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RESEARCH ARTICLE

Seed coating with phages for sustainable plant biocontrol of plant pathogens and influence of the seed coat mucilage

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Abstract

Pathogens resistant to classical control strategies pose a significant threat to crop yield, with seeds being a major transmission route. Bacteriophages, viruses targeting bacteria, offer an environmentally sustainable biocontrol solution. In this study, we isolated and characterized two novel phages, Athelas and Alfirin, which infect *Pseudomonas syringae* and *Agrobacterium fabrum*, respectively, and included the recently published Pfeifenkraut phage infecting *Xanthomonas translucens*. Using a simple immersion method, phages coated onto seeds successfully lysed bacteria post air-drying. The seed coat mucilage (SCM), a polysaccharide–polymer matrix exuded by seeds, plays a critical role in phage binding. Seeds with removed mucilage formed five to 10 times less lysis zones compared to those with mucilage. The podovirus Athelas showed the highest mucilage dependency. Phages from the *Autographiviridae* family also depended on mucilage for seed adhesion. Comparative analysis of *Arabidopsis* SCM mutants suggested the diffusible cellulose as a key component for phage binding. Long-term activity tests demonstrated high phage stability on seed surfaces and significantly increasing seedling survival rates in the presence of pathogens. Using non-virulent host strains enhanced phage presence on seeds but also has potential limitations. These findings highlight phage-based interventions as promising, sustainable strategies for combating pathogen resistance and improving crop yield.

INTRODUCTION

Transmission of microbial diseases via seeds is a significant concern in agriculture and can lead to considerable yield loss (Morris et al., 2007; Darrasse et al., 2010, 2018; Burdman & Walcott, 2012; Mansfield et al., 2012; Shade et al., 2017; Giovanardi et al., 2018; Johnston-Monje et al., 2021). Some estimates predict that the usage of contaminated seeds can lead to yield reductions ranging from 15% to 90% (Vishunavat et al., 2023). This issue becomes especially critical in the face of a growing global population with an increasingly urgent demand for food, coupled with the looming threat of climate change that puts conventional agricultural methods' productivity at risk. Bacteriophages as specialized viruses of bacteria could, in this context, offer a promising basis for developing targeted and sustainable biocontrol strategies.

Phages were discovered over a century ago by d'Herelle and Twort and were used for the first biocontrol trials shortly after that (Mallmann & Hemstreet, 1924). Nevertheless, with the discovery of a broad range of antibiotics, phages fell into oblivion due to their high specificity and lack of detailed knowledge. With the current rise of antibiotic or copper-resistant bacteria, classical methods

to fight the disease are becoming less effective (Pedroncelli & Puopolo, 2023; Sagar et al., 2019; Zhang et al., 2015). This has sparked a renaissance in phage research and moved them into the focus of researchers, policymakers and companies over the past decade (Salmond & Fineran, 2015). In this context, biocontrol strategies centred around phages show significant promise, given the vast diversity of naturally occurring viruses (Dion et al., 2020).

In agriculture, different phage application methods have been explored recently, including spraying phage suspensions on the phyllosphere (Balogh et al., 2008), treatment of irrigation water in pot experiments (Álvarez et al., 2019), treating seed tubers (McKenna et al., 2001) and coating leaves with formulations to protect the phages from radiation (Balogh et al., 2003). However, it has been reported by different studies that transmission via seeds appears as a major route for plant pathogen transmission, and effective plant biocontrol via seed coating has been addressed by only a few studies in recent years. This includes, for example, the protection of melon plants from *Acidovorax citrulli* by phage application (Rahimi-Midani et al., 2020) or the decontamination of seeds from *Xanthomonas campestris* (*Xcc*) (Holtappels et al., 2022). Importantly, the establishment of effective phage coatings requires an understanding of the binding mechanism, enrichment strategies and phage stability on seed surfaces, which has not been systematically addressed thus far.

One important seed product of many plant families is the seed coat mucilage (SCM), which is present in economically relevant plant families like *Lamiaceae* and *Solanaceae*, as well as in the model plant *Arabidopsis thaliana*, among many others (Western, 2012; Yang et al., 2012). The SCM is a layer of pectin, hemicelluloses, cellulose and proteins produced by the epidermal cells during seed development. It is released after imbibing the mature seed with water and subsequently starts to swell and cover the seed with a glycopolymer-matrix. Although the composition can differ among ecotypes of the same species, the major sugar-building blocks are fucose, arabinose, rhamnose, galactose, glucose, mannose, xylose and galacturonic acid (Voiniciuc et al., 2016). In the literature, the presence of mucilage was linked to securing anchorage in the soil, managing water levels around the seed and providing a benefit in the process of dispersal (Kreitschitz et al., 2021; Western, 2012). In this study, we assessed the influence of the SCM on phage binding and stability.

Different phage morphotypes are characterized by their specific receptor-binding proteins (RBPs), encompassing tail fibres and tail spikes. These proteins play a crucial role in recognizing chemical patterns on the surface of the host bacterium (Taslem Mourosi et al., 2022; Witte et al., 2021). While certain receptors bind to the sugar moieties of polysaccharides, others target proteins in the cell envelope (Bertozzi Silva et al., 2016). Consequently, phage adhesion to seeds may be facilitated by physical properties like the mesh-like polymer structure of the mucilage entrapping the phage particles or through direct chemical interaction between the phage RBPs and specific sugar residues.

In this study, we systematically assessed phage binding and the relevance of the SCM by focusing on the model plant *Arabidopsis thaliana* as well as representative bacterial plant pathogens. We describe two newly isolated phages infecting the prominent plant pathogens *Pseudomonas syringae* and *Agrobacterium fabrum* and further included members from an *E. coli* phage collection in our tests (Maffei et al., 2021). While all phages tested showed binding to wild-type *Arabidopsis* seeds, several phages showed significantly reduced binding to seeds with a removed mucilage layer. This included particularly phages of the *Autographiviridae* family, which were highly dependent on the presence of a mucilage. Testing several *Arabidopsis* seed mutants suggested a particular importance of the cellulose component of the mucilage. Further experiments confirmed a high stability of phages on seed surfaces without significant loss of infectivity. We are therefore confident that this study will serve as an important step towards establishing future phage-based seed applications in agriculture.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

Agrobacterium fabrum (strain C58) formerly known as *Agrobacterium tumefaciens* and *Pseudomonas syringae* (DSM 50274) (Young et al., 1978) were used as host strains for phage isolation in this study. *A. tumefaciens* cultures were grown on Lysogeny Broth (LB), whereas *P. syringae* (DSM 50274) and *Xanthomonas translucens* pv. translucens (DSM 18974) (Sapkota et al., 2020) cultures were grown on a nutrient agar (5.0 g peptone, 3.0 g yeast extract and 15.0 g Agar in 1000 mL of dH_2O.). All cultures were inoculated from single colonies in the respective liquid media for overnight cultures. The cultivation of the bacterial strains was performed at 30°C on a shaker at 150 rpm.

Phage isolation

The soil samples for *Agrobacterium* phage Alfirin were retrieved from the rhizosphere of a winter wheat plant at the IBG-2 crop garden (50.909277, 6.413403—Jülich, Germany) and phage Athelas was isolated from a wastewater sample donated by the Forschungszentrum Jülich wastewater plant (50.902547168169825,

6.404891888790708—Jülich, Germany). The isolation of the phages was performed as previously described (Erdrich et al., 2022). Briefly, the virus particles within the environmental samples were solubilized using 10 mL phosphate-buffered saline (100 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$; pH 7.5) and incubated for 3 h at room temperature. Afterwards, the samples were centrifuged at 5000 *g* for 15 min to remove solid particles. The supernatants were filtered through 0.2 μm pore size membrane filters (Sarstedt; Filtropur S, PES). A 1-mL aliquot of the filtered supernatant was mixed with 3.5 mL 0.4% NB soft agar and 100 μL of a densely-grown overnight culture (OD_{600} of 1) of the host and directly plated using the double agar overlay method (Kauffman & Polz, 2018). Plates were incubated at 30°C overnight. Purification of the phage samples was carried out by re-streaking single plaques with an inoculation loop on a fresh double agar overlay containing the host bacterium. This procedure was repeated at least three times.

All phages will be available to the public via the German Collection of Microorganisms and Cell Cultures (DSMZ) after publication. The annotated genomes were deposited on NCBI and are available under the accession number OR997969 (Alfirin) and OR997970 (Athelas).

DNA isolation

Phage DNA was isolated according to the manufacturer's protocol of the Norgen Biotek Phage DNA Isolation Kit (Norgen Biotek, Thorold, Canada). Briefly, 2 mL of Phage suspension (10^8 pfu/mL) was treated with 1 U/μL DNAse (Invitrogen, Carlsbad, CA, USA) to remove free DNA, followed by DNase I inactivation at 75°C for 5 min. After that the viral particles were lysed within the provided kit buffer. After incubation at 65°C for 15 min, 320 μL isopropanol was added. After that the sample was bound to a column and washed twice, before eluded with buffer and sored at −20°C until further usage.

DNA sequencing and genome assembly

Assembly of the DNA library was performed using the NEBNext Ultra II DNA Library Prep Kit for Illumina, according to the manufacturer's instructions, and shotgun-sequenced using the Illumina MiSeq platform with a read length of 2 × 150 bp (Illumina, San Diego, CA, USA). For each phage, a subset of 100,000 reads was sampled, and a de novo assembly was performed using CLC genomics workbench 20.0.4 (QIAGEN, Hilden, Germany). Finally, the obtained contigs were manually curated and checked for gene coverage.

Gene prediction and functional annotation

The phage open reading frames (ORFs) were predicted with Pharokka v 1.3.2 (Bouras et al., 2023) using default settings in terminase reorientation mode using PHANOTATE (McNair et al., 2019), tRNAs were predicted with tRNAscan-SE 2.0 (Chan et al., 2021), tmRNAs were predicted with Aragorn (Laslett, 2004) and CRISPRs were checked with CRT (Bland et al., 2007). Functional annotation was generated by matching each CDS to the PHROGs (Terzian et al., 2021), VFDB (Chen, 2004) and CARD (Alcock et al., 2019) databases using MMseqs2 (Steinegger & Söding, 2017) and PyHMMER (Larralde & Zeller, 2023). Contigs were matched to their closest hit in the INPHARED database (Cook et al., 2021) using mash (Ondov et al., 2016). Plots were created with the pyCirclizen package. Additionally, all identified sequences were later curated, usually manually, using online NCBI Blast against the non-redundant (NR) database 45. Conserved protein domains were further predicted using the batch function of NCBI Conserved Domain Database (CDD) 46 with the e-value cut-off of 0.01.

Electron microscopy of phage virions

For electron microscopy of single phage particles, 3.5 μL purified phage suspension was fixated on a glow discharged (15 mA, 30 s) carbon-coated copper grid (CF300-CU, carbon film 300 mesh copper) and stained with 2% (w/v) uranyl acetate. After air drying, the sample was analysed with a TEM Talos L120C (Thermo Scientific, Dreieich, Germany) at an acceleration of 120 kV.

Sterilization of Arabidopsis thaliana seeds

The seed coat was surface-sterilized by vortexing for 5 min in 50% ethanol (EtOH) containing 0.5% Triton x-100. Afterwards, the 50% EtOH was removed and replaced by 96% EtOH; the samples were inverted once. Afterwards, all EtOH was removed immediately. The seeds were transferred within a small volume of 96% EtOH onto sterile filter paper using a pipette. Finally, they were air-dried.

Phage binding to wild-type seeds of *Arabidopsis thaliana*

Approximately 1000 surface-sterilized *Arabidopsis thaliana* Col-0 seeds were incubated in a sterile Eppendorf tube with 1 mL bacteriophage suspension of a concentration of 10^8 Pfu/mL or higher for 30 minutes. This was followed by two subsequent washing steps in ddH_2O to

remove non-bound phage particles. Afterwards, evaporated (approx. 30–45 min).

Influence of seed coat mutants on phage binding

The following seed coat mutants were required from Nottingham Arabidopsis Stock Centre (NASC): ttg1-21 (GK-580A05); csla2-3 (SALK_149092); rhm2 (SALK_076300) and muci70-1 (SALK_129524). The cesa5 and sbt 1.7 mutants were present at the IBG-2. All seeds were germinated on sterile ½ MS plates and subsequently propagated on soil using a 16 h day/8 h night regime.

Afterwards, the harvested seeds were checked using ruthenium red staining (Voiniciuc, 2016) for their typical morphological appearance under the microscope. The seeds were subsequently used for phage-binding assays, as described above.

For mechanical removal of the mucilage layer, approx. 1000 EtOH surface sterilized wild-type seeds were incubated for 5 min in 1 mL ddH_2O, followed by two rounds of 15 min 30 Hz/s shaking in a ball-mill (Retsch MM200, Retsch, Germany) without beads (Voiniciuc, 2016). The mucilage containing supernatant was removed, and the seeds were washed in in two subsequent steps with ddH_2O. Afterwards, the seeds were placed on sterile filter papers and air-dried for 30 min under a laminar flow bench. Complete removal of the mucilage was verified by ruthenium red staining and microscopy before further use.

Phylogeny of PRB proteins and in silico protein folding

Ancestral states were inferred using the maximum likelihood method (Nei & Kumar, 2000) and the JTT matrix-based model (Jones et al., 1992). The tree (Figure 5.) shows a set of possible amino acids (states) at each ancestral node based on their inferred likelihood at site 1. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model and then selecting the topology with superior log likelihood value. The rates among sites were treated as being uniform among sites (Uniform rates option). This analysis involved 24 amino acid sequences. There were a total of 1386 positions in the final dataset. 1000 Bootstrap trees were generated for the final tree. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

The 3D protein structures of phage RBPs were predicted using the ColabFold v1.5.3 webserver with AlphaFold2 using MMseqs2 with the default settings (Mirdita et al., 2022).

Shelf life of phages coated onto plant seeds

To evaluate the stability and activity of coated phages on seeds, the *Agrobacterium* phage Alfirin, the *Pseudomonas* phage Athelas and the *Xanthomonas* phage Pfeifenkraut (Erdrich et al., 2022) were bound to *Arabidopsis thaliana Col-0* seeds as described above. The coated seeds were then stored within microcentrifuge tubes at 4°C for up to 28 days. At defined time points, a subset of seeds was taken from the tubes and incubated at 28°C for 24 h on a double agar overlay containing the respective host bacterium. Development of a lysis zone around the seed was indicative of the presence of infectious counted as phage particles activity. Finally, the total amount of seeds as well as the proportion exhibiting lysis zones around them were counted. We compared the stability to phages stored in SM buffer at the same conditions and over the same timeframe.

Survival of seedlings in presence of the pathogen and co-inoculation with a non-pathogenic host 'Booster'

The survival in the presence of the pathogen was assessed by infecting surface sterilized Col-0 seeds artificially by imbibing them in a bacterial solution of *Agrobacterium fabrum C58* at an OD_{600} of 0.4 for 30 min and subsequent air-drying on sterile filter paper (condition pathogen-Atum). The same procedure was used for the phage as described above (condition phage-only control). For the 'Booster'-condition, we sought to explore the efficacy of co-incubating seeds with a non-pathogenic strain devoid of the Ti-Plasmid (Morton et al., 2014). Our objective was to evaluate this approach as a means of locally enrich the presence of phages. In this case, phage Alfirin ($1*10^9$ pfu/mL) was coated together with the avirulent *Agrobacterium fabrum C58* ΔpTi onto Col-0 seeds at a MOI of 5 (condition 'booster'). Multiplicity of infection (MOI) is the ratio of infectious phage particles to target cells (bacteria) in a specific volume, offering a quantitative measure of the infection dynamics within a defined biological system.

Avirulent *Agrobacterium fabrum C58* ΔpTi at an OD_{600} of 0.01 ~ $5*10^7$ cfu/mL, was coated onto seeds as a negative control for the booster. All seeds were sown on ½ MS Agar plates and placed into the climate chamber with 12/12 h day/night regime at 22°C at day and 19°C at night. Scans of the plates were taken at 14 days after sowing to evaluate the plant growth by calculating the leaf area per plant. All germinated seedlings surpassing the 2-cotyledon stage without signs of necrosis were counted as alive. Subsequently, the seedlings were transferred to a LB medium-based double agar overlay, containing the

wildtype *Agrobacterium fabrum* C58, at an OD_{600} of 0.2 to assess the presence of the phage in the different conditions.

RESULTS

Phage isolation, morphology, annotation and taxonomy

The novel phages were isolated from winter wheat rhizosphere and wastewater on the campus of the Forschungszentrum Jülich. The *Agrobacterium* phage Alfirin was retrieved from the rhizosphere sample at the IBG-2 crop garden using *Agrobacterium strain C58* as a host. *Pseudomonas* phage Athelas was isolated from a wastewater sample at Forschungszentrum Jülich wastewater plant using *Pseudomonas syringae* pv. *lapsa* (DSM 50274) (Figure 1A). Phage Alfirin formed clear plaques with a mean diameter of 0.96 mm. Phage Athelas formed large and clear plaques with an average diameter of 6.86 mm (Figure 1B).

The isolated phages were sequenced using Illumina MiSeq short-read technology, and the genomic features of phage Alfirin, Athelas and Pfeifenkraut are summarized in Table 1, and all other phages used in this study in Table S1. Briefly, the genomes of the novel phages Alfirin and Athelas are 46 and 40 kb in size, with a GC content of 53% and 57%, respectively (Figure S1A,B).

While Alfirin is predicted to follow the headful packaging mechanism (Leffers & Basaveswara Rao, 1996), phage Athelas has short directed terminal repeats (DTRs) of 221 bp. The genomic ends were determined using Phage Term (Garneau et al., 2017). A prerequisite for phage biocontrol is a lytic lifestyle of the bacteriophage, therefore the lifestyle was predicted using PhageAI, a machine-learning tool which compares the genomes of over 20,000 publicly available phages (Tynecki et al., 2020). Both newly isolated phages were classified as virulent. This is further supported by the absence of genes coding for an integrase within the genomes.

A comparison of the genomes of our isolates with their closest relatives revealed that phage Athelas is part of a described species and phage Alfirin is its own new species. With an average nucleotide identity of 99%, Athelas is a member of the phage NOI species and belongs to the family of *Autographiviridae*. When compared with the closest relatives, phage Athelas clusters with phages isolated on *Pseudomonas syringae pv tomato*, as shown in nucleotide and coding sequence comparison (Figures S2 and S3). The genome of phage Alfirin shares a 58% sequence identity with

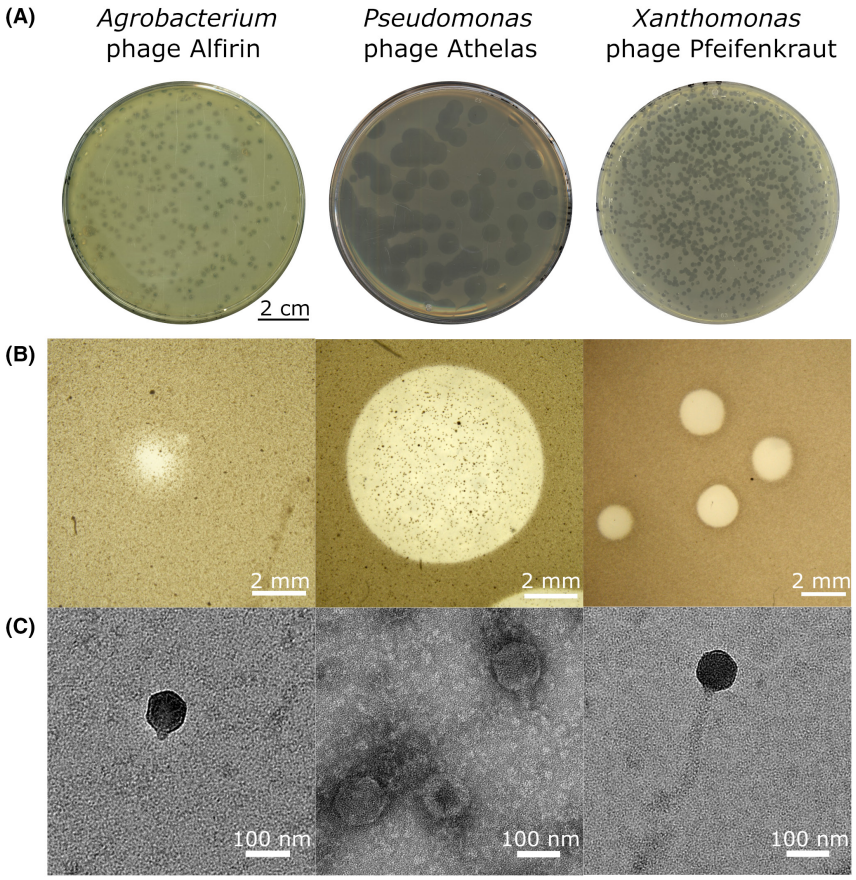

FIGURE 1 Phage morphology of the novel *Agrobacterium* phage Alfirin, and the *Pseudomonas* phage Athelas as well as *Xanthomonas* phage Pfeifenkraut, which was previously described (Erdrich et al., 2022). (A) Plaque morphologies of phages on 0.4% soft agar. Scale Bar: 2 cm; (B) Stereo microscopy of single plaques. Scale bar: 1 mm; (C) Transmission electron microscopy (TEM) images of virion particles. The phage isolates were negative stained with uranyl acetate. Scale bar: 100 nm.

TABLE 1 Basic genomic features of phage Alfirin, phage Athelas and other phages used in this study.

Phage name	Accession number	Reference host	Genome size (Bp)	GC content (%)	ORF number[a]	Genome termini class[b]	Lifestyle prediction[c]	Reference
Alfirin	OR997969	*Agrobacterium fabrum str. C58*	46,051	53.8	65	Headful (pac)	Virulent	This study
Athelas	OR997970	*Pseudomonas syringae* DSM 50274	40,850	57.1	56	DTR (short)	Virulent	This study
Pfeifenkraut	ON189044	*Xanthomonas translucens DSM 18974*	43,791	53.3	72	Headful (pac)	Virulent	Erdrich et al. (2022)

[a]Open reading frames (ORFs) were predicted with Pharokka v 1.3.2 (Bouras et al., 2023), described in more detail in the Material and Methods section.
[b]Genome termini classes were determined using PhageTerm (Garneau et al., 2017).
[c]Phage lifestyle was predicted by the machine-learning-based program PhageAI (Tynecki et al., 2020). Overall, both phages contain a relatively high fraction of ORF encoding proteins of unknown functions (hypothetical proteins/CDS; 38/65 for Alfirin and for Athelas 26/56), reflecting once more the significant amount of 'dark matter' harboured in phage genomes.

Agrobacterium phage Atu_02 and therefore forms a new species (Figure S3).

Binding of phages to *Arabidopsis* seeds and influence of the SCM

In the following, the binding of the newly isolated phages, as well as the previously described *Xanthomonas* phage Pfeifenkraut, to *Arabidopsis* seeds, was investigated.

To test the ability of phages to adhere to plant seeds, we used surface sterilized *Arabidopsis thaliana* Col-0, which is well-known to produce mucilaginous seeds (Francoz et al., 2015) and has the ability to generate large amounts of seeds in the relatively short time of 2–3 months (Boyes et al., 2001). To discriminate between binding and random co-translocation of the phages on the seeds, we washed the seeds twice in ddH_2O. To detect infectious phage particles bound to *Arabidopsis* seeds, we harnessed one of the hallmarks of phage biology—plaque assays—by placing the seeds, after treatment, onto a bacterial lawn containing the respective host species. A visible lysis zone which manifests as clearance of the bacterial lawn is consequently indicative of the binding of phage particles to the seed surface (Bacteriophages Methods and Protocols, Volume IV, 2019), Figure 2A.

After a first observation of phage binding to seeds of *A. thaliana* we asked the question, which mechanism is responsible for binding of the phages. Given that *Arabidopsis*, like other SCM-producing plants (Francoz et al., 2015), is known to release a matrix of sugars, pectin and cellulose upon contact with water, we conducted tests to evaluate whether the mucilage plays a role, either structurally or chemically, in the attachment of phages to the seeds. Using wildtype seeds and seeds were the mucilage has been removed (Voiniciuc, 2016), (Figure 2B), we could show that the mucilage is crucial for seed binding for *Pseudomonas* phage Athelas (73% reduction) and for *Xanthomonas* phage Pfeifenkraut (94% reduction) (Figure 2B,C). From this initial set of phages, only phage Alfirin was not significantly dependent on the presence of the mucilage.

Phages of the *Autographiviridae* family significantly depend on the presence of the mucilage

In the initial set of phages, phages Pfeifenkraut and Athelas showed a clear dependency on the presence of the mucilage. The strongest effect was reproducibly observed for podovirus Athelas. To test whether this trend holds for morphologically similar viruses, we tested all podoviruses from the *E. coli* BASEL collection (Maffei et al., 2021) and also included the model *E. coli* phage T7. We could show that T7, as well as Bas64-Bas68,

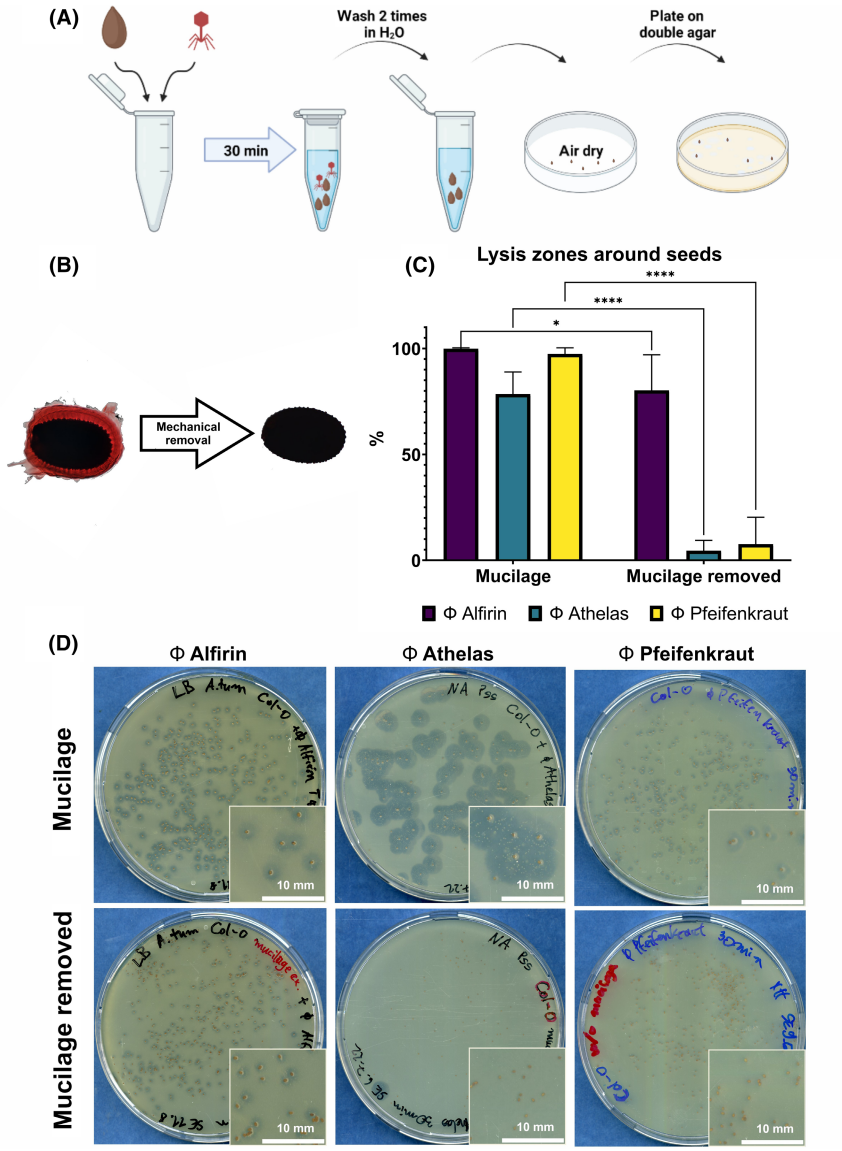

FIGURE 2 Phage binding to seeds and influence of artificial removal of the seed mucilage. (A) Seed-coating-workflow; binding of phage particles on seeds of *Arabidopsis thaliana*. (B) Wild-type Col-0 seed stained with 0.01% ruthenium red solution before and after mechanical removal of the mucilage. (C) Percentage of lysis zones detected around seeds for *Agrobacterium* phage Alfirin, *Pseudomonas* phage Athelas or *Xanthomonas* phage Pfeifenkraut incubated on seed with or without mucilage. The bar plot shows means of seeds from 3 independent experiments for each condition, where for each plate in an experiment the number of seeds showing a lysis zones is expressed as percentage from the total number on the respective plate.; $n = 50–300$ seeds per plate. Error bars represent standard deviation. A two-way ANOVA was significant $F(2.49) = 45.94$, a subsequent HSD was performed: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. (D) Double agar overlay with phage-coated seeds; upper lane unchanged *Arabidopsis* seeds, lower lane *Arabidopsis* seeds where the mucilage was mechanically removed. White boxes, display close-ups of individual seeds with lysis zones.

belonging to the *Autographiviridae* family, showed a strong dependence on the mucilage (Figure 3). The podovirus Bas69 belonging to the *Schitoviradae* showed no significant difference in seed adhesion with or without mucilage. These results indicate that the size (surface cross-section) alone cannot explain the differences in binding behaviour among different phages of similar size. The observed pattern suggests that taxonomically related phages also show similar adhesion properties to the mucilage of plant seeds.

Influence of seed coat mutants on phage binding

To further elucidate which components of the SCM are relevant for phage binding, we set out to test different *Arabidopsis* seed coat mutants with phage Athelas because it was affected most strongly by the presence or absence of the mucilage and produces large plaques, enabling robust quantification (Figure 4). As a control, we used phage Alfirin as a

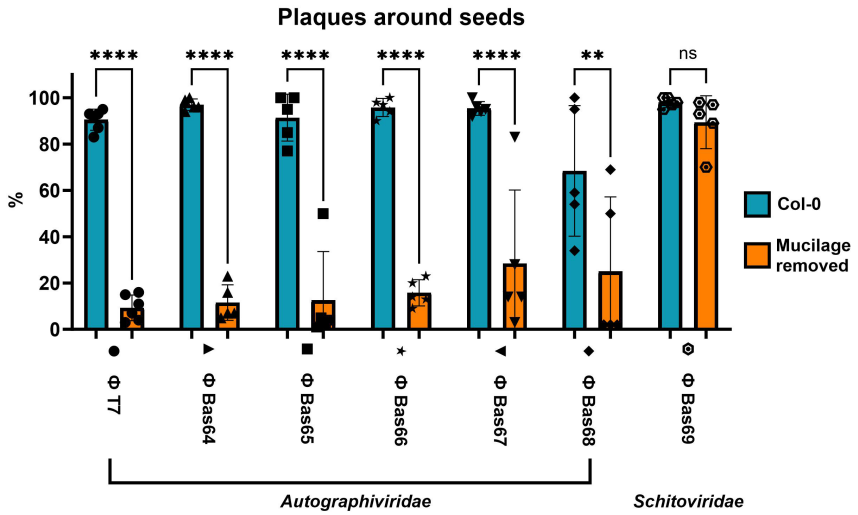

FIGURE 3 Binding of podovirions to seeds and influence of artificial removal of the seed mucilage. All BASEL featuring podovirus morphology were selected, as well as model phage T7. Percentage of plaques around seeds with and without mucilage. At least three independent experiments were performed for each phage and the number of seeds showing a lysis zones as well of the total number of seeds per plate was counted; N 50–300 seeds per plate. Data is presented as % of seeds surrounded by a lysis zone. A two-way ANOVA was significant F (6, 58) = 10,07; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns = not significant.

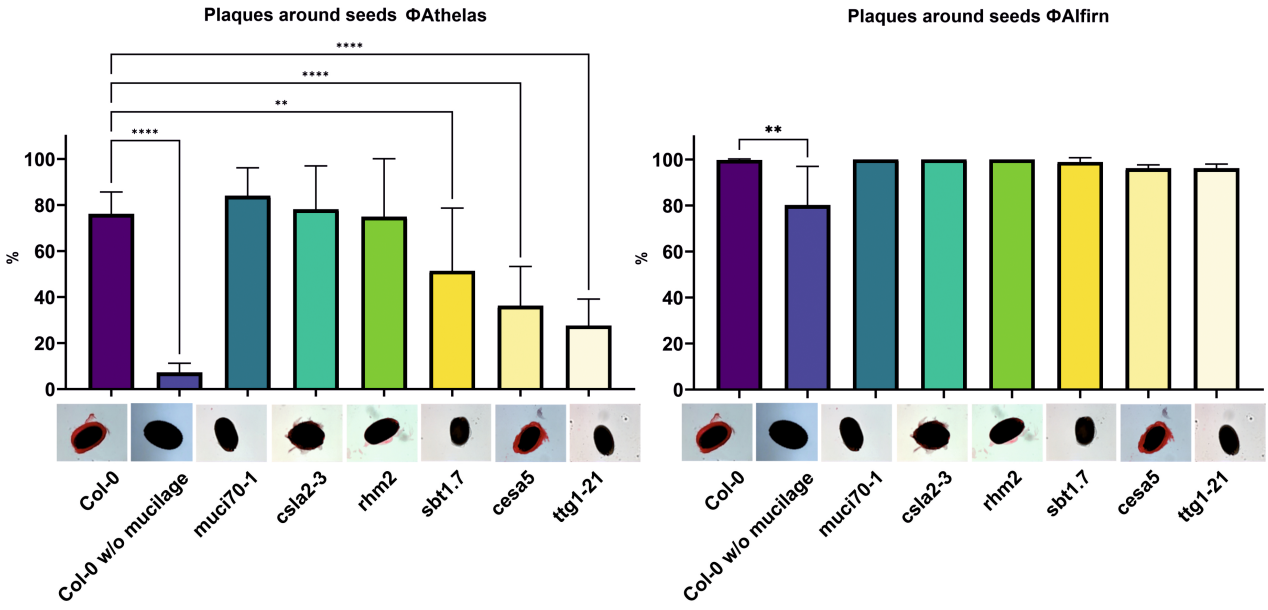

FIGURE 4 Phage seed coating on different *Arabidopsis* mutants. Left panel phage Athelas; right panel phage Alfirin. Shown is the amount (%) of plaques detected on a double agar overlay post seed coating of the *Arabidopsis* mutants: *muci70-1, csla2-3, rhm2, sbt1.7, cesa5* as well as the wild type *A. thaliana (Col-0)* and mechanically removed wild-type seeds. Below each column, the respective seed stained with 0.01% ruthenium red solution is depicted. The experiment was performed in three independent replicates for each SCM mutant and the number of seeds showing a lysis zones as well of the total number of seeds per plate was counted; N 50–300 seeds per plate. A two-way ANOVA was significant for phage Athelas F (7, 83) = 27,37 and showed one significant difference for phage Alfirin F (5, 17) = 3569. For both a subsequent HSD followed: **p < 0.01; ***p < 0.001; ****p < 0.0001.

control that does not require mucilage. The *rhm2, clsa2-3* and *muci70-1* (Table 2) mutants showed no significant influence on phage binding, indicating that pectin does not seem to be required for phage binding. TRANSPARENT TESTA GLABRA1 (TTG1) is a master regulator involved in many processes and is required for mucilage production. The mutant *ttg1-21* had a significant impact on phage binding for phage Athelas, which confirms the observation that removal of the mucilage impacts phage Athelas binding to seeds. The second strongest impact was observed for the cellulose synthase five mutant (*cesa5*) that is required for the production of cellulose within the mucilage and for the correct layering of the mucilage (Sullivan et al., 2011). Deletion of *cesa5* was reported to cause a reduction of diffusible cellulose within the

TABLE 2 *Arabidopsis* mutants used in this study and their physiological effects.

Mutant name	Gene name	NASC number	Physiological role	Reference
ttg1-21	Transparent testa galbra 1	GK-580A05	Master regulator with pleiotropic roles in, e.g. trichome initiation, anthocyanin biosynthesis, and seed coat mucilage biosynthesis. Deletion leads to the absence of SCM and the name giving transparent testa phenotype.	Tian and Wang (2020)
sbt1.7-1	Subtilisin-like serine protease 1.7	n.a	Plays a role in seed mucilage maturation by degradation of pectin methylesterase inhibitors. Deletion leads to severe mucilage extrusion defects.	Rautengarten et al. (2008)
cesa5-1	Cellulose synthase 5	N2106719	Cellulase synthase subunit 5 is expressed specifically in epidermal cells and coincides with the accumulation of mucilage polysaccharides in the SCM. Deletion leads to the repartitioning of mucilage pectin and the absence of diffusible cellulose within the mucilage, while the crystalline cellulose is not affected.	Griffiths and North (2017)
csla2-3	Cellulose synthase-like a2	SALK_149092	It is involved in the biosynthesis of mucilage glucomannan and the structuring of the crystalline cellulose in the adherent mucilage. Deletion leads to the repartitioning of mucilage pectin and the absence of crystalline cellulose rays within the mucilage, while the diffusible cellulose and total sugar content are not affected.	Yu et al. (2014)
rhm2/mum4	rhamnose biosynthesis 2/mucilage modified 4	SALK_076300	Required for the synthesis of pectinaceous rhamnogalacturonan I, the major component of *A. thaliana* mucilage Deletion leads to the reduction of pectin synthesis and, thereby to smaller total amounts of mucilage.	Usadel et al. (2004); Oka et al. (2007)
muci70-1	Mucilage related 70	SALK_129524	It is a pectin-related galacturonosyltransferase located in the Golgi apparatus? Deletion leads to a reduction of pectin in the SCM, shorter rhamnogalacturonan I (RGI) chains and, xylan substitution and over all smaller mucilage capsules around the seeds.	Voiniciuc et al. (2018)

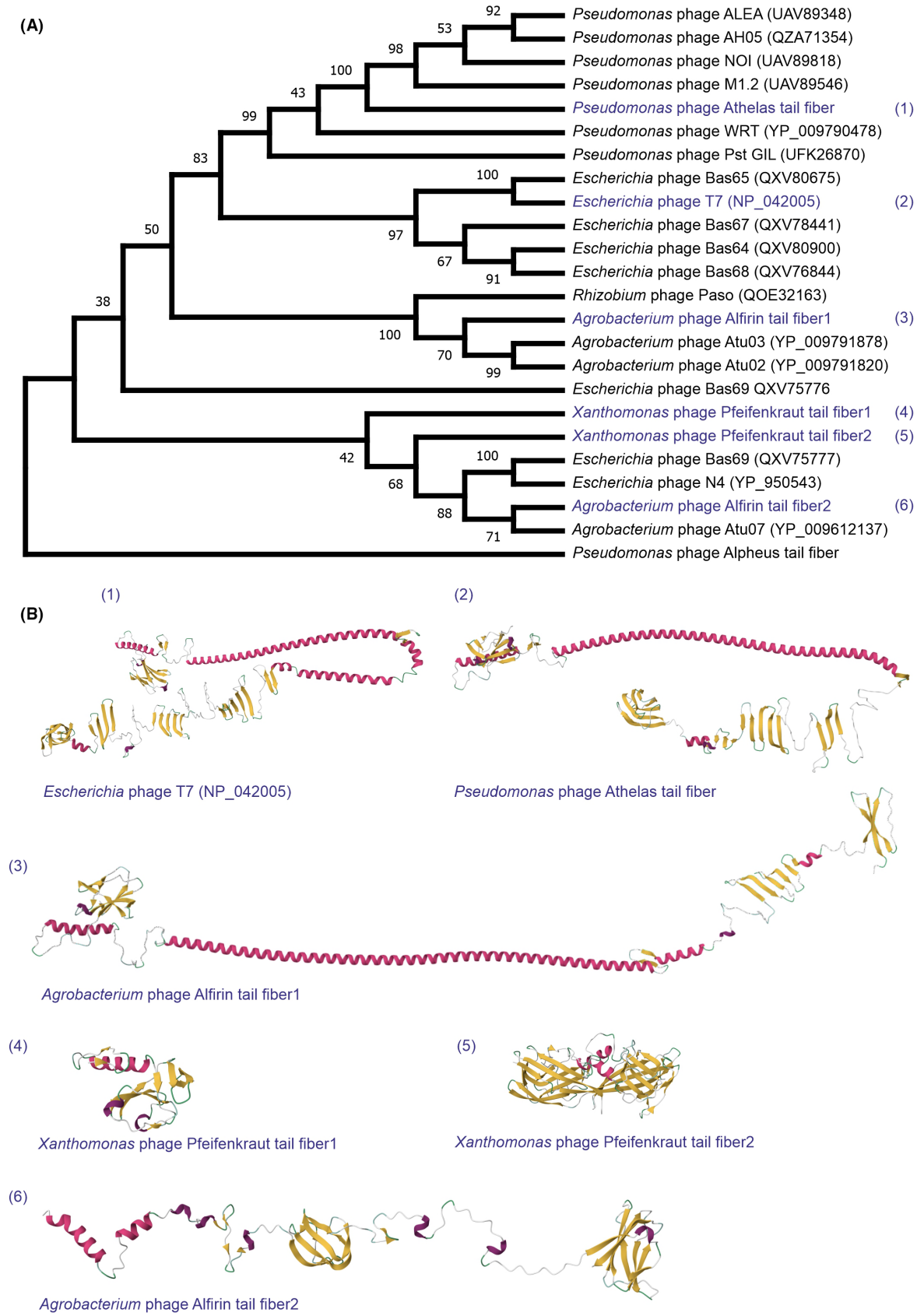

FIGURE 5 Phylogenetic tree of phage receptor binding proteins (RBPs) and in silico folding. (A) Phylogenetic tree of phage RBPs. Ancestral states were inferred using the Maximum Likelihood method (Nei & Kumar, 2000) and the JTT matrix-based model (Jones et al., 1992). Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021). (B) Protein structures of selected phage RBPs. The 3D protein structures were predicted using the ColabFold v1.5.3 webserver (Mirdita et al., 2022). N-termini are displayed in the left upper corner of each protein model.

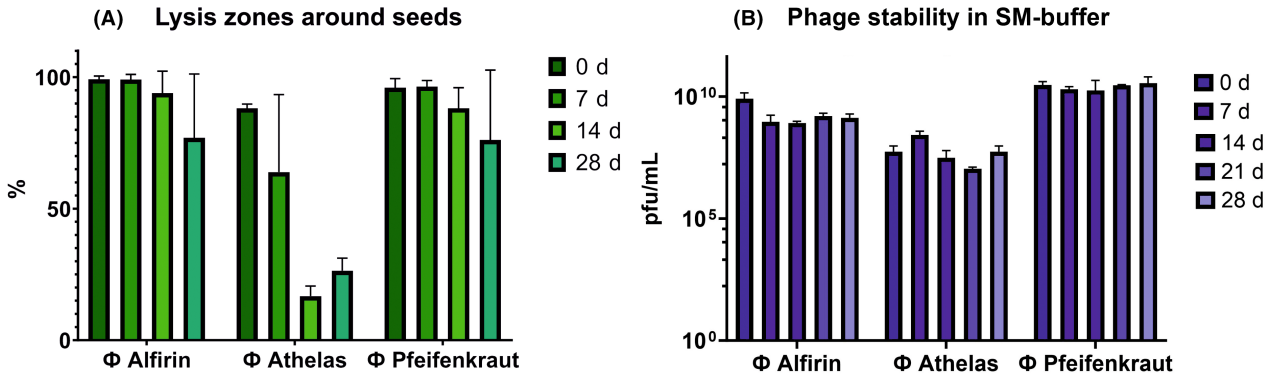

FIGURE 6 Stability of phage on *Arabidopsis* seeds stored at 4°C. (A) Shown is the percentage of seeds showing plaques when plated on a double agar lawn containing the host bacterium (*A. tumefaciens* for phage Alfirin, *P. syringae* for phage Athelas, and *X. translucens* for phage Pfeifenkraut). Portions of 50–200 seeds were tested at every time point indicated for each phage. (B) Control of phage storage in phage buffer. Displayed are the means of the three biological replicates, error bars indicate standard deviation.

Arabidopsis mucilage. Also, the *subtilisin-like serine proteases 1.7* (*sbt1.7*) mutant did impact Athelas adhesion to the seed. This result hints at the importance of accessible mucilage sugars for Athelas since sbt1.7-mutant does not release mucilage properly upon hydration (Rautengarten et al., 2008). Transmission electron microscopy further supported our hypothesis that Athelas directly interact with polymeric fibre structures within the mucilage of *Arabidopsis* (Figure S4).

As expected, phage Alfirin was not affected by the mutants tested in this study, which is consistent with previous experiments showing that the absence of mucilage only weakly affected phage Alfirin.

Comparative analysis of phage tail fibres

Testing of phage binding to different *Arabidopsis* mutants suggested the relevance of the mucilage polysaccharide fraction, more precisely, the diffusible cellulose, on phage binding. From previous studies, it is known that *E. coli Autographiviridae* (T3, T7 and Bas64-Bas68) recognize components of bacterial lipopolysaccharides (LPS) as a receptor (Ando et al., 2015; Maffei et al., 2021). This might indicate a similar mechanism for phage Athelas. To gain more insights into a potential specific chemical interaction with the seed mucilage, a set of RBP from the phages in this study and close relatives was compared phylogenetically and structurally by in silico folding of the proteins (Figure 5). To further investigate the cause of this differential binding behaviour of phages with a similar capsid size as well as a short tail, we compared the host binding proteins of those phages. The phylogenetic tree of the tail fibres revealed that the Basel *Autographiviridae* are a sister group to *Pseudomonas* phage Athelas, which was also highly dependent on the mucilage Figure 5A. The similarity of the tail fibres of these groups can also be seen in the structural 3D model computed with Alphafold (Figure 5B 1 and 2). The *E. coli Autographiviridae* (T3, T7 and Bas64-Bas68) were found to be dependent on the bacterial LPS in previous studies (Ando et al., 2015; Maffei et al., 2021). This hints into the direction of a similar mechanism for phage Athelas. Direct sequence comparison revealed that the N-terminal region showed higher conservation between those two regions than the C-terminal fraction. Another interesting observation is that phage Alfirins second tail fibre clusters together with phage Bas69 tail fibre which also was not significantly impacted by the removal of the mucilage.

Shelf life of phages on seed surfaces

High stability of infectious phage particles on seed surfaces is a prerequisite for the establishment of effective phage-based biocontrol strategies. To test for this, we conducted experiments to determine the storage stability of phages when attached to *A. thaliana* Col-0 seeds. For a timespan of more than 4 weeks, phages Alfirin and Pfeifenkraut showed high levels of stability when stored at 4°C. Phage Alfirin showed a binding of 99% and showed lysis zones for over 28 days and beyond (Figure 6A,B). A similar pattern was observed for phage Pfeifenkraut, with an initial average binding of 96%. Phage Athelas showed a lower initial binding with 88% and a notable reduction after 14 days (Figure 6 left). As phages were initially in SM buffer before coating, we performed a control experiment and could show that lysis zone creation was stable for all three phages for 28 days and beyond (Figure 6 right).

'Boosting' local phage amplification at plant seeds

We further investigated the potential of locally increasing the amount and longevity of phages *in planta*, by harnessing the phage's self-propagating ability. We co-inoculated phages together with a non-pathogenic

version of the host bacterium in low concentrations in addition to the phage. Provision of host cells for the phage was expected to lead to an amplification of the phage population, to keep phages in the system from the seed to the seedling. This, if successful, we hypothesized, would reduce the need for large-scale production of phage lysate prior to field application, which is impractical when envisioning phage usage by farmers. Additionally, the non-pathogenic bacterium might inhabit a somewhat similar niche as the pathogens in the soil microbiome, providing further competition for resources outside the plant. We tested this approach with *Agrobacterium* phage Alfirin, by adding a non-virulent *Agrobacterium* strain without the tumour-inducing plasmid (delta Ti) required for infection of the plant (Morton et al., 2014). We tested this local 'boost' of phage production with *Arabidopsis*. We could show that the survival rate and the leaf area are not significantly decreased in plants treated with the phage (Booster MOI5) compared to the control condition (Figure 7A,B). Nevertheless, we have to state that inoculation with the non-virulent bacterial strain still harmed the growth of the plants. This observation proposes a potential response to the 'non-virulent' bacterium as well. The leaf area production was affected significantly by the non-virulent as well as virulent strains. After 14 days in the climate chamber, we checked how many plants still had active phage particles on their surface and therefore transferred the seedlings to a bacterial lawn of *A. tumefaciens* and checked for occurring lysis (Figure 7C). Only plants in the booster condition still showed clear lysis zones, this is indicating that the additional delivery of a non-virulent-host in combination with the phage can improve phage longevity *in planta*, and should be investigated further as an application strategy, whereby the effect of the bacterium used to propagate the phage on the plant must be carefully considered.

DISCUSSION

Bacteriophages are still an untapped resource that could advance sustainable biocontrol strategies of plant pathogenic bacteria. This is due to the main characteristics of lytic phages: host specificity and the ability to self-propagate. In this study, we investigated the binding of phages to *Arabidopsis* seeds with a special emphasis on the influence of the SCM during this interaction. We confirmed the binding of all phages tested and observed that for some, the SCM is crucial for successful seed binding. We linked this dependence to specific mucilage components. Finally, we move towards more application-oriented questions, affirming the stability of phages on mucilage-producing seeds. Additionally, we observed enhanced seed/seedling viability under pathogenic pressure.

The importance of protecting seeds and young plant parts against pathogenic microbes cannot be overstated. In fact, bacterial transmission via seeds was reported with significant yield losses in many cases (Burdman & Walcott, 2012; Darrasse et al., 2010, 2018; Giovanardi et al., 2018; Johnston-Monje et al., 2021; Mansfield et al., 2012; Morris et al., 2007; Shade et al., 2017). The application of phages as a treatment strategy has gained special interest in recent years (Holtappels et al., 2022; Ogunyemi et al., 2019; Voronina et al., 2019). Successful treatment of plant seeds has recently been demonstrated for *Xanthomonas* (*Xcc*) in cabbage, for example, as a potential treatment in plant nurseries. Here, the authors showed significant symptom reduction and seed cleaning of artificially contaminated seeds when applying high phage concentrations (Holtappels et al., 2022). A further study showed the protection of rice seedlings with phages against *Xanthomonas oryzae* (Xoo), with an emphasis on pre-infection phage treatment, since this showed the strongest protection (Ogunyemi et al., 2019). Apparently, also the pre-treatment of seed tubers increased plant germination, as shown for potatoes (Voronina et al., 2019).

Altogether, these studies emphasize the high potential of seed- and pre-treatment strategies, but the mechanisms by which bacteriophages are kept in close proximity to the seeds or young plant parts and their interaction with surface components remain to be understood. In this study, we demonstrated that for certain types of phages, the SCM has a crucial part in the binding process to the seed. Our experiments have further validated the remarkable stability of infectious phage particles on seed surfaces, extending beyond a period of 4 weeks, which likely can be further improved by optimizing seed coating formulations. This will open up a variety of options for future applications on seeds that do not produce mucilage naturally.

Mucilage shows multiple independent origins throughout plant evolution (Yang et al., 2012), probably due to its functions like maintaining a moist environment for the seedling in a microenvironment, anchorage to soil and increased dispersal (Kreitschitz et al., 2021). On top of that, the mucilage could also be an additional layer of defence against unwanted bacteria by entrapping bacteriophages in close vicinity of the seeds and root tips. In our study, all tested phages bound to *Arabidopsis* seeds with mucilage. When the mucilage was mechanically removed, phage binding decreased significantly. However, phage-specific differences were noted. While phage Athelas and other members of the *Autographiviridae* family showed a very strong dependence on mucilage for binding, phage Alfirin also showed interaction with the seed surface in the absence of the mucilage. It would be interesting to investigate if the phage-mucilage dependency emerged as an adaptive trait for some phages that have become

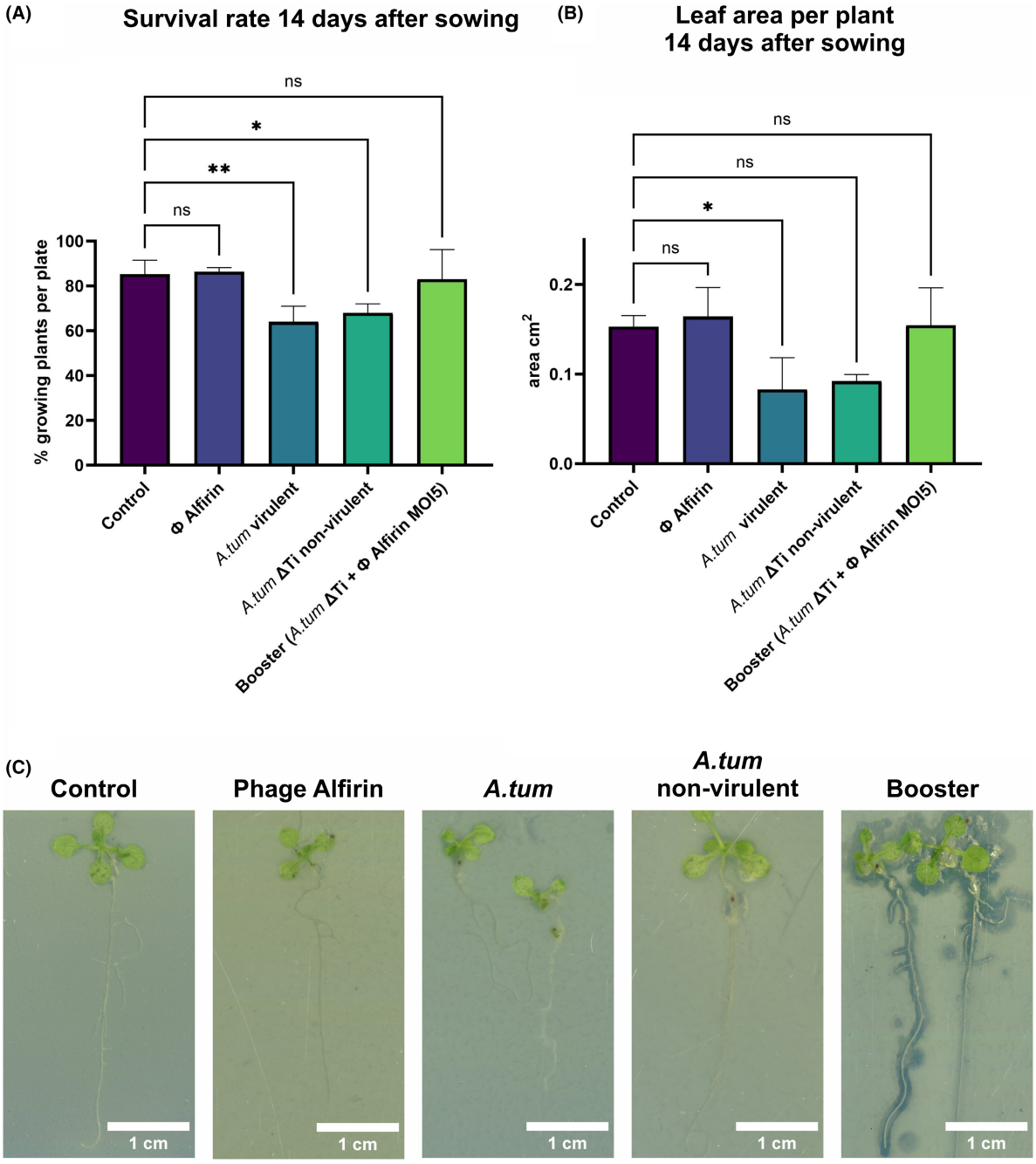

FIGURE 7 Local phage amplification acceleration with a non-virulent bacterial host. (A) Leaf area after 14 days ($N = 25$) the experiment was performed in triplicates. ANOVA; $F(4, 23) = 5734$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns = not significant. (B) Amount of plants with active phages after 14 days, quantified were plants showing lysis zones upon plating on a double agar containing *Atum*. Shown is the mean of 25 plants, with standard deviation. (C) Images of seedlings transferred to a bacterial lawn. The treatment conditions consisted of Control: plants alone; Phage Alfirin: seeds treated with the phage only; Atum OD_{600} 0.4 is using a virulent version of the pathogen; Atum ΔpTi OD 0.01—*Agrobacterium fabrum C58* ΔpTi, 'Booster' uses a non-virulent version of Atum ΔpTi OD_{600} 0.01 and phage Alfirin at an MOI 5. Lysis zones indicate phage presence.

integral to the plant-microbiome through co-evolution during the process of plant domestication (Cordovez et al., 2019).

In the study of the adhesion process, we differentiated between two mechanisms: (i) adhesion based on a physical structure of the polymer-matrix, where the matrix would function as a mesh with a pore size between 2 and 50 nm (Sanka et al., 2017) or (ii) adhesion based on chemical interactions between phages and seed mucilage components. The latter was approached by the systematic testing of seed coat mutants of the model plant *A. thaliana*. Here, phage Athelas showed the strongest effect on the *transparent testing galba 1* (*ttg1*) mutant, followed by the

cellulose synthase 5 (*cesa5*) and *subtilisin protease 1.7* (*sbt 1.7*) mutants. Both the *ttg1* and the *sbt1.7* mutants are indicative of the fact that mucilage formation/release is a prerequisite for adhesion of phage Athelas. TTG1 is a master regulator in *Arabidopsis* involved in many processes, including the production of the SCM (Ranocha et al., 2014), and its deletion leads to seeds that produce no mucilage. The deletion of *sbt 1.7* is described as a non-release phenotype because it is needed for the regulation of pectin methylesterases, which are crucial for mucilage release in *A. thaliana* seeds (Rautengarten et al., 2008). Most interestingly *cesa5* which is still releasing the mucilage, but possesses less diffusible cellulose in the mucilage (Griffiths & North, 2017), shows a significant reduction in binding of phage Athelas. This result suggested that phage Athelas requires this diffusible cellulose fraction for binding to the seed surface. This interaction could potentially be based on the attachment of phage tail fibres to the glucose units of the diffusible cellulose. Further evidence for this hypothesis is supported by the fact many bacterial genes are capable to produce cellulose as part of their biofilm and phages interact with them (Visnapuu et al., 2022). This was also reported for the plant pathogen *Pseudomonas syringae* (Dutta et al., 2019; Pérez-Mendoza et al., 2019). A similar observation was recently made *for Erwinia amylovora* phage S6 (Knecht et al., 2022). Nevertheless, further studies will have to test whether this hypothesis holds true and to identify the specificity determinants for this transient interaction.

While phages Alfirin and Pfreifenkraut showed high stability on seeds, it remains unclear why the infectivity of phage Athelas dropped drastically on seeds already after 14 days of storage, while it showed high stability in phage buffer (Figure 6). One possible explanation could be that the mucilage-polysaccharides are able to trigger the DNA ejection. A similar observation was described for bacterial LPS-triggered release of the phage genome in podovirions (Andres et al., 2010; González-García et al., 2015; Molineux, 2001). Another possibility could be that the drying process impacts phage Athelas stability, which could be theoretically overcome by adding stabilizers used in classical phyllosphere-phage formulations (Balogh et al., 2010).

The stability and titre of phages on seed surfaces can certainly be improved by optimizing seed coating formulations. Here, knowledge gained regarding the specificity determinants of chemical interactions will provide a powerful basis to improve the composition of seed coatings. This could be especially useful for plants that do not produce mucilage naturally. First attempts into the direction of artificial seed coating with chemical formulations have been reported, for example, in maize by chemical deployment of phages with polyvinylalcohol (Kimmelshue et al., 2019). Further, the application of non-virulent host species could serve as a way to amplify the effective phage titre in the proximity to the plant and thereby enhance protection. Nevertheless, the effect of the bacterium used for phage amplification must be considered carefully as emphasized by the results of this study. Apparently, the presence of a bacterium that lacks its virulence clusters can still have a negative effect on plant growth, potentially by activating a more general plant response to the detection of microbe associated molecular patterns, for example, flagellin or LPS (DeFalco & Zipfel, 2021; Newman et al., 2013).

In summary, the results reported in this study show effective binding of phages to *Arabidopsis* seeds and further emphasized that some phages, particularly podoviruses belonging to the *Autographiviridae*, strongly depend on chemical interactions with the SCM. Phage-based biocontrol on the seed level certainly has great potential for application. The chemical universality of some carbohydrates present in bacterial LPS might allow the targeted binding of phages to plant surfaces displaying similar sugar moieties. A better understanding of the molecular basis for these transient interactions, therefore, has a high potential for the establishment of targeted phage delivery strategies with a high relevance for applications in agriculture and medicine. Further clearing of seeds with phages was shown to be an effective strategy for selective seed cleaning against pathogenic bacteria, leaving the beneficial microbiota intact.

The ecological significance of our discovery that mucilage can bind phages raises important questions that merit further investigation. Is this binding merely coincidental, or is there a conserved chemical nature in the mucilage-microbe interface across different kingdoms of life? Would this also lead to a transmission of phages from plants to the next generation? These questions are certainly highly relevant in the context of plant-microbe interactions but also in the context of effective phage-based biocontrol strategies.

AUTHOR CONTRIBUTIONS

Sebastian H. Erdrich: Conceptualization; formal analysis; investigation; methodology; validation; visualization; writing – original draft; writing – review and editing. **Ulrich Schurr:** Conceptualization; funding acquisition; supervision; writing – review and editing. **Julia Frunzke:** Funding acquisition; supervision; writing – original draft; writing – review and editing. **Borjana Arsova:** Conceptualization; funding acquisition; project administration; supervision; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The annotated genomes were deposited on NCBI via Bankit and are available under the accession number OR997969 (Alfirin) and OR997970 (Athelas). Further data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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