairs are not predicted as top candidates (Pain et al. 2015), which strongly suggests that there are unknown crucial factors determining sRNA-mRNA specificities, or that current computational algorithms need further optimization. Many RNA-seq-based approaches have recently been developed to identify sRNA targets (reviewed in Saliba et al. 2017), but these approaches are costly and time consuming for extensive optimization and data analyses. Undoubtedly, additional experimentally validated ncRNA–target interactions will improve ncRNA target prediction algorithms in the future, but it is important to expand the search algorithms to include protein databases because some ncRNAs directly interact with protein targets.

As post-transcriptional regulators, some ncRNAs do not dramatically alter target gene transcript levels, whereas others only affect target mRNA translation without altering mRNA stability (Storz et al. 2011). In addition, as demonstrated by Levine et al. (2007), ncRNA-mediated gene regulation is largely affected by the rate of transcription of the target genes. Therefore, it is crucial to define the conditions under which a specific ncRNA exerts regulatory effects on target gene expression. Moreover, a high level of heterogeneity exists among the individual cells in bacterial colonies (Martins and Locke 2015), but standard procedures measure only the average levels in a population. In this regard, single-cell-based analyses may provide useful platforms to measure precisely the regulatory effects of ncRNAs on target gene expression. Recent advances in single-cell-based RNA-seq and proteomics look promising to provide more accurate genome-wide pictures of complex regulatory networks, including ncRNA regulators (Shapiro et al. 2013; Martins and Locke 2015).

5 Conclusions

RNA-seq approaches allowed identification of 1534 candidate ncRNAs from *A. tumefaciens* C58 (Table 2). This is, however, only the beginning of the regulatory ncRNA era, and a number of questions remain unanswered. For example, how many ncRNAs are true regulators? Do asRNAs represent important regulators or

mere by-products of transcriptional noise? Which ncRNAs, if any, modulate *Agrobacterium*–plant interactions? Although there are many challenges for ncRNA research, accumulating evidence has solidified the importance of ncRNA regulators for many aspects of biological reactions and stress responses. Technical advances, such as single-cell-based RNA-seq and proteomics, will provide new tools to reveal how ncRNAs specify targets, both RNAs and proteins, and how multiple layers of regulatory networks interact harmoniously with one another to maximize bacterial fitness. This in turn offers an excellent opportunity to improve the efficiency and host-range of *A. tumefaciens*-mediated plant genetic transformation.

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