Introduction

Within the last decade it has become increasingly clear that small RNAs (sRNAs) are equally efficient and versatile regulators of gene expression as protein-based transcription factors. Most trans-encoded sRNAs act at the post-transcriptional level by base-pairing to target mRNAs and can have a positive or negative effect on gene expression by affecting translation and/or RNA decay. Small RNAs typically offer only limited complementarity to their targets. A segment of only seven contiguous bases, the so-called seed region, can be sufficient to confer specificity. Therefore, sRNAs are well suited for regulation of multiple mRNAs. Another level of complexity is reached when a single mRNA is subject to regulation by several sRNAs. Overall, this can lead to large sRNA-based regulatory networks that sense and respond to the nutritional status of the cell.

The fundamental importance of sRNAs is reflected by their involvement in numerous cellular processes, like cell division (DicF), transcription (6S RNA), photosynthesis (PcrZ), stress adaption (OxyS), virulence, quorum sensing (Qyr), carbon storage (CarBC), and phosphosugar metabolism. A class of genes frequently controlled by sRNAs codes for periplasmic substrate binding proteins of bacterial ABC transporters. This transporter superfamily uses periplasmic solute-binding proteins to take up a wide range of substrates (sugars, amino acids and their derivatives, as well as proteins and drugs). Most of our knowledge on sRNAs derives from studies with Escherichia coli and Salmonella. However, deep sequencing-assisted approaches have revealed numerous sRNAs in any given bacterium or archaeon. Experimental evidence for a regulatory function of these small-sized RNAs has been provided in a limited number of cases, for example, in Bacillus subtilis and other Gram-positives, in cyanobacteria, archaea, Rhodobacter, and Xanthomonas.

Genome-wide surveys have recently revealed hundreds of sRNAs in the plant pathogen Agrobacterium tumefaciens, the plant symbiont Sinorhizobium meliloti, and the human pathogen Brucella abortus. A combination of proteomic and bioinformatic approaches suggested dozens of AbcR1 targets in A. tumefaciens. Several of these newly discovered targets are involved in the uptake of amino acids, their derivatives, and sugars. Among the latter is the periplasmic sugar-binding protein CheV, a component of the virulence signal transduction system. We examined 16 targets and their interaction with AbcR1 in close detail. In addition to the previously described mRNA interaction site of AbcR1 (M1), the CopiRNA program predicted a second functional module (M2) as target-binding site. Both M1 and M2 contain single-stranded anti-SD motifs. Using mutated AbcR1 variants, we systematically tested by band shift experiments, which sRNA region is responsible for mRNA binding and gene regulation. On the target site, we find that AbcR1 interacts with some mRNAs in the translation initiation region and with others far into their coding sequence. Our data show that AbcR1 is a versatile master regulator of nutrient uptake systems in A. tumefaciens and related bacteria.
results in cell proliferation and tumor formation. Additionally, plant metabolism is re-programmed to produce opines serving as carbon and nitrogen source for \textit{A. tumefaciens}. Perception of plant-derived signals involves several bacterial factors, including the two-component system VirA/VirG, which mediates the activation of the virulence cascade, and ChvE, a periplasmic substrate binding protein that binds host-derived sugars and plays a role in activation of the virulence cascade (ChvE). Other putative substrate-binding proteins are involved in attachment (AvrA), two-dimensional (OipA), and agrocinopine (AcrV). In addition to these specialized functions in plant-microbe interaction, ABC transport systems are required for regular nutrient acquisition in \textit{A. tumefaciens} like in other free-living bacteria.

At least three ABC transporters in \textit{A. tumefaciens} are under negative control of the sRNA AbcR1 (ABC transporter regulator 1). Among the targets is \textit{atu2422} encoding the binding protein for GABA (\(\gamma\)-amino butyric acid), a plant-derived defence molecule that interferes with quorum sensing in \textit{Agrobacterium}. AbcR1 is encoded in an intergenic region in tandem with the related sRNA AbcR2. Both are maximally expressed in the late exponential phase. Currently, there is no evidence that AbcR2 plays a regulatory role in \textit{A. tumefaciens}.

Like \textit{Agrobacterium}, various \\textit{Rhizobium} species encode numerous sRNAs, including homologs of AbcR1 and AbcR2. In contrast to \textit{Agrobacterium}, AbcR1 and AbcR2 in \textit{Sinorhizobium} are divergently expressed, namely the first is present in exponentially regulated by AbcR1 and AbcR2. In \textit{Brucella abortus}, both AbcR1 and AbcR2 seem to have at least some redundant function. Microarray analysis revealed about 25 elevated transcripts, several coding for ABC transporters, in the double mutant. At least three of these transcripts can be controlled by AbcR1 or AbcR2 alone. Moreover, only the double mutant but neither single mutant was attenuated in macrophages and in mice. The commonalities and differences in AbcR1-mediated gene regulation in these model organisms certainly warrant further studies to understand the role of this conserved sRNA in a plant pathogen, a plant symbiont, and a human pathogen.

AbcR1 belongs to the large group of Hfq-associated sRNAs. Hfq is an RNA chaperone that facilitates base-pairing between sRNAs and their targets. About 10 ABC transporter genes were found to accumulate in an \textit{A. tumefaciens} \(\Delta\text{Hfq}\) mutant and we wondered whether more than the previously identified three targets \textit{atu2422}, \textit{atu1879}, and \textit{fvrC} were controlled by AbcR1. We used a combination of proteomics and bioinformatics approaches to identify numerous new targets of AbcR1. RNA–RNA interactions studies revealed that AbcR1 uses two separate regions to address mRNAs either in the translation initiation region (TIR) or far downstream in the coding region. Our results support the function of AbcR1 as versatile master regulator to control \textit{Agrobacterium} physiology.

\section*{Results}

\textbf{AbcR1 regulates periplasmic binding proteins of several ABC transporters}

To identify new targets of AbcR1, we compared the proteomes of the marker-less \textit{AbcR1} mutant (\(\Delta\text{AbcR1}\)) and the wild-type (WT) strain by two-dimensional PAGE. Cultures were grown to stationary phase (OD600 of 1.5) when AbcR1 is maximally expressed. Total protein extracts from three biological replicates were subjected to two-dimensional PAGE, and protein abundance was visualized by dual-channel images (Fig. S1). Proteins equally abundant in the WT and mutant appear as yellow spots, whereas proteins overexpressed in WT or \(\Delta\text{AbcR1}\) are green or red, respectively. Overall, 68 proteins were affected by the presence of \(\Delta\text{AbcR1}\), indicating potential targets of AbcR1 (Table S1). Twenty-five were up and 43 downregulated. Twenty candidates were extracted from the gel, digested with trypsin, and subjected to mass spectrometry (Table S1). The presence of the known targets \textit{Atu2422} and \textit{Atu1879} among them validated this approach. Northern blot experiments revealed that the increased protein levels in the \(\Delta\text{AbcR1}\) mutant correlated with increased mRNA levels of \textit{atu2422} and \textit{atu1879} in stationary phase (Fig. 1A and B).

\section*{Validation of eight new AbcR1 targets}

The 18 other AbcR1-dependent proteins were so far as-unknown candidates (Table S1). To recapitulate AbcR1-mediated regulation at the mRNA level, eight of the new candidates were chosen for northern blot analysis with \textit{Agrobacterium} WT and \(\Delta\text{AbcR1}\) mutant grown to exponential (OD600 of 0.5) and stationary (OD600 of 1.5) phase. The mRNAs of five periplasmic binding proteins of ABC transporters (\textit{Atu0857}, \textit{MalE}, \textit{Atu4678}, and \textit{DppA}) showed clear AbcR1-dependent regulation consistent with elevated protein levels in the \(\Delta\text{AbcR1}\) strain (Fig. 2A–E). The same was true for \textit{Atu0857}, an annotated oxidoreductase (Fig. 2F). The \(\text{frcB}\) transcript appears to be downregulated by AbcR1 in the exponential growth phase but, consistent with 2D PAGE, upregulated in stationary phase (Fig. 2G). Reduced transcript levels of \textit{apfl} in \(\Delta\text{AbcR1}\) in exponential growth supported positive regulation by AbcR1 as seen on the protein level (Fig. 2H). Transcripts of \textit{apfl} and \textit{dppA} (Fig. 2B) were only detectable in exponential growth phase suggesting that they undergo a rapid turnover in later growth phases.

\section*{Overlap between AbcR1- and Hfq-dependent mRNAs}

The 18 AbcR1-dependent genes \textit{matE}, \textit{atu4678}, and \textit{dppA} have recently been shown to be affected by Hfq. In that study, several proteins overrepresented in the \textit{A. tumefaciens} \(\Delta\text{Hfq}\) mutant were isolated from 1D SDS-PAGE gels and identified as ABC transporters. This led us to assume that a more comprehensive profile of the \(\Delta\text{Hfq}\) proteome might reveal additional AbcR1 targets. Upon separation by 2D-SDS-PAGE, 31 putative Hfq-dependent proteins were selected and identified by mass spectrometry (Fig. 3A). Among them were periplasmic binding proteins of ABC transporters (Table S2) and 10 proteins identified as AbcR1 targets were also affected by the absence of Hfq (Fig. 3B) indicating that AbcR1 acts through Hfq as previously shown for the target \textit{Atu2422}.
Validation of six more AbcR1 targets

Northern blot experiments with probes against the two Hfq targets atu4432 (Fig. 3) and atu4259 confirmed regulation by AbcR1 as their mRNAs accumulated in the sRNA mutant (Fig. 4A and B; note that migration of the very abundant 16S rRNA to a similar position in the gel interferes with detection of the mRNAs and results in two bands).61 One particularly interesting protein affected by Hfq was ChvE, a periplasmic sugar-binding protein involved in host sensing of A. tumefaciens.43,45,64 Its regulatory pattern resembles that of FrcB. Both proteins were less abundant in the hfq deletion strain than in the WT (Fig. 3A; Table S2). In contrast to most other AbcR1 targets, but like the frcB transcript (Fig. 2G), the chvE mRNA was slightly downregulated in the absence of AbcR1 in exponential phase but clearly upregulated in stationary phase (Fig. 4C) suggesting growth phase-dependent regulation by AbcR1. Regulation of ChvE by AbcR1 raised our interest in NocT and AttC, substrate binding proteins of putative virulence-related ABC transporters required for the uptake of plant-synthesized nopaline (NocT) or for the transport spermidine and putrescine (AttC).46 They were not detected by 2D PAGE analysis. However, northern

Table 1. Potential AbcR1 targets in A. tumefaciens identified by 2D proteomics

| Protein | Locus tag | Product | ΔAbcR1/WT |
|---------|-----------|---------|-----------|
| Atu4577 | atu4577   | ABC transporter substrate binding protein | 66,90 |
| PykA    | atu1762   | pyruvate kinase | 36,86 |
| RbsB    | atu3827   | ABC transporter substrate-binding protein (ribose) | 25,64 |
| Atu0857 | atu0857   | oxidoreductase | 13,45 |
| Atu1188 | atu1188   | oxidoreductase | 9,42 |
| MalE    | atu2607   | ABC transporter, substrate binding protein (maltriose) | 8,05 |
| Fpg     | atu0404   | glucose-6-phosphate isomerase | 6,94 |
| Atu4046 | atu4046   | ABC transporter substrate-binding protein (glucose-6-phosphate isomerase) | 6,22 |
| MurF    | atu2099   | UDP-N-acetylmannosaminyl-D-glutamate-2,6-β-D-mannopyranoside ligase | 5,87 |
| Atu1879 | atu1879   | ABC transporter, substrate binding protein (amino acid) | 4,50 |
| Atu1157 | atu1157   | ABC transporter, substrate binding protein | 3,87 |
| Atu4678 | atu4678   | ABC transporter substrate-binding protein (amino acid) | 3,85 |
| Atu2422 | atu2422   | ABC transporter, substrate binding protein (amino acid GABA) | 3,66 |
| FrcB    | atu0063   | ABC transporter, substrate binding protein (sugar) | 2,13 |
| DppA    | atu4113   | ABC transporter substrate-binding protein (dipeptide) | 2,07 |
| Atu3259 | atu3259   | dehydrogenase | 0,20 |
| RipE    | atu1088   | 50S ribosomal protein L9 | 0,18 |
| Atu2422 | atu2422   | ATP synthase delta chain | 0,16 |
| MurB    | atu2092   | UDP-N-acetylglucosaminylglycosamine reductase | 0,08 |
| RipY    | atu2227   | 50S ribosomal protein L23 | 0,05 |

List of proteins with altered abundance in three replicates of the ΔabcR1 strain in comparison to the WT (fold changes < 0.5 or > 2, respectively). Quantitative proteomics was performed by two-dimensional PAGE with total protein samples from stationary growth phase (OD600: 1.5) of the A. tumefaciens wild-type (WT) and the AbcR1 deletion mutant (ΔabcR1) followed by MALDI-TOF analysis. The entire list of all proteins significantly accumulated in WT or in ΔabcR1 can be found in Figure S1.
blot analysis revealed that they clearly are AbcR1 targets. nocT is a typical negatively controlled AbcR1 target (Fig. 4D) whereas regulation of attC varies depending on the growth condition (Fig. 4E). The final potential AbcR1 target was predicted by the CopraRNA algorithm (Comparative Prediction Algorithm for sRNA Targets, see below). Atu3114 was not identified by our proteomics approaches but northern blot analysis showed AbcR1-dependent regulation (Fig. 4F).

CopraRNA predicts two functional AbcR1 modules and variable target-binding regions

Having identified at least 16 AbcR1-dependent genes, we wondered whether they are all regulated by base pairing of the RBS with the first exposed loop of AbcR1 as documented for att2422 and S. meliloti livK.26,57 To computationally predict interaction regions between AbcR1 and its target mRNAs, we made use of the recently established CopraRNA program.8 It integrates phylogenetic information to predict sRNA–mRNA interactions on the genomic scale. An alignment of orthologous AbcR1 sequences from A. tumefaciens, Agrobacterium radiobacter, Rhizobium etli CFN42, Rhizobium leguminosarum bv. viciae, Rhizobium etli 652, Sinorhizobium meliloti 1021, and Sinorhizobium medicae WSM419 revealed long almost identical sequence stretches (Fig. 5A). The secondary structures were compared using the ClustalW2 program prior to calculation of a consensus structure with the RNAalifold webserver.65,66 Regions highly conserved in sequence are equally conserved in structure (Fig. 5B). Like the experimentally mapped structure of A. tumefaciens AbcR1, the sRNAs fold into three hairpins.26 Apart from the att2422 interaction site (module 1 = M1), a second conserved single-stranded region (M2) was found between the first and second hairpin. Both regions contain a UCCC motif potentially able to interact with SD-like sequences (Fig. 5A and B). A domain prediction of putatively interacting sites between AbcR1 and 15 of the target mRNAs validated in this study.
Figure 3. For figure legend, see page 629.
region (the SD sequence but several sites are located far into the coding 

M2-target mRNA interactions in AbcR1 region M1 (100 to 150 nucleotides containing the predicted interaction region). As expected, band shifts with AbcR1 and AbcR1 region M2 including the adjacent hairpin 2 were shown to interact with AbcR1 region M2 (Figs. 6 and 7). Underrepresentation of AtpH protein and attC transporter components were underlined. (+) or (-) indicate over- or underrepresentation of proteins in deletion mutants.

AtpH mutant reveals new putative AbcR1 targets. (Fig. 8A). In vivo verification of target binding by AbcR1 modules M1 and M2. To validate the in vitro results on the interaction of AbcR1 with its target mRNAs in vivo, we used an A. tumefaciens ΔAbcR1/2 double mutant complemented with the empty vector (+v in Fig. 8) or a plasmid constitutively expressing one of the four AbcR1 variants (+AbcR1, +Mut1, +Mut2, or +Mut1+2). Production of the AbcR1 transcripts was confirmed by northern blot analysis (Fig. 8A). The mRNA levels of four different AbcR1 targets were determined by northern blot analysis. Consistent with this assumption, the Mut1-2 RNA was unable to shift the malE fragment.

In vivo verification of target binding by AbcR1 modules M1 and M2.

(note that atu4577 could not be used because it is not a conserved gene) suggested that both M1 and M2 are involved in target recog- 

nition (Fig. 3C, for visualization of detailed AbcR1 M1- and M2-target mRNA interactions in A. tumefaciens see Table S3). The predicted mRNA interaction sites preferentially lie around the TIR. The target RNAs consisted of 100 to 150 nucleotides containing the predicted interaction region. As expected, band shifts with AbcR1 and AbcR1 region M2 including the adjacent hairpin 2 were shown to interact with AbcR1 region M2 (Figs. 6 and 7). Underrepresentation of AtpH protein and attC transporter components were underlined. (+) or (-) indicate over- or underrepresentation of proteins in deletion mutants.

AtpH mutant reveals new putative AbcR1 targets. (Fig. 8A). In vivo verification of target binding by AbcR1 modules M1 and M2. To validate the in vitro results on the interaction of AbcR1 with its target mRNAs in vivo, we used an A. tumefaciens ΔAbcR1/2 double mutant complemented with the empty vector (+v in Fig. 8) or a plasmid constitutively expressing one of the four AbcR1 variants (+AbcR1, +Mut1, +Mut2, or +Mut1+2). Production of the AbcR1 transcripts was confirmed by northern blot analysis (Fig. 8A). The mRNA levels of four different AbcR1 targets were determined by northern blot analysis. Consistent with this assumption, the Mut1-2 RNA was unable to shift the malE fragment.
Figure 5. For figure legend, see page 631.
responsible for regulation of *att*2422 and *att*3114 (Fig. 8B and D). In accordance with the band shift results (Fig. 6C), *att*6678 was predominantly controlled via M2 (Fig. 8C). The same was true for *att*4431, which was predicted to bind the M2 region *AbcR1* in its coding sequence (Fig. 8E).

**Binding sites of AbcR1 in the CDS contain SD-like sequences**

To precisely map the AbcR1-binding positions in the CDS of selected target mRNAs, we used an in vitro reverse transcription approach. The principle is illustrated in Figure 9A. Target mRNA fragments of 100 or 150 nt length were annealed to end-labeled primers complementary to regions upstream of the predicted interaction region followed by cDNA synthesis. Transcripts of two different concentrations of *AbcR1* prior to reverse transcription were mapped in reference to a sequence reaction run on the same gel. In a control experiment (Fig. 9B), the mapped *att*2422–*AbcR1* interaction site corresponded to the previously reported site overlapping the SD sequence of the mRNA. As an example for an mRNA targeted far within the CDS, *att*3114 was used. The CopraRNA-predicted region around 516 nt in the open reading frame was found to interact with *AbcR1*, thus resulting in prematurely terminated cDNA fragments (Fig. 9C). With the mude RNA, the presence of *AbcR1* led to truncated cDNA products corresponding to a CDS region around +207 (Fig. 9D). The mapped interactions sites show that *AbcR1* addresses SD-like UGGAGAG motifs (see sequence to the left of Fig. 9B–D) regardless of their position in the mRNA.

*AbcR1* promotes degradation of target mRNAs when bound to the TIR or CDS

The previously identified target mRNAs of *att*2422 and *att*6678 were significantly stabilized in the absence of *AbcR1* in vivo. This provided evidence that interaction of *M1* with *att*2422 or CDS (*att*1879) accelerates mRNA turnover and led us to study the effect of *AbcR1* on the stability of various target mRNAs. We selected one example each for M1-TIR, M2-TIR, M1-CDS, M2-CDS, and M1/M2-CDS interactions and determined mRNA degradation in the presence or absence of *AbcR1* after transcription was stopped by addition of rifampicin.

Interaction of *AbcR1* with the TIR via M1 (*fcbB*, Fig. 10A) or M2 (*att*6678, Fig. 10B) destabilizes the target mRNAs as shown by their elevated stability in the absence of the sRNA. The same is true when the CDS is bound by *AbcR1* either by M1 (*att*3114, Fig. 10C), M2 (*att*4431, Fig. 10D), or M1 or M2 (*maid*, Fig. 10E) suggesting that negative regulation by *AbcR1* involves RNA degradation regardless of whether the TIR or CDS is targeted. Contrary to these negatively regulated transcripts, stability of the positively regulated *att*H transcripts was not influenced by *AbcR1* (Fig. 10F).

**Discussion**

Global approaches like proteomics or microarrays and bioinformatic predictions are commonly used for sRNA target identification. In this study, we employed a combination of global proteomics and comparative biocomputational predictions for identifying targets of *AbcR1* in the plant-pathogen *A. tumefaciens*. Validation of 14 targets via northern blot hybridization enlarged the set of currently known *AbcR1* targets to 16 mRNAs. Although several target mRNAs of *AbcR1* have been reported in *Brucella* and *Sinorhizobium*, the mode of action of this conserved sRNA has not yet been studied. Our study uncovered two distinct target-binding sites in *AbcR1* and variable interacting loci in the controlled transcripts.

*AbcR1* targets different sites of mRNAs through two functional modules

Many Hfq-associated sRNAs contain a single-stranded domain able to interact with multiple target mRNAs. Other sRNAs have several functional domains that base pair with different sets of target mRNAs in *E. coli*, *Salmonella*, and *Vibrio Harveyi*. Previously, only one conserved target-binding region strategically positioned in the first exposed hairpin loop of *AbcR1* has been reported. Preceding this, the recently established comparative target prediction tool CopraRNA, which has been previously used to predict the two and three interaction regions of *GcvB* and *Spork2*, respectively. Strikingly, the two computationally predicted and experimentally verified modules M1 and M2 are highly conserved among *Rhizobiales* suggesting that the two functional modules are conserved in *A. tumefaciens*. The more distantly related *AbcR1* sequence from *Brucella* was not included in the CopraRNA predictions because it exhibits less sequence identity to *A. tumefaciens* *AbcR1* than...
Figure 6. For figure legend, see page 633.
Figure 6 (See opposite page). Binding of target mRNAs at the translation initiation region by two distinct functional modules. (A) Secondary structures of WT AbcR1 and the variants Mut1, Mut2, and Mut1+2. Band-shift experiments with AbcR1 variants and atu2422 (A), frcB (B), atu4678 (C), chvE (C), and atpH (D) mRNA fragments (−50/+100 nt relative to the AUG start codon). Predicted IntraRNA duplexes formed by AbcR1 and target mRNAs are shown to the left. Numbering of mRNA nucleotides is given relative to the AUG/GUG start codon. 32P-labeled AbcR1 variants (< 0.05 pmol) were incubated with increasing concentrations of unlabeled target RNAs at 30 °C for 20 min. Final concentrations of unlabeled RNA were added in 100 (lanes 2), 200 (lanes 3), and 400 (lanes 4) fold excess. Samples shown in lanes 1 were incubated with water (control).

Figure 7. Binding of target mRNAs in the coding sequence by AbcR1. (Top) Secondary structures of AbcR1 wild-type, the variants Mut1, Mut2, and Mut1+2. Band-shift experiments with AbcR1 variants and atu1879 (A), atu3114 (A), atu4678 (B), chvE (C), and atpH (D) mRNA fragments (−50/+150 nt relative to the AUG start codon). Predicted IntraRNA duplexes formed by AbcR1 and target mRNAs are shown to the left. Numbering of mRNA nucleotides is given relative to the AUG/GUG start codon. 32P-labeled AbcR1 variants (< 0.05 pmol) were incubated with increasing concentrations of unlabeled target RNAs at 30 °C for 20 min. Final concentrations of unlabeled RNA were added in 100 (lanes 2), 200 (lanes 3), and 400 (lanes 4) fold excess. Samples shown in lanes 1 were incubated with water (control).
the homologs from *Sinorhizobium* and *Rhizobium* species. The existence of two single-stranded M1- and M2-like regions in the predicted secondary structure of *B. abortus* AbcR1, however, suggests that two functional AbcR1 modules are not restricted to plant-associated bacteria.39

On the target site, our study revealed that AbcR1 binding regions are scattered throughout the TIR and CDS. Although interference with translation by mRNA binding around the SD sequence is considered the most common control mechanism of sRNAs, targeting of coding sequences has been described in enterobacteria, for example, RyhB and ompD, ArcZ-tpx, RyhB-fadL, SgsS-manX, and SdrR-ompF.51-53,77-79 Two exposed UC-rich interaction regions in AbcR1 and the potential to interact with SD-like regions in the TIR or CDS allows pervasive gene regulation by this sRNA in *A. tumefaciens*.

**AbcR1: A conserved master regulator of ABC transporters**

There is increasing evidence that sRNAs are more than single target regulators, but rather act on multiple trans-encoded targets and rewire entire transcriptional networks.2,77,78 Many well-studied sRNAs in enterobacteria control large sets of functionally related target mRNAs; for example, RyhB regulates mRNAs encoding iron-binding proteins involved in iron homeostasis, OmrA/OmrB regulate mRNAs encoding proteins for outer membrane protein synthesis, and GcvB controls genes for amino acid biosynthesis and transport.7,22-24,69,70,79-83

Homologs of AbcR1 from *S. meliloti*, *R. etli*, and *B. abortus* are similar in sequence and structure.52-54,56,59,84 A functional classification of target mRNAs (ABC transport system) was initially described for the AbcR sRNAs in *A. tumefaciens* and *B. abortus* 2308.26,59 The experimental verification of 14 AbcR1 targets encoding periplasmic transport proteins carrying sugars, amino acids, and opines supports the function of AbcR1 as a key regulator for these transport systems (Fig. 11).

Our previous study described a potential role of AbcR1 in plant defense, quorum sensing, and virulence of *A. tumefaciens* because the AbcR1 target *atu2422* codes for binding protein of an importer of GABA, a plant defense molecule.26,85-88 We now find that AbcR1 also silences synthesis of ChvE (Fig. 4C), a regulator in sugar-dependent activation of the virulence cascade as well as other virulence-related ABC transporters (NocT and ArcC).43,45 This strengthens the hypothesis that AbcR1 is involved in plant–microbe interactions and post-infection nutrient acquisition.

Although most currently known sRNAs block translation of target mRNAs by interfering with ribosome binding, several sRNAs can activate gene expression.89 They can, for example, bind upstream of the TIR and remodel an intrinsic inhibitory mRNA structure such that the sequestered ribosome binding site is liberated (DsrA and RprA).90-92 Recently, new translation-independent pathways of mRNA activations have been reported for *cfa* through RydC, and for *yigL* through SgrS in *Salmonella*.19,93 In enterobacteria, well-characterized sRNAs like RyhB and ArcZ repress some target mRNAs, but activate translation of *shIA* (RyhB) and *rpoS* (ArcZ).69,74,79,80,94 In addition to the many negatively controlled AbcR1 targets in *A. tumefaciens* we found *atpH* as positively regulated gene. It is predicted to encode the delta subunit of the ATP synthase.95 Although it remains unknown how AbcR1 controls *atpH* expression, for instance, AbcR1 does not alter *atpH* mRNA stability.
directly, the extensive interaction region between AbcR1 M2 and the TIR of atpH is indicative of a direct mechanism. Control of multiple ABC transporters and the ATP synthase suggests that AbcR1 coordinates nutrient acquisition and energy conversion in *A. tumefaciens.*

**Experimental Procedures**

**Bacterial growth conditions**

Bacterial strains and antibiotics used in this study are listed in Table S5. *E. coli* was grown in LB medium at 37 °C. *A. tumefaciens* strains were cultivated in YEB medium at 30 °C.

**Strain and vector constructions**

The ΔAbcR1 and Δhfq mutant strains were constructed in previous studies. Runoff plasmids as templates for in vitro transcription of AbcR1 or target mRNA fragments were flanked by the T7-promoter sequence (GAAATTAATAGCACTCAGTTAGGG) and an EcoRV site PCR-amplified with primers listed in Table S4 and subcloned into pUC18. AbcR1 variants were constructed via site-directed mutagenesis using the primers listed in Table S4.

**Protein preparation**

Cells of *A. tumefaciens* wild-type, ΔAbcR1, and Δhfq were grown in 30 ml YEB medium at 30 °C to an OD600 of 1.5. Culture volumes of 30 ml were harvested, washed three times in 30 ml of TE-buffer (100 mM Tris and 1 mM EDTA), and finally resuspended in 4 ml of TE-buffer with 1.39 mM PMSF and 0.2 mM DTT. Cells were disrupted by three passes through a chilled French press. The lysates were centrifuged at 10 000 × g for 30 min to remove the cell debris. Protein concentrations were determined by Bradford assays.

**Two-dimensional PAGE and Mass Spectrometry**

Total proteins extracts of *A. tumefaciens* wild-type, ΔAbcR1, and Δhfq cells were concentrated by chloroform/methanol precipitation up to 600 μg μl⁻¹. Isoelectric focusing and SDS-PAGE were performed as described previously. Protein solutions were loaded on Immobiline DryStrip pH 4–7, 24 cm (GE Healthcare). After isoelectric focusing, proteins were subjected to 12.5% SDS-PAGE, and the spots were visualized using RuBPS (C₁₂H₂₂N₆Na₄O₁₈Ru₆S₆) staining. Protein spots were scanned using a Typhoon TRIO (GE Healthcare) and were quantified with the Delta two-dimensional software (version 4.0, Decodon). Selected protein spots were excised from the gel, and protein identification

**Figure 9.** Precise mapping of AbcR1 binding sites in target mRNAs. (A) Principle of AbcR1 binding-site mapping by toeprinting analysis. -AbcR1, without AbcR1; reverse transcription (RT) starting from a primer complementary to the target mRNA sequence transcribes a full-length product. +AbcR1, pairing of AbcR1 with the target sequence terminates reverse transcription (truncated product). AbcR1 binding-site mapping on atu2422 (B), atu3114 (C), and malE (D) RNA fragments was performed as described in Experimental procedures. The position of truncated products is indicated to the right. mRNA nucleotides involved in M1 binding are shown to the left. Concentrations of AbcR1 RNAs were 1.5 pmol μl⁻¹ (lane 2) and 2.5 pmol μl⁻¹ (lane 3).
Figure 10. AbcR1 M1 and M2 target mRNAs in the TIR and the CDS for degradation. Northern blot analyses of frcB (A), atu4678 (B), atu3114 (C), atu4431 (D), malE (E), and atpH (F) transcripts from cultures treated with rifampicin. Cultures of the A. tumefaciens wild-type (WT) or the ΔAbcR1 deletion mutant (ΔR1) were grown to exponential or stationary (in case of frcB) growth phase in YEB medium and treated with rifampicin (250 mg ml⁻¹). Total RNA fractions were collected at the indicated time points. Eight μg of total RNA were separated on 1.2% denaturing agarose gels. Ethidiumbromide-stained 16S rRNAs were used as loading control. Quantification of transcript stabilities and their calculated half-lives are given to the right.
using mass spectrometry was performed by MALDI-TOF mass spectrometry as described previously. RNA preparation and northern analysis

Cells were harvested, washed, and frozen in liquid nitrogen as described previously. Isolation of total RNA was done by using the hot acid phenol method. Northern analyses were performed as previously described. To measure mRNA stability, rifampicin was added to the cell cultures in a final concentration of 250 mg ml\(^{-1}\) and samples for RNA isolation were collected before (0 min) and 1, 2, 3, 4, and 6 min after addition of the transcriptional inhibitor rifampicin. In order to determine the half-life of the specific mRNAs, the amount of transcripts present at each time point was quantified using the Image software Alpha Ease FC (Alpha Innotech). The primers used for RNA probe generation are listed in Table S4 in the supplemental material.

Gel shift experiments

The sRNAs AbcR1 WT, QC1, QC2, and QC1+2 and the target mRNA fragments (comprising ~150 nucleotides in the TIR or in the CDS) were synthesized in vitro by runoff transcription with T7 RNA polymerase from the linearized plasmids listed in Table S4. 5’ end labeling of AbcR1 WT or AbcR1 variants (QC1, QC2, and QC1+2) with \(^{32}\)P was performed as described. RNA band shift experiments were performed in 1x structure buffer (Ambion) in a total reaction mixture volume of 15 µl as follows. 5’ end labeled AbcR1 (corresponding to 5000 c.p.m.) and 1 µg of tRNA were incubated in the presence of unlabeled target mRNA fragments (~150 nt) at 30 °C for 20 min. The final concentrations of added unlabeled RNA fragments are given in the figure legends. Prior to gel loading, the binding reactions were mixed 4.5 µl of native loading dye (50% glycerol, 0.5× TBE, 0.1% bromophenol blue and 0.1% xylene cyanol) and run on native 6% polyacrylamide gels in 0.5× TBE buffer at 300 V for 1.5–3 h.

Mapping of sRNA-binding sites

Mapping of AbcR1-binding sites were performed like previously described “toeprint analysis” with some modifications. Annealing mixtures contained 0.5 pmol unlabeled atu2422 (50 nt upstream from the AUG start codon), malE (+100/+250 relative to AUG start codon), or atu3114 (+437/+588 relative to AUG start codon) and one mol of 5’ end labeled primer runoff_atu2422_rv, runoff_malE2_rv, and runoff_atu3114_rv in VD buffer without magnesium. Annealing mixtures were heated for 3 min at 80 °C and snap frozen in a frozen plastic box. After incubation on ice for 20 min, different concentrations (listed in figure legends) of AbcR1, WT, or water (as negative

Figure 11. The AbcR1 regulon of A. tumefaciens. AbcR1 controls mRNAs of periplasmic substrate-binding proteins of 14 ABC transporters (sugars and amino acids to the left and right, respectively), an annotated oxidoreductase (atu0857) and Atph. Module 1 (red) and module 2 (blue) dependent genes are sorted toward the top and bottom of the schematic cell, respectively. The interaction region (TIR or CDS) and the mode of action (repression or activation) are indicated. Dashed lines refer to computationally predicted interactions.
control) were added and incubated at 37 °C for 20 min. After addition of 2 μL RNAse A (20 mg/mL), 4 μL BSA, dNTPs, and MIVL reverse transcriptase (USB), cDNA synthesis were performed at 37 °C for 10 min. Reactions were stopped by adding formamide loading dye and reaction aliquots were separated on a denaturing 8% polyacrylamide gel. Reverse transcriptase cDNA products were identified by comparison with sequences generated with the same 5' end labeled primer.

Biostatistical tools

Alignments and structures were generated by the ClustalW software obtained from http://www.ebi.ac.uk/Tools/msa/clustalw2/. Secondary structures and consensus structures were predicted by comparison with sequences generated from formamide loading dye and reaction aliquots were separated on a denaturing 8% polyacrylamide gel. Reverse transcriptase cDNA products were identified by comparison with sequences generated with the same 5' end labeled primer.

Bioinformatic tools

Alignments and structures were generated by the ClustalW software obtained from http://www.ebi.ac.uk/Tools/msa/clustalw2/. Secondary structures and consensus structures were predicted by comparison with sequences generated from formamide loading dye and reaction aliquots were separated on a denaturing 8% polyacrylamide gel. Reverse transcriptase cDNA products were identified by comparison with sequences generated with the same 5' end labeled primer.
agrobacterium tumefaciens.

29. Hynes AH, Piddock VJ. Baculovirus ABC transporters of ampicillin resistance. Rev Microbiol 2001; 55:29-50.
30. Shi, Z., Zhang, M., Zhang, H., Zhang, H., Xu, J., Xu, H. 2010. 48:478-83. PMCID:2523719, http://dx.doi.org/10.1186/1471-2164-11-53

31. 44. Gröbner V, Klibanov LV, Lazareva B, Zelenina D, Adly-Yulina M, Vysy T, Tolkach A, Telfa M, Pitzschke A, Hirt H. RNA-targeting of amino acids. Res Microbiol 2001; 152:259-66; PMID:11838202; http://dx.doi.org/10.1016/s0034-5288(01)00517-x

32. 45. Narberhaus F. Deep sequencing uncovers numerous predicted non-coding RNAs in the genome of Agrobacterium tumefaciens. J Bacteriol 2010; 96:135-44; PMID:20444087; http://dx.doi.org/10.1128/jb.00557-09

33. 46. Narberhaus F. Hfq influences many small non-coding RNAs. Nat Rev Microbiol 2010; 8:58-62; PMID:20082284; http://dx.doi.org/10.1038/nrmicro2615

34. 47. Pitzschke A, Hirt H. New insights into an old story: Hfq as a bacterial RNA chaperone. Biochem Biophys Res Commun 2013; 436:488-494; PMID:23554411; http://dx.doi.org/10.1016/j.bbrc.2013.08.032

35. 48. Vogel J, Leeu HW. Hfq and its constellation of RNA. RNA Biol 2011; 8:110-20; PMID:21535893; http://dx.doi.org/10.4161/rna.8.1.16187

36. 49. Guoqin M, McWilliam H, Liu Y, Wiater A, Grüber AR, Stettler PP. RNAfold: improved common secondary structure prediction for RNA sequences. Nucleic Acids Res 2008; 36:47-9; PMID:18040959; http://dx.doi.org/10.1093/nar/gkm855

37. 50. Vogel J, Wage WP. Target identification of small noncoding RNAs in bacteria. Proc Natl Acad Sci U S A 2011; 108:9699-704; PMID:21599703; http://dx.doi.org/10.1073/pnas.1104793108

38. 51. Barry EU, MacKintosh FC, Broughton WS, Neale D. Expressional analysis of the Vinc gene products of Agrobacterium tumefaciens. J Bacteriol 1987; 169:517-24; PMID:2476600

39. 52. Wu J, Zhou S, Xu Y, Wang B, Wang W. Herpes simplex virus type I virulence in cell culture. Virus Res 2011; 156:177-88; PMID:21760622; http://dx.doi.org/10.1016/j.virusres.2011.05.014

40. 53. Vogel J, Leuliette WE, Chau I, Mack S, Kudla J, Weidner F. Identification of potential small non-coding RNAs in the genome of Agrobacterium tumefaciens. RNA 2008; 14:1046-61; PMID:18660946; http://dx.doi.org/10.1261/rna.196270

41. 54. 55. Guoqin M, McWilliam H, Liu Y, Wiater A, Grüber AR, Stettler PP. RNAfold: improved common secondary structure prediction for RNA sequences. Nucleic Acids Res 2008; 36:47-9; PMID:18040959; http://dx.doi.org/10.1093/nar/gkm855

42. 56. Vogel J, Wage WP. Target identification of small noncoding RNAs in bacteria. Proc Natl Acad Sci U S A 2011; 108:9699-704; PMID:21599703; http://dx.doi.org/10.1073/pnas.1104793108

43. 57. Barry EU, MacKintosh FC, Broughton WS, Neale D. Expressional analysis of the Vinc gene products of Agrobacterium tumefaciens. J Bacteriol 1987; 169:517-24; PMID:2476600

44. 58. Wu J, Zhou S, Xu Y, Wang B, Wang W. Herpes simplex virus type I virulence in cell culture. Virus Res 2011; 156:177-88; PMID:21760622; http://dx.doi.org/10.1016/j.virusres.2011.05.014

45. 59. Guoqin M, McWilliam H, Liu Y, Wiater A, Grüber AR, Stettler PP. RNAfold: improved common secondary structure prediction for RNA sequences. Nucleic Acids Res 2008; 36:47-9; PMID:18040959; http://dx.doi.org/10.1093/nar/gkm855

46. 60. Vogel J, Wage WP. Target identification of small noncoding RNAs in bacteria. Proc Natl Acad Sci U S A 2011; 108:9699-704; PMID:21599703; http://dx.doi.org/10.1073/pnas.1104793108

47. 61. Barry EU, MacKintosh FC, Broughton WS, Neale D. Expressional analysis of the Vinc gene products of Agrobacterium tumefaciens. J Bacteriol 1987; 169:517-24; PMID:2476600

48. 62. Wu J, Zhou S, Xu Y, Wang B, Wang W. Herpes simplex virus type I virulence in cell culture. Virus Res 2011; 156:177-88; PMID:21760622; http://dx.doi.org/10.1016/j.virusres.2011.05.014

49. 63. Guoqin M, McWilliam H, Liu Y, Wiater A, Grüber AR, Stettler PP. RNAfold: improved common secondary structure prediction for RNA sequences. Nucleic Acids Res 2008; 36:47-9; PMID:18040959; http://dx.doi.org/10.1093/nar/gkm855

50. 64. Vogel J, Wage WP. Target identification of small noncoding RNAs in bacteria. Proc Natl Acad Sci U S A 2011; 108:9699-704; PMID:21599703; http://dx.doi.org/10.1073/pnas.1104793108

51. 65. Barry EU, MacKintosh FC, Broughton WS, Neale D. Expressional analysis of the Vinc gene products of Agrobacterium tumefaciens. J Bacteriol 1987; 169:517-24; PMID:2476600

52. 66. Wu J, Zhou S, Xu Y, Wang B, Wang W. Herpes simplex virus type I virulence in cell culture. Virus Res 2011; 156:177-88; PMID:21760622; http://dx.doi.org/10.1016/j.virusres.2011.05.014

53. 67. Guoqin M, McWilliam H, Liu Y, Wiater A, Grüber AR, Stettler PP. RNAfold: improved common secondary structure prediction for RNA sequences. Nucleic Acids Res 2008; 36:47-9; PMID:18040959; http://dx.doi.org/10.1093/nar/gkm855

54. 68. Vogel J, Wage WP. Target identification of small noncoding RNAs in bacteria. Proc Natl Acad Sci U S A 2011; 108:9699-704; PMID:21599703; http://dx.doi.org/10.1073/pnas.1104793108

55. 69. Barry EU, MacKintosh FC, Broughton WS, Neale D. Expressional analysis of the Vinc gene products of Agrobacterium tumefaciens. J Bacteriol 1987; 169:517-24; PMID:2476600
Salvail H, Lanthier-Bourbonnais P, Sobota JM, Caza A, Rice JB, Vanderpool CK. The small RNA SgrS activates three distinct promoters in intact E. coli. Nucleic Acids Res 2011; 39:3806-19; PMID:21245045; http://dx.doi.org/10.1093/nar/gkq1219

Freidlikh KS, Papenfort K, Berger AA, Vogel J. A conserved Rhs-dependent small RNA controls the synthesis of major protein DonQ. Nucleic Acids Res 2012; 40:4343-60; PMID:22385552; http://dx.doi.org/10.1093/nar/gks1229

Papenfort K, Storz G. Multiple target regulation by small RNA: a conserved Hfq-dependent small RNA. Mol Microbiol 2009; 64:1260-73; PMID:17542919; http://dx.doi.org/10.1111/j.1365-2958.2008.06395.x

Desnoyers G, Morissette A, Prévost K, Massé E. Small RNA-induced differential degradation of the polycistronic Shine-Dalgarno mRNA in E. coli. J Biol Chem 2011; 286:25880-94; PMID:22180532; http://dx.doi.org/10.1074/jbc.M110.140715

Brzury JR, Qiu J, Lai EM, Narberhaus F. Proteomic and transcriptomic characterization of a virulence-deficient Sinorhizobium meliloti mutant. Mol Genet Genomics 2010; 283:575-93; PMID:20457975; http://dx.doi.org/10.1007/s00438-010-0702-4

Jaguar E. Locus of Coordinate expression: a computational tool for the comprehensive analysis of microarray data. Bioinformatics 2002; 18:1154-62; PMID:12089821; http://dx.doi.org/10.1093/bioinformatics/18.6.1154

Tabatabai GM, Jenson AR, Huffman JA, Rhee SY. Rapid isolation of DNA from fresh or frozen tissues. Anal Biochem 2007; 365:286-94; PMID:17276375; http://dx.doi.org/10.1016/j.ab.2007.01.031

Nester EW. Comparative transcriptome analysis of Agrobacterium tumefaciens and other organisms. Plant Physiol 2006; 141:113-24; PMID:16800123; http://dx.doi.org/10.1104/pp.105.071891

Boschetti S, Morse MA, Thompson JA. Intact cell, native level protein detection using anti-protein antibodies. Anal Biochem 1998; 260:101-6; PMID:6323249; http://dx.doi.org/10.1006/abio.1998.5037

Shi A, Morita S, Hasegawa S, Nakamura Y, Takeda E, Abe H. Nonspecific, low-affinity binding of small RNAs to protein targets. Genes Cells 2005; 10:959-70; PMID:16311170; http://dx.doi.org/10.1111/j.1365-2443.2005.00924.x

51. Pfenninger T, Papenfort K, Brzury JR, Jenson AR, Horwich AL. The H(+)-translocating unit of Escherichia coli F(O)F1 ATP synthase. J Biol Chem 2013; 288:25880-94; PMID:23864656; http://dx.doi.org/10.1074/jbc.M304406200

52. Rudolph M, Hain G, Flügge UI. A method for the quantitative determination of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1975; 62:268-74; PMID:258107; http://dx.doi.org/10.1016/0003-2697(75)90213-3

53. Dwek RA, Higgs DR. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal Biochem 1994; 186:1-5; PMID:775386; http://dx.doi.org/10.1006/abio.1994.1214

54. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harb Perspect Biol 2011; 3:3; https://doi.org/10.1101/pdb.a003798

55. Hillers T, Eggens R, Padduk, K, Friedrich, K. Hinkelhoff-Hennemann B, Becker E, Deckert-Müller G, Takaishi T. Spheroplast lysis in the context of the H(+)–translocating unit of Escherichia coli. FEBS Lett 2009; 583:1766-72; PMID:19459451; http://dx.doi.org/10.1016/j.febslet.2009.04.002

56. Takahashi T, Fujita Y, Goto S, Maruyama Y, Abe H. dU overnight culture of E. coli cells. J Mol Cell Biol 2011; 29:1086-107; PMID:20660454; http://dx.doi.org/10.1089/molc.2010-1179

57. Zaker M, Milad NA, Khatibi S, et al. CopraRNA and IntaRNA: producing small RNA targets, network and expression analysis. Nucl Acids Res 2014; 42:2630-40; PMID:24838564; http://dx.doi.org/10.1093/nar/gkt867