Insights into the diversity of φRSM phages infecting strains of the phytopathogen Ralstonia solanacearum *complex: regulation and evolution*

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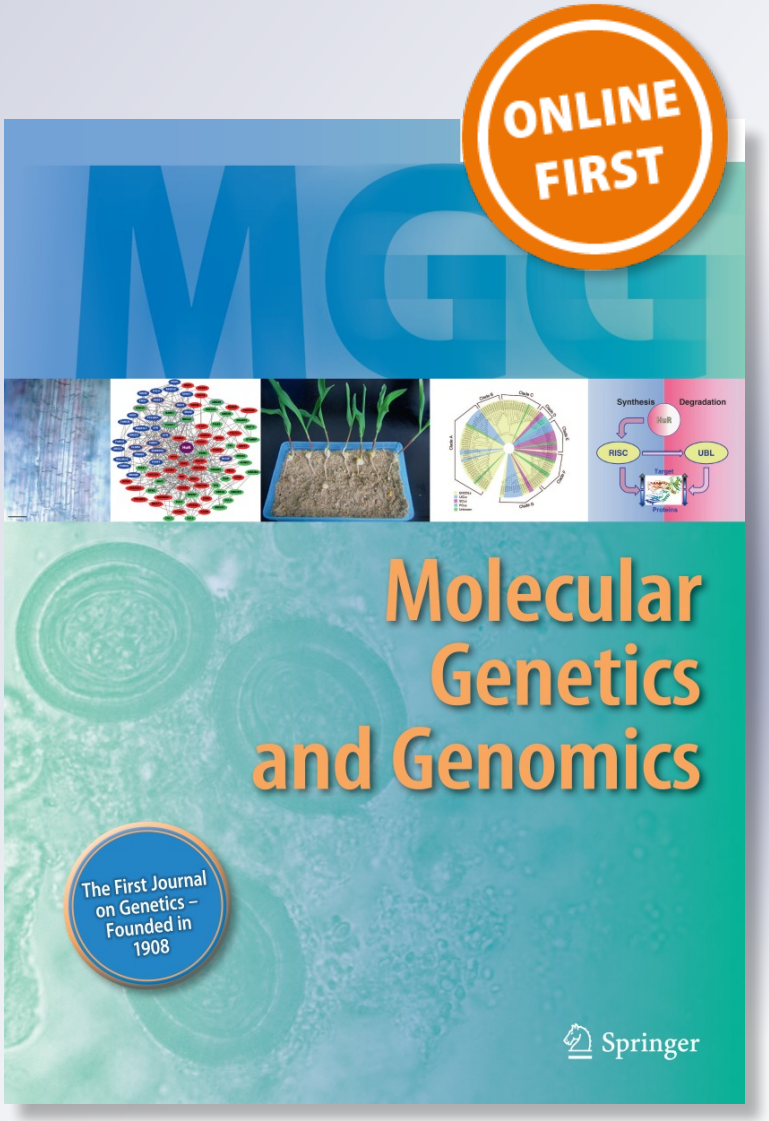

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ORIGINAL PAPER

Insights into the diversity of φRSM phages infecting strains of the phytopathogen *Ralstonia solanacearum* complex: regulation and evolution

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Abstract The filamentous φRSM phages (φRSM1 and φRSM3) have integration/excision capabilities in the phytopathogenic bacterium *Ralstonia solanacearum*. In the present study, we further investigated φRSM-like sequences present in the genomes of *R. solanacearum* strains belonging to the four major evolutionary lineages (phylotypes I–IV). Based on bioinformatics and comparative genomic analyses, we found that φRSM homologs are highly diverse in *R. solanacearum* complex strains. We detected an open reading frame (ORF)15 located upstream of the gene for φRSM integrase, which exhibited amino acid sequence similarity to phage repressor proteins. ORF15-encoded protein (a putative repressor) was found to encode a 104-residue polypeptide containing a DNA-binding (helix-turn-helix) domain and was expressed in *R. solanacearum* lysogenic strains. This suggested that φRSM3-ORF15 might be involved in the establishment and maintenance of a lysogenic state, as well as in phage immunity. Comparison of the putative repressor proteins and their binding sites within φRSM-related prophages provides insights into how these regulatory systems of filamentous phages have evolved and diverged in the *R. solanacearum* complex. In conclusion, φRSM phages represent a unique group of filamentous phages that are equipped with innate integration/excision (ORF14) and regulatory systems (ORF15).

Keywords Filamentous phage · Integration/excision · Self-regulation · Phytopathogen

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Introduction

Ralstonia solanacearum is a phytopathogenic bacterial species that causes severe wilting of many important crops (Yabuuchi et al. 1995; Hayward 2000; Alvarez 2005). Strains of *R. solanacearum* can be divided into four main phylotypes: phylotype I, which includes strains originating primarily from Asia; phylotype II, from America; phylotype III, from Africa and surrounding islands in the Indian Ocean; and phylotype IV, from Indonesia (Fegan and Prior 2005; Villa et al. 2005). In our previous studies, we demonstrated a high level of diversity between phages infecting *R. solanacearum* strains (Kawasaki et al. 2007; Yamada et al. 2007; Fujiwara et al. 2008; Askora et al. 2009). One of these phages, φRSM1, was characterized as an Ff-like phage (Inovirus) based on its particle morphology, genomic single-stranded DNA (ssDNA), and infection cycle. The φRSM1 genome (9,004 bp) contains two inserts that are absent from the M13 gene organization; a short region containing open reading frames (ORF) 2 and 3 (*orf2–orf3*), located between the replication module and the structural module, and a 2.0-kb cluster containing reversely orientated *orf13* and *orf14*, along with *attP*, in the replication module (Kawasaki et al. 2007). φRSM1-related DNA sequences have occasionally been found integrated into the genomes of certain strains of *R. solanacearum* (Kawasaki

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et al. 2007). Askora et al. (2009) characterized two additional φRSM prophages, φRSM3 and φRSM4, which are closely related to φRSM1 and carry the same unit of integration elements as φRSM1. Recently, using in vivo assays, Askora et al. (2011) characterized the integration/excision recombination abilities of the φRSM genome in host cells and showed that it was strictly dependent on the presence of φRSM1 *orf14*. Integration is thought to be accomplished by a phage integrase (ORF14), which has significant homology to resolvases/DNA invertases (small serine recombinases), and contains *attP*/*attB* sites corresponding to the 3′ end of the host serine tRNA (UCG) gene in the reverse orientation (Kawasaki et al. 2007; Askora et al. 2011). It is noteworthy that the same unit of integration (φRSM Int/*attP*) was also found in a *Ralstonia pickettii* 12J phage and a *Burkholderia pseudomallei* 668 prophage (Askora et al. 2011). These observations suggested the frequent occurrence of prophages like φRSM in natural populations of *R. solanacearum*, as well as in related bacterial species. Our previous results clearly demonstrated the lysogenic cycle of φRSM phages (Kawasaki et al. 2007; Askora et al. 2009, 2011). However, how the lysogeny of these φRSM phages is regulated in host cells remains unknown. To elucidate the regulatory mechanism for the lysogenization process of φRSM in *R. solanacearum*, we identified and examined similar sequences in the rapidly accumulating collection of complete *R. solanacearum* complex genomes in the public databases (Remenant et al. 2010; Li et al. 2011). In addition, we characterized an ORF (*orf15*) upstream of *orf14* in φRSM3 that may function as a regulator of φRSM lysogeny.

Materials and methods

Bacterial strains and phages

R. solanacearum strains MAFF106603 and MAFF730139 (Yamada et al. 2007) were cultured in CPG medium containing 0.1 % casamino acids, 1 % peptone, and 0.5 % glucose (Kelman 1954) at 28 °C with shaking at 200–300 rpm. When required, the medium was supplemented with kanamycin and/or ampicillin at 50 μg/mL. Phage φRSM3 was propagated as described previously (Askora et al. 2009).

RNA isolation and DNA manipulation

Standard molecular biological techniques for DNA isolation and digestion with restriction enzymes and other nucleases such as DNase I and RNase were as described by Sambrook and Russell (2001). Phage DNA was isolated from purified phage particles by phenol extraction. In some cases, extrachromosomal DNA (RF DNA) was isolated from phage-infected *R. solanacearum* cells by the mini-preparation method (Ausubel et al. 1995). Total bacterial RNA was isolated from 3 mL of a culture of φRSM3-infected MAFF106603 cells at the exponential phase (1×10^8 colony forming units/ml) using an RNAprotect Bacteria Reagent kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's protocol. Total RNA was treated with 10 U RNase-free DNase I (Takara Bio., Kyoto, Japan) for 30 min at 37 °C to remove any genomic DNA contaminants. DNase I was inactivated by phenol/chloroform extraction. PCR was performed under standard conditions in a MyCycler (Bio-Rad Laboratories, Hercules, CA). PCR conditions were as follows: 2 min at 94 °C; followed by 25–40 cycles of 98 °C for 10 s, 68 °C for 1 min/kb, and 72 °C for 15 s. At the end of the program, the specificity of the primer set was confirmed by melting curve analysis (65–95 °C with a heating rate of 0.5 °C/min). MAFF 106603 genomic DNA was used as a positive control in the PCR reaction. DNA sequencing was performed using a BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) in an Applied Biosystems 3700 DNA sequencer.

Quantification of phages

Phage susceptibility assays were based on standard agar overlays using a dilution series (Yamada et al. 2007). Small turbid plaques typical of Ff phages indicated that *R. solanacearum* was sensitive to a φRSM phage. The phage stock dilutions were usually adjusted so that 300–600 plaque-forming units (pfu) were obtained per plate for the purpose of titration (see below). The phage titer in the supernatant was determined by a standard plaque assay with the indicator strain MAFF 106603 for φRSM3. In the phage infection experiments, we used different strains of *R. solanacearum* as hosts. Each strain was used for a different purpose: MAFF 730139 was used as a lysogenic strain with φRSM3, and a non-lysogenic strain MAFF 106603 was used as a host strain for φRSM3 propagation.

Real-time quantitative reverse transcription polymerase chain reaction

Real-time qRT-PCR was performed as described previously (Alemzadeh et al. 2006). First-strand cDNA was synthesized from 1 μg of total RNA using a ReverTra Ace reverse transcriptase kit (Toyobo, Osaka, Japan) and gene-specific primers according to the manufacturer's instructions.

Specific gene primers were designed using Primer3 (v. 0.4.0) software (http://frodo.wi.mit.edu/primer3/#PRIMER_MAX_TEMPLATE_MISPRIMING). Duplicate reactions with the omission of reverse transcriptase were carried out as a negative control to eliminate the possibility of residual DNA

amplification. Real-time PCR was performed using a SYBR premix Ex *Taq* kit (Takara Bio.) with a Line Gene Fluorescence quantitative detection system (BioFlux, Tokyo, Japan). The 10-μL reaction mixture contained 5 μL of SYBR premix Ex *Taq*, 1 μL of diluted cDNA, and 0.5 μM each gene primer (Table S1). PCR was performed under the following conditions: initial heating for 3 min at 95 °C and 45 cycles of 95 °C for 10 s, 62 °C for 10 s, and 72 °C for 15 s. At the end of the program, the specificity of the primer set was confirmed by melting curve analysis (65–95 °C with a heating rate of 0.5 °C/min). Relative expression levels were calculated as the ratio of expression of each gene against that of the 16S rRNA gene in *R. solanacearum.*

Construction of ΔRSM3 mutant phage lacking ORF15

To determine the role of *orf15*, located in the φRSM3 genome, we constructed a φRSM3 mutant lacking *orf15* and some of its upstream region (designated ΔRSM3). *orf15* was deleted from φRSM3 DNA by generation of a φRSM3 genomic DNA fragment missing *orf15* by PCR. Amplification was achieved using the primer pair: forward, 5′-GAT GAG AAC TCC TAT CAT GGC GAA ACA CTT-3′ (corresponding to φRSM3 DNA position 8,821–8,850), and reverse, 5′-ACA AGG TGT TCA GCC CGG CAC GCT GAA CG-3′ (corresponding to φRSM3 DNA position 8,549–8,521). These primers generated a φRSM3 DNA fragment lacking the entire *orf15* region (position 8,524–8,820) from the φRSM3 DNA template. The PCR product (about 8.56 kb) was purified from agarose gel following electrophoretic separation and then circularized with T4 DNA ligase (Ligation High, Toyobo) overnight at 16 °C. The resulting DNA was introduced into *R. solanacearum* MAFF 106603 competent cells by electroporation. Following incubation for 2 h at 28 °C, bacterial cells were subjected to plaque assay. Single plaques were isolated, and phage-containing cells were cultivated to obtain replicative-form phage DNA (RF). The ΔRSM3 DNA sequence was confirmed by complete genomic DNA sequencing.

In vivo integration/excision of φRSM3 and ΔRSM3 DNA in the *R. solanacearum* chromosome

To examine the status of the φRSM prophage in the host after phage infection, the φRSM3 prophage and the ΔRSM3 mutant were examined by colony PCR assay following infection into *R. solanacearum* MAFF 106603. Following infection with either wild-type φRSM3 or ΔRSM3, random single colonies of *R. solanacearum* MAFF 106603 were used as a template for PCR using a 36-base forward primer, 5′-GAA GGA CAC AT CCG GGT ACA CGC CCA AGG CCA TGA C-3′ (primer p1, corresponding to a sequence 350-bp upstream of *attL*), and a 29-base reverse primer, 5′-ACC TAT CGC GAG ATC AGC TCG TGC TCC AA-3′ (primer p4, corresponding to a sequence 450-bp downstream of *attR*). To determine the junction regions upstream and downstream of the attachment sites (*attL* and *attR*), chromosomal DNA was extracted from each of the selected infected cells and subjected to PCR to amplify fragments containing the attachment sites (*attL* and *attR*). The *attL* region was amplified using a 33-base forward primer, 5′-GGA GTA TCT TGG CGG AGA GGG TCC TTT TTG AAC-3′ (primer P1), and a 28-base reverse primer, 5′-GAG CGC CGA ATT CGC AAA CCG CCT CTC C-3′ (primer P2), while *attR* was amplified using a 29-base forward primer, 5′-CCT GGA TCC TGT TGT TGG GCG GAA TTA TG-3′ (primer P3), and a 28-base reverse primer, 5′-TTC GAA TCC CAC CCT CTC CGC CAT ACG C-3′ (primer P4). The primer sequences were based on the *attB* region sequence of MAFF 301558 [DDBJ: AB585971]. The PCR products were then gel purified and sequenced.

Bioinformatics

Sequences homologous to that of φRSM were identified in the NCBI GenBank database using the FASTA, FASTX, BLASTP, and BLASTX programs (Altschul et al. 1997). Multiple sequence alignments were performed using the DNASIS program (version 3.6; Hitachi Software Engineering Co., Japan).

Protein sequence alignments were performed using ClustalX, where the coloring scheme depends on both residue type and the pattern of conservation within a column (http://www.cgl.ucsf.edu/chimera/docs/ContributedSoftware/multalignviewer/cxcolor.html).

Results

φRSM is highly diverse in strains of the *R. solanacearum* complex

The rapid accumulation of complete genomic sequences of *R. solanacearum* in databases provides us with the opportunity to identify further φRSM homologs in *R. solanacearum* strains, which can be used to characterize the regulatory mechanism for lysogenization of these filamentous phages. In this study, we further identified φRSM-like sequences in the genomes of different *R. solanacearum* phylotypes, including *R. solanacearum* Y45 (phylotype IB, GenBank: AFWL00000000), IPO1609 (phylotype IIB, GenBank: CU914168), and CMR15 (phylotype III, GenBank: FP885895), and *R. syzygii* strain R24 (phylotype IV, GenBank: FR854086.1), using each of the φRSM ORFs as query sequences. As a result, we detected many φRSM

homologs integrated into the genomes of the *R. solanacearum* strains. At the current state, 13 whole genomic sequences of *R. solanacearum* are available, among which six strains contained φRSM sequences. In this study, five strains of *R. solanacearum* and related species containing an entire copy of φRSM were subjected to analysis (Fig. 1; Table 1). Interestingly, in all cases, an *int* gene, encoding a recombinase-like protein, accompanied by an *attP*-like sequence was located between the *gII* and *gV* genes, in the same organization as that seen in φRSM1 and φRSM3 (Askora et al. 2009).

Identification of a putative regulatory gene (*orf15*) in the *R. solanacearum* phage φRSM3

Using bioinformatic analysis, we examined the φRSM-like sequences for a gene that might encode a phage regulator. A small ORF, designated *orf15*, was identified upstream of *orf14* (Int) (φRSM3 positions 8,524–8,838, GenBank: AB434711), which was overlooked in our previous annotation of the φRSM3 genome (Addy et al. 2012; Askora et al. 2011) (Fig. 1). This ORF encodes a protein of 104 aa residues (also corresponding to *orf15* of φRSM1, GenBank: AB259123) with sequence similarity to putative phage repressors (e.g., phage repressor of *Pelobacter propionicus* DSM2379, exhibiting 40 % aa identity, GenBank: YP_899965, *E* value = 0.004). It is noteworthy that a sequence closely related to *orf15* was also found at the corresponding position in all the φRSM homologs integrated into the genomes of *R. solanacearum* strains (Fig. 1). Using ClustalX, the deduced amino acid sequence of φRSM3 ORF15 was aligned with that of putative lambda repressor-like transcriptional regulators (LacI family), as well as ORF15-like sequences found in φRSM-related phages in *R. solanacearum* strains, including *R. syzygii* R24 genomic contig 00001-1628 (exhibiting 93 % aa identity), *R. solanacearum* Y45 chr contig 178 (98 aa, 94 % identity), and *R. pickettii* 12J phage (98 aa, 92 % identity). However, the ORFs located at the same position in several *R. solanacearum* strains showed different amino acid sequences, for example, UW551 cont0570 (96 aa, 35 % identity), IPO1609 genome draft (96 aa, 35 % identity), and CMR15 (96 aa, 35 % identity) (Fig. 2). Motif analysis (http://bioinf.cs.ucl.ac.uk/psipred) of φRSM3-ORF15 revealed a DNA-binding helix-turn-helix motif in the N terminus of the protein, which is a characteristic of phage repressors (Fig. 2, boxed in red). These observations led us to propose that *orf15* of φRSM3 might be a strong candidate regulator gene for the process of φRSM lysogeny in *R. solanacearum*.

Fig. 1 Comparison of the genomic organization of φRSM1 with ▶ that of other related phages and prophages found in various organisms. Linear ORF maps were compared according to a previously reported model (Kawasaki et al. 2007; Askora et al. 2009). ORFs are represented by *arrows* oriented in the direction of transcription. The putative functional modules for replication (R), structure (S), and assembly–secretion (A–S) are indicated. ORF sizes (in amino acid residues) are in parentheses. The region containing the *orf14-attP* sequence and a large intergenic (IG) region are also indicated on the map. The region containing *orf15* (shaded in *red*) is indicated as an insert because it is variable among phages. Sequence data: φRSM1 [DDBJ/EMBL/GenBank: AB259123]; φRSM4 (prophage in *R. solanacearum* UW551) [GenBank: NZ_AAKL00000000.1]; φRSM5 (prophage in *R. solanacearum* IPO1609) [GenBank: CU914168]; φRSM6 (prophage in *R. solanacearum* CMR15) [GenBank: FP885895]; φRSM7 (prophage in Y45) [GenBank: AFWL00000000]; φRSM8 (prophage in *R.* syzygii strain R24) [GenBank: FR854086.1]; *R. pickettii* 12J prophage (φRSM9) [GenBank: ACD27678]

Function of φRSM3-ORF15

The lysogenic state of filamentous phages (chromosomally integrated) such as CTXφ of *Vibrio cholerae* is maintained and tightly controlled. Those phages encode transcriptional regulators whose role is to inhibit transcription of the replication protein and downstream virion genes (Waldor and Friedman 2005; Rakonjac et al. 2011). To test a possible regulatory role of ORF15 in φRSM3 lysogeny, the transcription of some major genes, including *orf12* (replication), *orf14* (Int), and *orf15* (putative repressor), was assayed by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) in the φRSM3-lysogenic strain. As a negative control, total RNA was analyzed from strain MAFF 106603, which

Table 1 Main features of φRSM homologs in the genome of *R. solanacearum* complex

Strain	Phylotype	φRSM	% GC	Accession no.	Reference
MAFF211270	I	ϕRSM1	59.9	AB259123	Kawasaki et al. (2007)
MAFF730139	I	ϕRSM3	59.6	AB434711	Askora et al. (2009)
UW551	II	ϕRSM4	60.8	NZ_AAKL00000000	Remenant et al. (2010)
IPO1609	IIB	ϕRSM5	60.8	CU914168	Remenant et al. (2010)
CMR15	III	ϕRSM6	59.6	FP885895	Remenant et al. (2010)
R. syzygii R24	IV	ϕRSM7	59.9	FR854086.1	Remenant et al. (2010)
Y45	IB	ϕRSM8	59.6	AFWL00000000	Li et al. (2011)
R. pickettii 12J	ND	ϕRSM9	60.3	ACD27678	Askora et al. (2011)

ND not determined

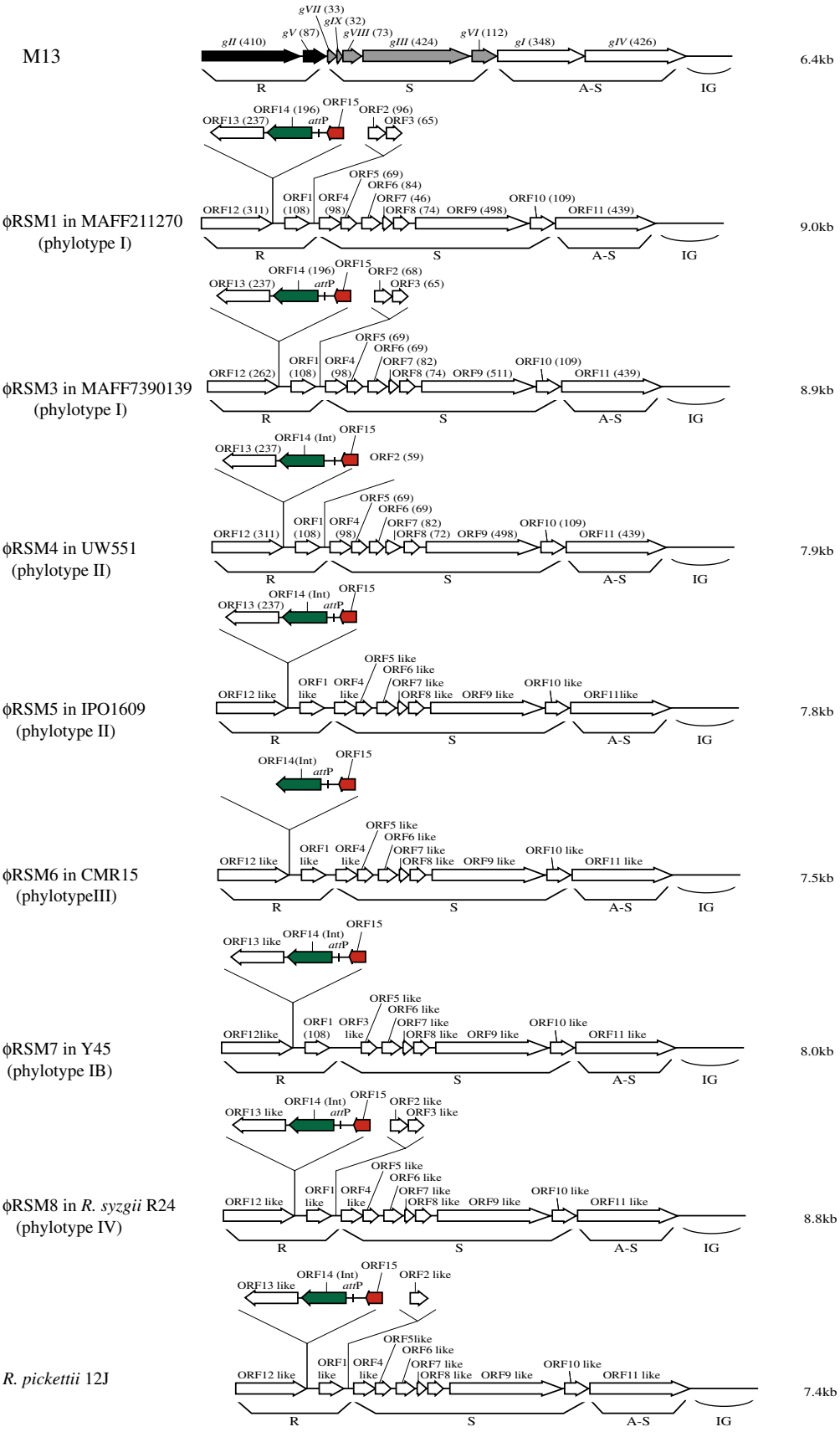

Fig. 2 Comparison of the amino acid sequence of ORF15 (putative repressor) of φRSM3 with various repressor proteins using ClustalX software. The region of highest homology among these repressor proteins and ORF15 corresponded to the 45 amino acid residues boxed in *red*. The amino termini of these repressors contain helix-turn-helix DNA-binding domains (boxed in *red*). An *asterisk* indicates the conserved Gly residue at position 31 in all repressor proteins. The ClustalX coloring scheme depends on both residue type and the pattern of conservation within a column (http://www.cgl.ucsf.edu/chimera/docs/ContributedSoftware/multalignviewer/cxcolor.html). Sequence data: φRSM3 [UniProt: F8WK51]; φRSM1 [UniProt: A0JC19]; *R. syzygii* R24 [UniProt: G2ZYP6]; IPO1609 [UniProt: B5SFT9]; CMR15 [UniProt: D8N910]; *R. pickettii* 12J [UniProt: B2U9N]; LacI repressor [accession no.: 3524456MAB] (Color figure online)

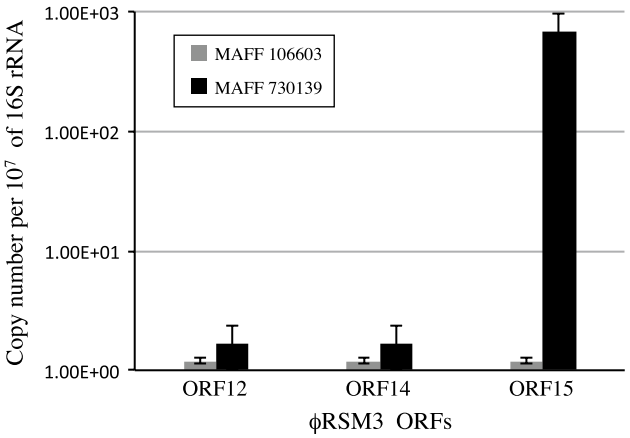

Fig. 3 Transcript analysis in lysogenic bacteria. The transcription levels of some major genes, *orf12* (replication), *orf14* (Int), and *orf15* (putative repressor) in φRSM3-lysogenic strain MAFF 730139 were assayed by real-time reverse transcription PCR. Non-lysogenic strain MAFF106603 was used as a control. Expression values were normalized against the 16S rRNA gene values for each cell. Mean expression and standard deviation values were calculated from the results of three independent experiments

does not contain any φRSM prophages. MAFF 730139 is a stable lysogenic strain with φRSM3 (Askora et al. 2009). The results shown in Fig. 3 indicated high expression of *orf15*, but undetectable levels of *orf12* and *orf14* in the lysogenic strain. This suggested that in the stable lysogenic strain MAFF 730139, high levels of ORF15 (putative repressor) might repress the expression of *orf12* (encoding DNA replication protein) and inhibit phage DNA synthesis (episomal replication), hence contributing to low phage yield (phage release from prophage-containing cells). These results coincided with the fact that the φRSM3-lysogenic strain MAFF 730139 seldom produced φRSM3 particles (Askora et al. 2009), which is consistent with the very low phage yield of many chromosomally integrated filamentous phages (Davis et al. 2002).

Table 2 Susceptibility of *R. solanacearum* strains to different phages

Strain	Prophage-type[a]	φRSM3	φRSM3Δ15[b]
MAFF106603	–	+(2.4×10^7)	+(3×10^{10})
MAFF730139	φRSM3	–	+(1.6×10^2)

(+) indicates that the strain is susceptible to the phage, with titers in parenthesis derived from the same stock of either φRSM3 and φRSM3Δ15, while (−) indicates that no plaques were observed

[a] According to Askora et al. (2009)

[b] Mutant φRSM3 phage with a deletion of ORF15

φRSM3 ORF15 plays a regulatory role in φRSM3 integration and excision

In our previous work, we showed that the φRSM *int* gene, *orf14*, is required for both integration and excision of φRSM phages using in vivo assays (Askora et al. 2011). To determine whether ORF15 is involved in the regulation of integration/excision of φRSM phages, an *orf15* deletion mutant was constructed in φRSM3. *orf15* of φRSM3 was deleted, and the resulting phage DNA (designated ΔRSM3) was then introduced into non-lysogenic *R. solanacearum* MAFF106603 cells, which were then subjected to plaque formation assays (Table 2). The phage titer of ΔRSM3 was 3×10^{10} pfu/ml in the infection of a non-lysogenic strain. This result coincided with the fact that episomally replicating filamentous phages produce large numbers of phage progeny, reaching titers of up to 10^{13} pfu/mL of culture (Davis et al. 2002). To determine whether ΔRSM3 predominantly replicates episomally without integration into the host chromosome, the replicative form (RF) and integrated form of the phage were detected in host cells. We assayed for the presence of circular replicating ΔRSM3 as an episomal form. Restriction pattern (using the ClaI restriction nuclease) of the RF of DNA extracted from ΔRSM3 phage-infected cells always coincided with those expected from the ΔRSM3 DNA sequence, indicating that viable ΔRSM3 phage was

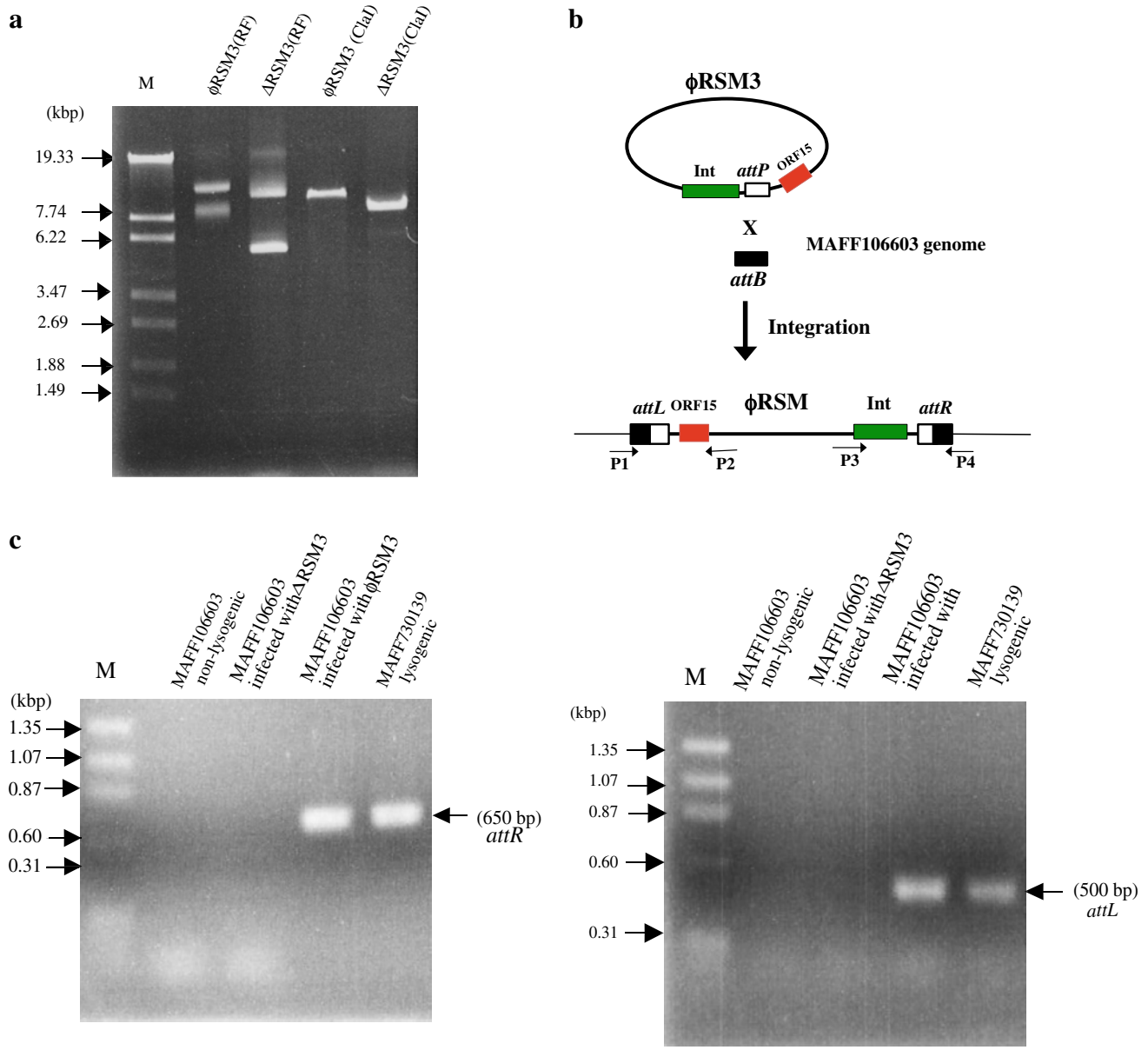

Fig. 4 φRSM3 ORF15 plays a regulatory role in φRSM3 excision and integration. **a** Restriction patterns of the replicative form of φRSM3 and ΔRSM3 DNA following infection of *R. solanacearum* MAFF106603 (undigested products, lane 1: φRSM3, lane 2: ΔRSM3). Restriction enzyme digestion of the DNA with *Cla*I confirmed the exact genomic structure of φRSM3 (8.9 kb) (lane 1) and ΔRSM3 (8.5 kb) (lane 2) recovered from the cells. **b** Schematic representation of φRSM3 integration/excision in *R. solanacearum*. The two attachment sites, *attL* and *attR*, are indicated after φRSM3 integration into the *attB* site of the bacterial chromosome. Upon excision from the chromosome by a site-specific recombination event between *attL* and *attR*, recombination was expected to generate a circular phage DNA and a chromosome lacking the phage genome. **c** PCR detection of prophage junctions in *R. solanacearum* MAFF106603. Following integration, the upstream junction, containing *attL*, was amplified by PCR using primers P1 and P2. The downstream junction, containing *attR*, was PCR amplified using primers P3 and P4. PCR products were separated by agarose gel electrophoresis. The positions corresponding to *attL* (500 bp) and *attR* (650 bp) are indicated. Lysogenic strain MAFF730139 was used as a positive control, according to (Askora et al. 2011)

produced (Fig. 4a). This result was confirmed by detecting the upstream and downstream junction sites of integration following transfection with φRSM3 and ΔRSM3 by a PCR assay using a set of specific primer pairs (Fig. 4b). The PCR assays yielded the predicted products of 500 bp for the *attL* region and 650 bp for the *attR* region when an exact integration took place between φRSM3-*attP* and *attB* of strain MAFF 106603 (Fig. 4c) (Askora et al. 2009, 2011). No PCR products for *attL* and *attR* were detected from chromosomal DNA extracted from ΔRSM3-infected cells, indicating that

ΔRSM3 does not integrate into *R. solanacearum* MAFF 106603 (Fig. 4c).

Diversity of the putative DNA-binding sites and immunity regions in φRSM-related prophages

As described above, φRSM-related prophage sequences were detected in various bacterial genomes. To determine whether similar regulation by ORF15 may also occur in these prophages, the upstream region containing regulatory elements of *orf15*-like sequences were compared with that of φRSM3 *orf15*. We detected two different types of corresponding upstream sequence: (1) those very similar to that of φRSM3 *orf15*, including sequences found in prophages of *R. solanacearum* Y45 chr contig 178 (at position 30,066–29,770, 99 % similarity) and *R. syzygii* R24 genomic contig 00001-1628 (GenBank: FR854086.1, at position 634,766–635,062, 89 % similarity), and (2) those completely different to the φRSM3-ORF15 sequence, including those found in UW551 cont0570 (GenBank: AAKL01000012, at position 73,087–73,377), IPO1609 genome draft (GenBank: CU914168, at position 2,278,161–2,278,451), and CMR15 (GenBank: FP885895, at position 1,111,110–1,110,820). The sequences are compared in Fig. 5a. It is noteworthy that these two types of upstream sequences correlated with the two types of *orf15*-like sequences, suggesting interaction between upstream elements and the ORF15 regulatory protein. As described above, ORF15 may be involved in establishing and maintaining the prophage state, as well as phage immunity. Therefore, these results indicate that there are at least two different lineages in φRSM phages, which differentially infect phylotypes of *R. solanacearum* strains. As seen in a phylogenetic tree based on the *orf15* upstream sequences (Fig. 5b), the φRSM3-type phages predominantly infected strains belonging to phylotype I, whereas the other type phages (such as a prophage found in strain UW551) infected phylotypes II, III, and IV. In addition, two other strains of phylotype I whose genomic sequences are available (FJAT-91, accession no. PRJNA199615, and PS107, accession no. PRJNA56539) contained the same type of orf15-upstream sequence as φRSM3 and were assigned to the upper clade of Fig. 5b (data not shown). Strains such as GMI1000, CFBR2957, FJAT-1458, K60-1, Molk2, and Po82 did not contain any φRSM-related sequences in their genomes.

Discussion

In this study, we demonstrated that φRSM-type filamentous phages (represented by φRSM1 and φRSM3) were found to be frequently integrated into genomes of *R. solanacearum* strains. The results suggest an extended host range for φRSM-type phages that is significantly larger than previously expected (Askora et al. 2011). In all cases, the phages encoded an int-*attP* integration system and integrated into the host genome at *attB* (tRNA-Ser) (Kawasaki et al. 2007; Askora et al. 2009). The unique unit of integration (*int-attP*) might have been horizontally acquired by a φRSM ancestor from other plasmids or phages (Campbell 1992; Lindqvist et al. 1993; Semsey et al. 2002) and facilitated the new lysogenic cycle in the φRSM phages. Together with φRSM phages, it is not surprising that other types of filamentous phages with the *int-att*P unit occur frequently in the natural environment (Askora et al. 2011).

In contrast to Ff phages such as coliphages M13 and fd, φRSM3 phage establishes a lysogenic state between the host and the phage (Askora et al. 2009, 2011, 2012). When φRSM3 phage infects a host, it can replicate and/or integrate its DNA into the bacterial DNA and form a lysogen. In the latter case, φRSM3 will replicate passively whenever the bacterium replicates (Askora et al. 2011; Addy et al. 2012). The genomes of lysogenic phages do not permanently stay in the host genome. Occasionally, prophage genomes can be excised from bacterial chromosomes and then integrated back into the genome at a later point (Nash 1996). These reversible processes are commonly driven by the *int-xis* system in temperate phages, and some cofactors may also participate in this process. In this light, an additional ORF (*orf15*) on the genome of φRSM3 phage was identified upstream of *orf14*, which encoded a putative repressor (Figs. 1, 2). It is well known that some phage repressor proteins (e.g., lambda repressor) interact with DNA through a helix-turn-helix DNA-binding motif located in the N-terminal region of the protein (Pabo and Sauer 1984; Sauer et al. 1990). Within proteins containing this motif, the turn is usually centered on a Gly residue surrounded by two hydrophobic amino acids (Sauer et al. 1990). ClustalX alignment of φRSM3-ORF15 and the φRSM homologs determined this conserved Gly residue to be at position 31 in all the cases (Fig. 2). Furthermore, ORF15 meets all the requirements suggested by Sauer et al. (1990), for helix-turn-helix motifs in a regulator protein. The high expression level of ORF15 in the φRSM3-lysogenic strain (Fig. 3) suggested that ORF15 might be involved in the regulation of ϕRSM3 phage and hence contributed to low phage yield. Furthermore, without *orf15*, phage predominantly replicated without integration, suggesting its involvement in integration and prophage maintenance, and could also infect φRSM-lysogenic strains (Table 2), indicating its involvement in phage immunity. All these results showed that *orf15* of φRSM3 might be the regulator gene for the process of φRSM lysogeny in *R. solanacearum*. It is worth mentioning that results shown in Table 2 raised the question of why the prophage repressor in MAFF 730139 did not inhibit infection of ΔRSM3. One possible explanation for this may be a failure of the φRSM3

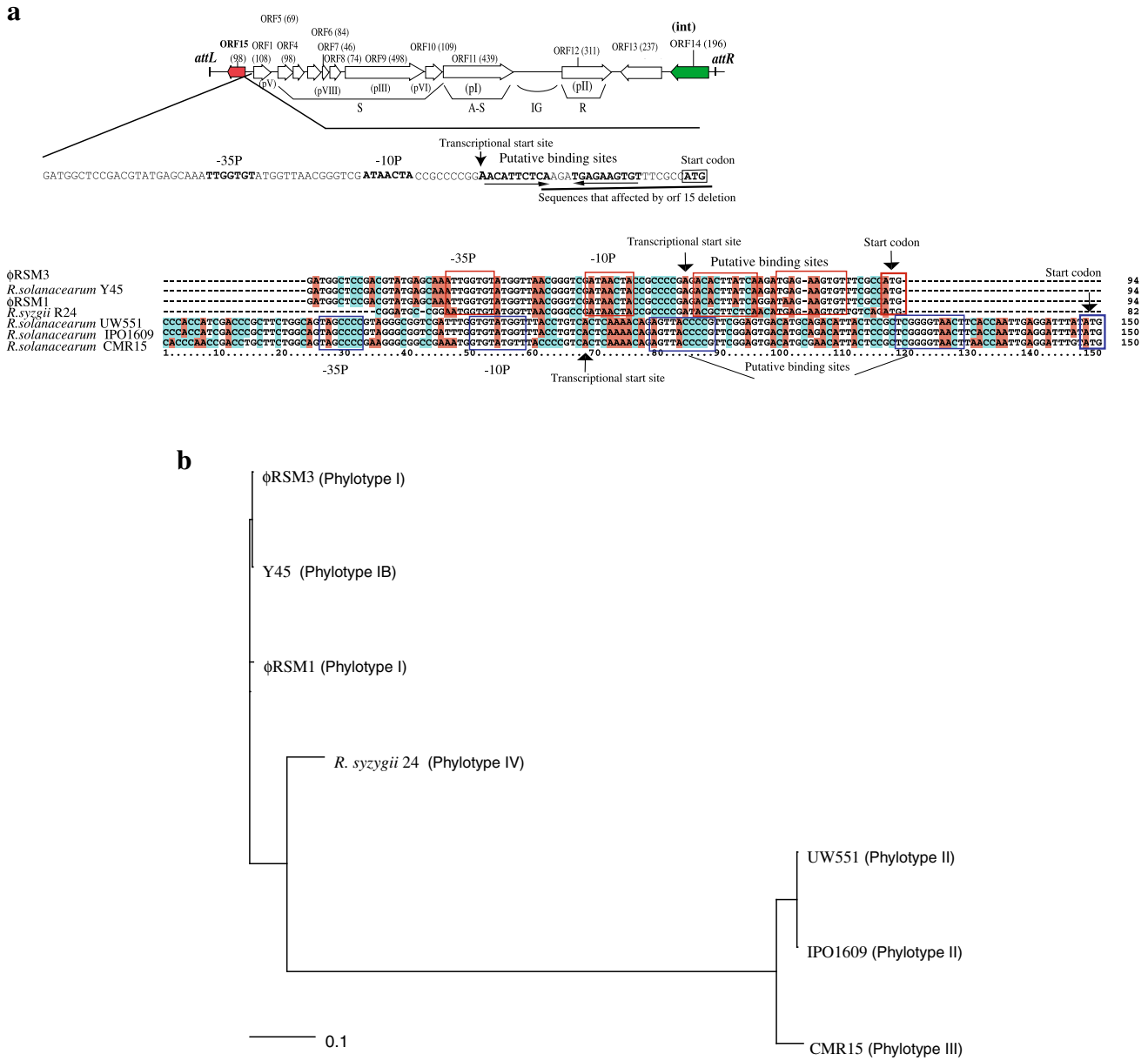

Fig. 5 Sequence analysis of the upstream region of φRSM-like prophages from different strains, and generation of a phylogenetic tree based on ORF15 protein sequences. **a** Alignment of the upstream DNA sequences of φRSM3-ORF15 and φRSM-related phages found in *R. solanacearum* strains. The putative promoter sequences and their binding sites (inverted repeats) for ORF15 of φRSM3 (infecting strains of phylotype I), φRSM1 (infecting strains of phylotype I), φRSM prophages found in *R. solanacearum* Y45 (phylotype IB), and *R. syzygii* 24 (phylotype IV) are boxed in red. The *blue box* indicates the divergent putative binding sites and the promoter sequences of φRSM prophages found in *R. solanacearum* UW551 (phylotype II), IPO1609 (phylotype IIB), and CMR15 (phylotype III). The deletion endpoint of *orf* 15 in ΔRSM3 is shown in the upper panel. **b** Phylogenetic tree illustrating the relationship between the upstream regions found in all φRSM prophages. The tree was constructed from multiple sequence alignment using the TreeView X phylogenetic trees program (Color figure online)

repressor to recognize the controlling region (operator) in ΔRSM3 because the deletion of *orf15* also included the regulatory sequences (Fig. 5a). No plaques were observed on the lawn of lysogenic strain MAFF 730139 following infection with the wild-type φRSM3 (Table 2).

In our study, we demonstrated that φRSM-related prophage sequences were detected in various bacterial genomes (Fig. 1). To see whether similar regulation by ORF15 may also occur in these prophages, the upstream region containing regulatory elements of *orf15*-like sequences were compared with that of φRSM3 *orf15*. The results in Fig. 5a showed that there were two types of ORF15-like protein and associated upstream sequences (regulatory) encoded by φRSM phages indicating that

there are at least two different lineages in φRSM phages, which differentially infect phylotypes of *R. solanacearum* strains. Different phylotypes were selected by these phages depending on the types of ORF15 upstream elements (Fig. 5b). Because φRSM phages represent a unique group of filamentous phages that are equipped with an integration/excision system encoded by an innate protein (ORF14), as well as a regulation system encoded by *orf15*, a study on the evolutionary and biological effects of these phages on host bacteria would be very interesting.

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