Understanding the dynamics of Plant – Bacteria – Bacteriophage interactions as a means to improve plant performance

Inaugural Dissertation

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‘‘Look at a plant in the midst of its range! Why does it not double or quadruple its numbers? We know that it can perfectly well withstand a little more heat or cold, dampness or dryness, for elsewhere, it ranges into slightly hotter or colder, damper or drier districts. In this case, we can clearly see that if we wish in imagination to give the plant the power of increasing in numbers, we should have to give it some advantage.’’

Charles Darwin (1809-1882)

‘‘... on opening the incubator, I experienced one of those rare moments of intense emotion which reward the research worker for all his pains: at first glance, I saw that the broth culture, which the night before had been very turbid, was perfectly clear: all the bacteria had vanished... as for my agar spread it was devoid of all growth and what caused my emotion was that in a flash I understood: what causes my spots was, in fact, an invisible microbe, a filterable virus, but a virus parasitic on bacteria. Another thought came to me also, If this is true, the same thing will have probably occurred in the sick man. In his intestine, as in my test-tube, the dysentery bacilli will have dissolved away under the action of their parasite. He should now be cured.’’

Felix D’Herelle (1873-1949)

Publications

The research presented in this dissertation has been published in the following manuscripts:

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Forschungszentrum Jülich GmbH. Matrix-vermittelte Beschichtung von Zielorganismen mit Bioeffektoren. German patent application ZUEV82050053160505022019 (2023). (Inventors: **Erdrich, S.H**., Arsova, B., Frunzke, J., & Schurr, U.)

Abbreviations

Atum	*Agrobacterium fabum C58*
DNA	Deoxyribonucleic acid
Cfu	Colony forming units
DAMP	damage associated molecular patterns
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
e.g.	exempli gratia
et al.	et alia
HR	hypersensitive response
MAMP	microbe associated molecular patterns
PCR	Polymerase chain reaction
Pfu	Plaque-forming units
Pss	*Pseudomonas syringae pv lapsa*
Pst	*Pseudomonas syringae pv tomato*
RNA	Ribonucleic acid
RNA-seq	RNA-sequencing
ssDNA	Single -stranded DNA
ssRNA	Single-stranded RNA
T3SS	Type 3 secretion system
TEM	Transmission electron microscopy
Xcc	*Xanthomonas campestris pv. campestris*
Xoo	*Xanthomonas oryzae pv. oryzae*
Xtt	*Xanthomonas translucens pv. translucens*

Further abbreviations not included in this section can be found in the JCB abbreviation list under the following link: `http://jcb.rupress.org/content/standard-abbreviations`.

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1 Summary

Plant protection is crucial in the context of a secure food supply. With antibiotic-resistant bacteria on the rise, we explore new, sustainable plant protection strategies and utilise naturally occurring bacterial viruses to counter pathogenic bacteria. These viruses, known as bacteriophages, are highly specific and outnumber bacteria by a factor of ten, and are present in every habitat on Earth. Despite their abundance, the number of available isolates for plant pathogenic bacteria is still very limited. The bacterial genus of *Xanthomonas* contains many well-known plant pathogens with the ability to infect some of the most important crop plants, causing significant economic damage. Unfortunately, classical pest control strategies are neither particularly efficient nor sustainable.

Investigating phage-based strategies, we set the foundation in our lab by isolating seven novel *Xanthomonas* phages (Langgrundblatt1, Langgrundblatt2, Pfeifenkraut, Laurilin, Elanor, Mallos, and Seregon). As part of this PhD project, we further characterised, classified and tested them for their biocontrol potential *in vitro*. Besides good prerequisites for subsequent *in planta* experiments, we established four taxonomic novel genera.

With seeds being one of the major transmission routes for bacterial pathogens in agriculture, we tested strategies to protect plants from the early stages. Therefore, phages for two important crop pathogens, *Pseudomonas syringae* and *Agrobacterium fabrum (tumefaciens),* were isolated and tested for their interaction with the seed coat mucilage, deepening the understanding of seed-based biocontrol. Some of the tested phages were highly dependent on mucilage for seed binding, whereas podophage Athelas showed the highest dependency. The significance of this observation was broadened by testing further podoviruses of the *Autographiviridae* family obtained from the systematic *E. coli* (BASEL) phage collection. These showed a similar dependence on the mucilage for seed adhesion.

Phage coating effectively increased the survival rate of plant seedlings in the presence of the pathogen. Long-term activity tests revealed a high stability of phages on seed surfaces. The utilisation of non-virulent host strains was further successfully applied to enrich the presence of infectious phage particles on seed surfaces. Altogether, our study highlights the potential of phage-based applications as sustainable biocontrol strategy at the seed level.

A further part of this work aimed at gaining a molecular understanding of the tripartite interaction between plants, bacteria, and phages in a novel tripartite transcriptomics approach. We aimed to fill the knowledge gap on how the plants gene expression is responding during phage-based biocontrol. For this purpose, a gnotobiotic system was used to study infection of *Arabidopsis thaliana* with the plant pathogen *Xanthomonas campestris*. Here, the application of the *Xanthomonas* phage Seregon could successfully counteract the bacterial infection almost to the level of the uninfected control. Additionally, we observed a significant variation in the expression of defence-related genes throughout the tripartite interaction. While *X. campestris* inoculation led to expression of several salicylic acid responsive genes like WRKY70 and WAK1, the treatment of *X. campestris* with phage Seregon led to a significantly reduced upregulation of these genes. We also identified GRP3.1 as uniquely upregulated in response to phage-based control of *X. campestris*. In summary, this thesis offers unprecedented insights into the molecular-level tripartite interactions between plants, bacteria, and phages, thereby establishing a crucial foundation for the development of sustainable biocontrol strategies in agriculture utilizing phages.

2 Scientific context of this thesis and key results

2.1 Biocontrol of pathogens

In the face of an ever-expanding global population estimated to exceed 9 billion people by 2050, the persistent rise in food demand presents an existential challenge (Gilland 2002). This challenge is exacerbated because most arable land is currently being put to use, leaving little room for expanding agricultural areas. Furthermore, extreme weather conditions resulting from climate change pose a significant threat to the already limited agriculturally usable areas. The necessity for increased yields cannot be met simply by expanding land use. Instead, it underscores the imperative to minimise losses throughout the production process. One important contributor to losses is the annual crop loss due to diseases, with approximately 10% succumbing to the destructive impact of pathogenic microbes. Pathogens such as fungi and pathogenic bacteria play a significant role in this loss (Ahmed et al. 2022; Strange and Scott 2005; Tillman et al. 1999), causing widespread damage to important crops like e.g. potatoes, tomatoes, beans, cereals, apples, and *Brassicaceae*-crops undermining global food security during larger outbreaks. Of notable concern is the alarming trend among pathogenic bacteria to increasingly aquire resistance to classical control strategies. These strategies, like antibiotics and copper, which have traditionally served as measures against agricultural diseases, are becoming less effective over time due to resistance emergence(s) in recent years (McManus et al. 2002; Zhang et al. 2015).

2.1.1 Bacterial pathogens

Pathogenic bacteria cause losses in many important crops with the genera of *Pseudomonas, Ralstonia, Agrobacterium, Xanthomonas, Erwinia, Xylella, Dickeya, Pectobacterium,* and *Clavibacter* being of large economical and scientific importance (Mansfield et al. 2012). Prominent examples are apple (*Erwinia amylovora*), potato (*Pectobacterium carotovorum*), kiwi (*Pseudomonas syringae pv. actinide*), olives (*Xylella fastidiosa*), rice (*Xanthomonas oryzae pv. oryzae*), and cabbage (*Xanthomonas campestris pv. campestris*)(Table 2.1). The genus *Xanthomonas* contains multiple plant pathogens causing devastating losses in a wide variety of plants, from the *Poaceae* family (including food staples like rice, sugar cane and wheat) to the *Brassicaceae* family (including food crops cabbages, broccoli and oil seed). Also, the genus *Pseudomonas* includes a vast array of plant pathogenic strains affecting many important crop species, infecting *Solanaceae* causing issues such as bacterial leaf spot in tomato. Citrus fruits, stone fruits and many other crops can also get infected by different *Pseudomonas* strains (Hoefte and De Vos 2007). Nevertheless, the list is not exhaustive; many more examples could be cited, with an excellent overview of the most relevant ones given by Mansfield et al. (2012).

Bacterial plant pathogens have evolved in many ways to exploit eukaryotic energy resources (Fatima and Senthil-Kumar 2015). While not all bacteria have evolved into this route, some can be important eukaryote allies. The bacteria discussed here use the plants more as a resource than a mutualistic partner. Originally, three main lifestyles for pathogens were defined: 1) the biotrophic, where the pathogens invade the host plant and live from its resources (Glazebrook 2005); 2) the necrotrophic, where they kill the plant and feed on the dead material (Laluk and Mengiste 2010); and 3) the semi-biotrophic, where a bacterium uses both strategies to exploit the plant before killing it. Recent studies indicate that these categories should be understood less as strictly divided categories and more as a continuum, falling between the extremes of killing the plant and living within it (Kraepiel and Barny 2016). For the sake of clarity, we are sticking with the classical categories here. Biotrophs, like *A. tumefaciens* and *P. syringae,* invade the plant through root cracks or stomata after reaching critical density. Once in the apoplast, the biotrophic bacterium represses the host defence response by expressing virulence factors (Xin et al. 2018) to establish their population within the niche. Some biotrophs like *Agrobacterium* go even a step further: after suppressing the host defence , they start simulating tissue growth (gall growth and hairy roots) and reprogram the plant to produce opines (Yamada and Nihira 1999), a nutrient

source preferred by the bacterium (Gohlke and Deeken 2014; Lee et al. 2009; Tarkowski and Vereecke 2014).

Semi-biotrophic pathogens like *Xanthomonas* start out in a similar way when not transmitted by seed. They also enter the plant through natural openings or wounds and accumulate in the mesophyll or the xylem (Ryan et al. 2011). For example, *Xanthomonas campestris* (Xcc), as a vascular pathogen of this genus, starts to build biofilms within the vascular bundles to feed on the plant's minerals. Eventually, the biofilm clogs the bundle completely, which leads to an undersupply of plant tissue above the clogged vessel; in addition, the bacteria alter the plant's hormone regulation, leading to the death of the tissue. This marks the transition from the biotrophic to the necrotrophic lifestyle, where *Xcc* feeds off dead plant cells to gain nutrients (Islam et al. 2021).

The necrotic pathogens have no need to enter the living plant since they only feed on dead plant material. Therefore, they skip the whole tissue invasion completely and destroy the tissue from the outside (Laluk and Mengiste 2010). To rapidly kill the plant cells, they overwrite the plant defence, not to suppress it, but to disrupt its balance and use the resulting self-destruction mechanisms of the plants to its advantage. This self-destruction mechanism, known as hypersensitive response (HR), is often associated with the rapid production of reactive oxygen species (BalintKurti 2019). After the plant cells, necrotrophic pathogens feed on the dead plant tissue. The plant's only way to defend against them is the heavy fortification of cell walls at the attack site and tight regulation of their own defence system. This necrotrophic nutrient acquisition strategy is predominant in fungi and oomycetes, but *Pectobacterium carotovum* also applies this strategy, infecting roots and tubers of many important crop plants (Charkowski 2018; Kunkel and Chen 2006).

The most important factor for the success of a bacterial infection is to have the right virulence genes for the host plant they are trying to invade. Virulence factors, which include proteins and toxins, are secreted into the plant cell, in many cases through a type 3 secretion system (T3SS), to suppress or alter the host defence signalling (Hacker and Kaper 2000). Other secretion systems have proved important for the delivery of compounds required for successful plant infection (Maphosa et al. 2023; Sole et al. 2015; Tian et al. 2015). One prominent example of these compounds delivered to the plant cell by bacterial secretion systems is the HRP (hypersensitive reaction and pathogenicity) virulence factor. It was first identified in 1990 in *Xanthomonas* (Kamoun and Kado 1990) and fostered research in the

field of plant bacteria crosstalk during susceptibility and resistance (Büttner 2016). In the centre of *Xantomodales* virulence lays the HrpG and HrpX regulon, which is responsible for the expression of a T3SS and delivery of Type III effectors amongst orchestrating many other processes involved in plant cell takeover e.g. production of degradative hydrolases delivered into the extracellular milieu (Teper et al. 2021).

To come up with effective control strategies, it is important to understand how the pathogen functions on the molecular level, how the bacterium causes damage to the plant and how the transmission to other plants is facilitated. All those points, if well understood, could give us leverage to control the pathogen. The main transmission routes for pathogenic bacteria are contaminated seeds, insects, and remnants of infected plants from the previous year within the soil (Gazdik et al. 2021). Additionally, weather conditions like wind and rain can spread bacteria to neighbouring plants. Also, contaminated agricultural machinery contributes to disease transmission from infected fields to new locations (Borkar and Yumlembam 2016).

2.1.2 Classical control strategies

One of the earliest strategies to limit the impact of diseases was the rotation of crops, where the choice of planted crop was altered annually. According to this crop rotation strategy, the same crop is introduced into the soil only once every few yearssulphur (Wezel et al. 2014). Thus, the impact of pathogenic bacteria in the soil (Gazdik et al. 2021) is limited by phasing them out due to the absence of their host. While this was a valid approach during the ages of small-scale decentralised farming, it would require much more effort with large-scale monoculture farming. In the global North, the cultivated agricultural area per farmer has significantly expanded in recent decades. This expansion has resulted in the utilisation of the same machinery across a much larger expanse, increasing the potential for pathogen contact. (Lowder et al. 2021). Also, the demand for profit maximisation has led to several identical crop rotations due to increased demand for individual varieties.

During the 1880s, one of the first major breakthroughs in chemical plant protection was introduced by Pierre-Marie-Alexis Millardet and his discovery of a lime-copper mixture later called the “Bordeaux mixture”. This innovative blend, composed of copper sulfate ($CuSO_4$) and quicklime (CaO), marked a pivotal moment in agricultural history with the introduction of the first fungicide (La Torre et al. 2018). In the following decades, various copper formulations were introduced in agriculture, consolidating copper’s position as a fundamental

Table 2.1: Overview of important bacterial pathogens

Agrobacterium spp.	Infects most dicotyledonous plants with economically relevant ones like: grapevine or stone fruit	crown gall	(Barton et al. 2018; Carlier et al. 2004; Kado 2014; Smit et al. 1996)
Clavibacter michiganensis	Tomato, eggplant, pepper and other members of the *Solanaceae*	bacterial canker	(Eichenlaub and Gartemann 2011; Peritore-Galve et al. 2021)
Dickeya spp.	Potato, tomato, sweet potato, banana, maize, and *Chrysanthemum spp.*	soft rot and vascular wilts	(Hugouvieux-Cotte-Pattat et al. 2023; Toth et al. 2011)
Erwinia amylovora	Apple, Pear and other members of the *Rosaceae*	fire blight	(Billing 2011; Pique et al. 2015)
Pectobacterium spp.	Potato, tomato, paprika, cabbage and displays a broad host range amongst agricultural crops	soft rot or black leg	(Charkowski 2018; Czajkowski et al. 2015)
Pseudomonas spp.	Kiwi, bean, leek, grains, rice, cherry and a broad range of other crops	Bacterial spot, bacterial canker, and leaf streak diseases	(Baltrus et al. 2017; Gullino 2021; Vanneste 2017; Xin et al. 2018)
Ralstonia solanaceum	Potato, tomato, banana and other members of the Solanaceae	Bacterial wilt and black rot	(Peeters et al. 2013; Vailleau and Genin 2023)
Xanthomonas spp.	Rice, crucifers, citrus, pepper, tomato as well as a vast variety of monocotyledonous and dicotyledonous plants	Bacterial blight, black rot, citrus canker, bacterial spot and leaf streak diseases	(Brunings and Gabriel 2003; Büttner and Bonas 2010; Naqvi et al. 2022; Sanya et al. 2022; Thieme et al. 2005; Vicente and Holub 2013; Zhang and Wang 2013)
Xylella facidosa	Olives, grapevines, citrus, coffee, almond, alfalfa and stone fruits	Pierce's disease, olive quick decline syndrome or citrus variegated chlorosis	(Morelli et al. 2021; Redak et al. 2004; Simpson et al. 2000)

tool for crop protection against a vast array of plant diseases (Lamichhane et al. 2018). The use of copper-based solutions provided farmers with an effective means to safeguard their crops even after the detection of the first disease symptoms. This was accomplished by the release of divalent or monovalent copper ions (Cu^{2+} or Cu^{+}) from the "copper solutions". These free copper ions are toxic to the microbes in larger amounts, either by reacting with water and oxygen to produce OH- and causing oxidative damage or by iron replacement in iron–sulphur clusters of crucial enzymes, rendering them inactive (Yu et al. 2023). However, reports of bacteria gaining resistance against copper treatments are dating back to the 1980s (Lamichhane et al. 2018) and have increased frequently in the following decades. This raised concerns and prompted discussions on whether escalating doses could counteract diminishing effectiveness (La Torre et al. 2018). Despite its power in inhibiting microbial threats, copper carries an inherent phytotoxic effect. The accumulation of copper in soil and water bodies, as a consequence of repeated applications, raised environmental alarms. This concern, coupled with a growing awareness of the potential adverse effects on non-target organisms (Karimi et al. 2021), resulted in the implementation of restrictions by regulatory bodies such as the European Union on the amount legally used by farmers. These measures aimed to limit the amount of copper introduced into agricultural practices, emphasising the need for sustainable and environmentally conscious approaches to crop protection (Tamm et al. 2021).

Another broadly applied form of chemical protection of plants is the use of antibiotics. First introduced in the 1950s, streptomycin was the first antibiotic used for plant pest control (Dekker 1963) in apple trees against *E. amylovora,* where it is still in use today. This paved the way for using other forms of antibiotics, such as oxytetracycline, gentamicin, and oxolinic acid. Antibiotics, albeit derived from the same synthesis process, can be differentiated into different classes based on their binding properties. Aminoglycosides like streptomycin inhibit protein synthesis by binding to the A-site on the 16S ribosomal RNA of the 30S ribosome, thereby interfering with functions crucial for bacterial survival (Kotra et al. 2000; Krause et al. 2016). They have become a major strategy in agricultural practices, and their usage has expanded for plant protection against many bacterial genera, e.g. *Erwinia*, *Pseudomonas* and *Xanthomonas*, protecting from diseases like fire blight in apples and pears, bacterial spot in tomatoes and peppers, and canker disease in citrus fruits (Verhaegen et al. 2023). Their biggest advantages are the innocuousness to the plant by only inhibiting prokaryotic growth, easy and quick application, as well as their broad range of efficiency against many strains of bacterial pathogens. Their relatively low price has spread their use even in the present days (Taylor and Reeder 2020). However, their constant usage has led to the emergence of

antibiotic-resistant bacterial strains not only in the hospital setting but also in agriculture, being once again a threat to food production.

2.1.3 Resistance emergence

Due to the aforementioned frequent use of antibiotics in agriculture, many bacterial genera have now acquired resistance to the commonly used antibiotics. Among these antibiotic-resistant bacteria are many that affect important crops. They range from the causal agent of fire blight in apple trees, *E. amylovora*, to *Pectobacterium*, which causes soft rot disease in various plants, resulting in a substantial economic impact on the potato industry. Additionally, different strains of *Pseudomonas* infect a variety of crops (McManus et al. 2002). Bacteria resist antibiotics, despite the variety of structures, biochemical properties, and mode of action of antibiotics, by encoding different resistance mechanisms. The major resistance strategies of bacteria rely on preventing the accumulation of critical amounts of the antibiotic compound at the cellular target. They avoid the consequences of the antibiotic by increased export via transporters or reducing uptake, enzymatic modification of the antibiotic and altering the target protein of the antibiotic via mutation (Munita and Arias 2016).

Resistant strains of *E. amylovora* have been reported in many places, such as the US and New Zealand (Chiou 1995; Palmer et al. 1997). It has forced Israel's agriculture to switch antibiotics completely, exchanging streptomycin with oxynitric acid. However, resistant strains against this antibiotic have already been found (Kleitman et al. 2005). The European Union has banned the use of antibiotics in plant disease control due to the frequent emergence of resistance; nevertheless, streptomycin is still in use for fire blight control in Austria, Germany, and Switzerland following strict regulations (Sundin and Wang, 2018). Also, pathogens like *Pectobacterium* and *Dickeya*, responsible for blackleg and soft rot diseases, are acquiring resistance against antibiotics like streptomycin (Fukasawa et al. 1980; Miller et al. 2022). *Pectobacterium* and *Dickeya* are already responsible for losses of approximately 46 million euros annually in potato production in the EU (Dupuis et al. 2021). With the spread of resistance, this financial damage might increase further. Additionally, there are reports of natural resistance to streptomycin in some *Pectobacterium* strains (Vu et al. 2022).

For *Xanthomonas*, there are several reports of resistance development against antibiotics: e.g., in peach orchards, resistant strains against streptomycin and oxytetracycline of *Xan-*

thomonas arboricola pv. pruni were reported (Herbert et al., 2022). The rice pathogen *Xanthomonas oryzae pv. oryzae* (*Xoo*) is gaining streptomycin resistance in China, with studies suggesting this is caused by mutation of the *rpsL,* a gene encoding the ribosomal protein S12, a protein required for flawless protein production, which is normally targeted by streptomycin (Zhang et al., 2011). Studies in *Xanthomonas campestris pv. vesicola* proved that transposons like Tn5393 are a natural reservoir for streptomycin resistance, which is exchanged between multiple plant pathogenic bacteria (Sundin and Bender 1995).

Making matters even worse, observations of occurring co-resistance to antibiotics and metals like copper are becoming more frequent. Often, multidrug efflux pumps are the cause of resistance. Multidrug efflux pumps function as transporters that can translocate a broad range of compounds from the cell, including molecules like antibiotics and metals like copper. Accumulating both components in soils can incentivise bacteria to maintain multidrug efflux pumps since it is evolutionary beneficial under these conditions, increasing the likelihood of co-resistance occurrence (Glibota et al. 2019; Zhang et al. 2015). Taken together, the prolonged mass use of antibiotics and copper formulations has contaminated our agricultural areas and driven natural selection in favour of resistant pathogen strains, which makes it hard to fight with our current measures. Hence, there is an urgent need for sustainable, adaptable, and environmentally friendly biocontrol strategies. One of the most promising solutions currently under consideration is the utilisation of bacteriophages - viruses which specifically infect bacteria.

2.2 Bacteriophages

Bacteriophages (or phages for short) were first independently discovered by Frederick Twort (1915) and Felix d'Hellere (1918) as bacteria-specific viruses. They earned their name, which translates from Greek (βακτήριον φαγεῖν) as "bacteria-eater", through the observation that they can lyse bacterial cultures or lawns. The potential of using phages for medical and agricultural treatment was recognised early on, with the first field trials conducted in the 1920s and 30s (Mallmann and Hemstreet 1924). Nevertheless, it was not until 1939 that phages were visualised for the first time (Ruska et al. 1939), displaying their morphological diversity. Phages occur in every environment (Dion et al. 2020), and they are estimated to outnumber their bacterial counterparts by a factor of ten, making bacteriophages the most abundant biological entity on Earth (Suttle 2005). Prokaryotic viruses occur not only in large numbers but also have a high morphological variety, from tailed viral particles (Figure

2.1), rod-shaped tubular viruses, and icosahedral tailless spheres, to the more exotic shapes of lemons and ampulla in archaeal viruses (Ackermann 2009, 2007; Dion et al. 2020; Krupovic et al. 2018; Ofir and Sorek 2018).

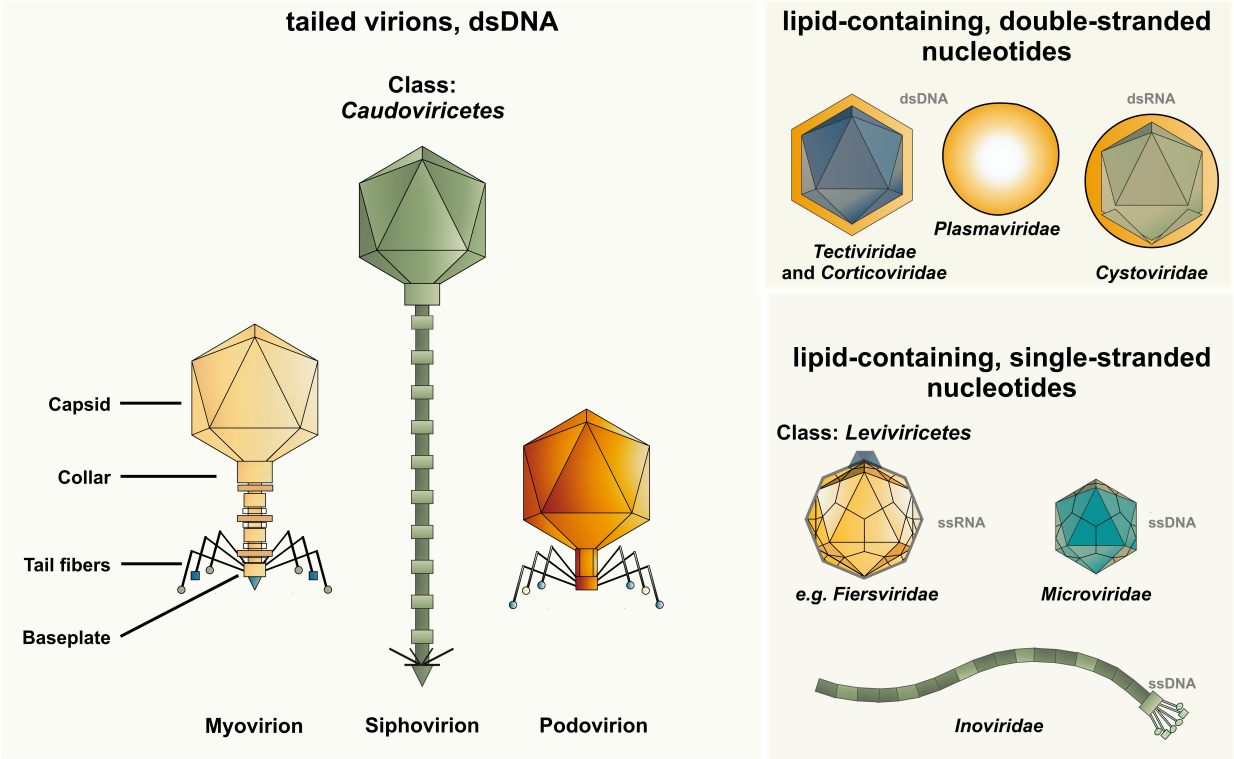

Figure 2.1: **Phage morphology and appearance of distinct virion types.** Phage families are classified by the type of their genetic material and hallmark genes (Lefkowitz et al. 2018). Before the genetic classification, phage families were grouped by the appearance of their virion. Provided is an overview of the immense diversity of phage morphologies, including tailed phages, dsDNA phages, lipid-containing phages with double-stranded genomes and phages with single-stranded genome of both nucleotide type RNA and DNA. The figure was designed based on Ofir and Sorek (2018) and Dion et al. (2020).

Phages, which are diverse in their morphology and genome, can also be classified by their replication strategies. These strategies are known as "lifestyles" and include lytic, lysogenic (also referred to as temperate phages), and chronic, which are the most prominent lifestyles (Figure 2.2). Phages with a lytic lifestyle inject their genetic material into the bacterial host after successfully attaching to the cell surface. They then inactivate or escape the host's defence mechanisms and use the bacterial resources to produce as many viral progenies as

possible before leaving the host. Lysis of the host cell is typically achieved by enzymatically degrading the cell wall from within. Once lysis has occurred, free phages can infect new host cells. Temperate phages also inject their genetic material into the host, but upon infection, they "decide" whether to integrate or directly complete the lytic reproduction cycle. The decision is made based on environmental clues like nutrient status of the bacterium, indicators of stress, and some phages even use their own quorum sensing (QS) molecules to influence this decision (Erez et al. 2017; Gallego Del Sol et al. 2019). If the balance of QS signals or metabolic status of the bacterium tips in direction of integration into the bacterial genome, bacteriophages use enzymes to insert their genome into the bacterial chromosome and replicate alongside the bacterium. Once the bacterial experiences stress the lysogenic phage re-enters the lytic cycle based on SOS-signalling of the host and kills the bacterium (Howard-Varona et al. 2017; Silpe et al. 2023). The phage enzymes used for sequence insertion into the bacterial genomes are called integrases, they mediate site-specific recombination between the bacterial and phage genome by detection of short motives (Groth and Calos 2004). Some temperate phages even have a quorum sensing system, as mentioned above, to keep track of the bacterial population (Erez et al. 2017; Aframian et al. 2021; Bruce et al. 2021).

Phages exhibiting a chronic lifestyle also inject their genome into their bacterial host, but in contrast to the other lifestyles, they continuously produce phage progeny without killing the bacterium. They disperse by secretion of the phage offspring (mostly Inoviruses or lipid membrane phages), but even when not killing the bacteria, they impose a heavy fitness burden on its bacterium (Roux et al. 2019). Essentially, the host is used as a living virus factory, which constantly secretes new viruses through the cell envelope. Even though this classification is useful for categorising bacteriophages, research in recent years uncovers a more nuanced picture, indicating that there is more of a continuum of lifestyles instead of distinct categories of replication (Mäntynen et al. 2021).

Besides the diversity in lifestyles and virion shape, bacteriophage diversity becomes even broader when accounting for genetic diversity, even within a morphologically similar virion class. The International Committee on Taxonomy of Viruses (ICTV) reclassified the prokaryotic viruses, in the past classified by a morphology-based taxonomy based on newly emerging genetic data (Turner et al. 2023). Thus, reclassification led to the abolishment of morphology-based families of tailed phages *Myoviridae*, *Podoviridae*, and *Siphoviridae,* leading to the formation of 2 families, 30 subfamilies, and 321 genera within the class of *Caudoviricetes*.

These developments show how active this field of research is even a century after the discovery of the first "bacteria eaters". Since then, the study of bacteriophages has led to many

observations crucial for our understanding of basic principles in life science and for the development of efficient molecular biology tools (Salmond and Fineran 2015).

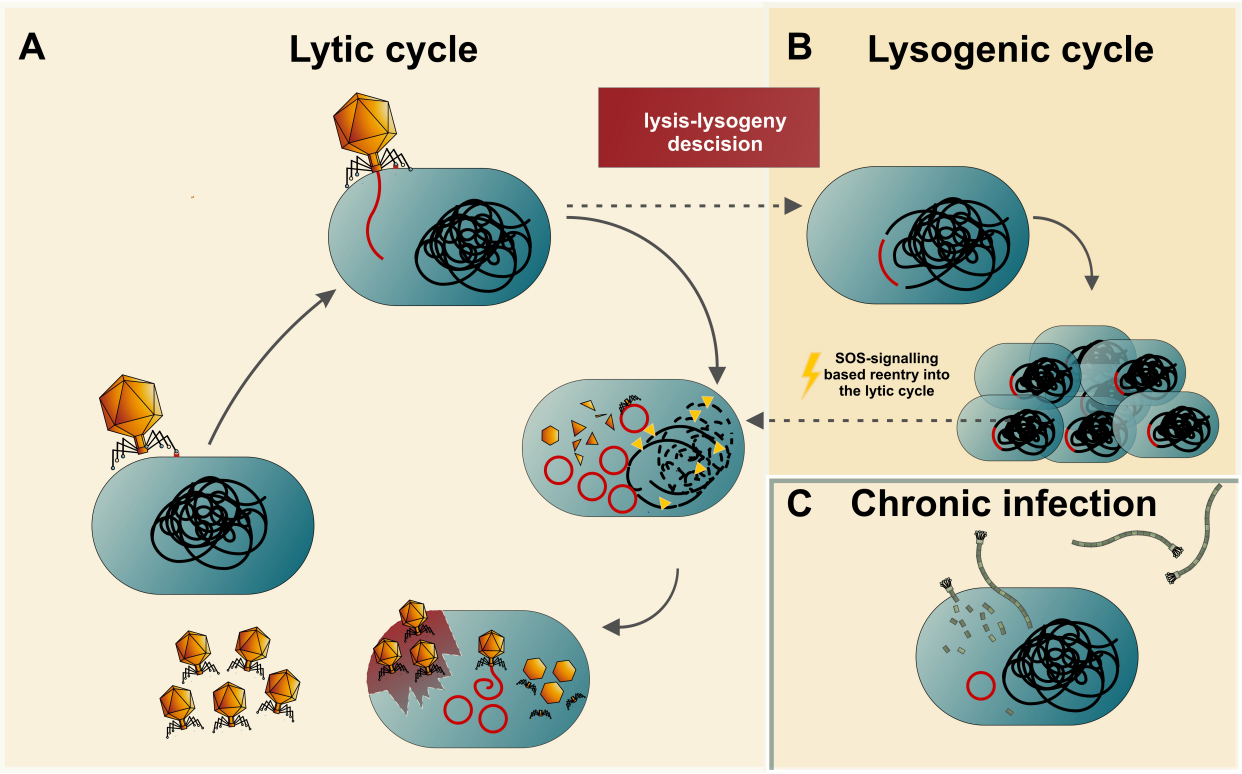

Figure 2.2: **Most common life cycles of bacteriophages**. Phages use a bacterial host as a resource for the production of new phage particles. However, it is important to note that not all phages operate in the same manner: **A) Virulent phages** have a straightforward lifecycle; they identify their host bacterium by binding to a receptor on the bacterial envelope, afterwards, they inject their genome into the host cell and start replicating directly, blocking host defences and degrading its genome at the same time. Once the host resources are used up, phage enzymes lyse the cell from within and release the newly assembled phage particles. Afterwards, the cycle can start over again. **B) Temperate phages** start their infection cycle in the same way as the virulent phages; however, upon injection of the genetic material, they are able to integrate into the bacterial genome and are maintained as prophage during replication of the host. Only when the bacterial host experiences stress, the prophage excises and enters the lytic cycle to produce viral particles. A different strategy is used by **C) chronic phages,** which, upon infection of the host, constantly produce viral particles that are then secreted through the bacterial membrane instead of killing the host by a lysis event.

Table 2.2: Recent phage biocontrol *in planta* selected studies.

Bacterium	**Phage**	**Outcome**	**Study**
Acidovorax citrulli	ACPWH	Showed that coating melon seeds with phage ACPWH leads to both higher germination- and survival-rate in presence of the pathogen.	(Rahimi-Midani et al. 2020)
Acidovorax citrulli	ACPWH	Phage-treated melon plants had an over 80% higher survival rate 20 days after treatment. Further, they showed an artificial translocation of the phage from the soil to the leafs of the plant.	(Rahimi-Midani and Choi 2020)
Acidovorax citrulli	ACF1, ACF8, and ACF12	Translocation of phage ACF1 from soil to different plant parts. The phage was detected in root, hypocotyl and leaf tissue after 24h. Phages remained both in hypocotyl and root tissue for ten days, but were not detectable in leaf tissue after 48h.	(Gasic et al. 2022)
Agrobacterium tumefaciens, *Pseudomonas syringae pv. syringae*, *Xanthomonas translucens*	ϕAlfirin, ϕAthelas, ϕPfeifenkraut	Phages can be stabily coated onto muculiage cntaining seeds for over 4 weeks. Phage Athelas depents on sugars present within the mucilage for adhesion to seeds.	(Erdrich et al. 2024)
Burkholderia glumae	FLC6	Jumbophage with cross-genus activity. They showed suppression of seedling rot disease by FLC6.	(Sasaki et al. 2021)
Burkholderia glumae	FLC8, FLC9, and FLC10	Treatment of rice seedlings leads to a substantial reduction of the disease severity index (DSI) with phage concentrations of 10ˆ7 pfu/mL, with a stronger effect for the Jumbo phages FLC8 and FLC9.	(Kanaizuka et al. 2022)

Table 2.2

Bacterium	**Phage**	**Outcome**	**Study**
Erwinia amylovora	PEar1, PEar2, PEar4, and PEar6	Testing a cocktail of four phages could significantly reduce infection (macerated area) in a pear-based bioassay.	(Akremi et al. 2020)
Erwinia amylovora	EAP1, EAP2, EAP3 and EAP4	In a pear slice assay, they showed that a cocktail of the phages performed similarly to the classically used antibiotic Streptomycin.	(Hassan et al. 2023)
Erwinia amylovora	phages pEa_SNUABM_27 (ϕ27), pEa _SNUABM_47 (ϕ47), Fifi318 (ϕ318), and Fifi451 (ϕ451)	Showed polysorbate and kaolin formulation enhances UV protection and phage durability on leafs for two weeks.	(Jo et al. 2023)
Pectobacterium atrosepticum	Nepra, Nobby, Slant, Lelidair, Gaspode and Momine	A cocktail of six phages was tested and showed a substantial reduction of disease incidence and disease severity of potato soft rot under storage-like conditions.	(Carstens et al. 2019)
Pectobacterium carotovorum	POP72	POP72 delayed the development of soft rot disease in a Chinese cabbage assay for up to 12 h and showed suppression of disease symptoms, especially at high MOIs.	(Kim et al. 2019)
Pectobacterium carotovorum	PCT27	Demonstrated that the cotreatment of phage and streptomycin (Agrepto ®) reduced the amount of antibiotic needed to a quarter of the conventional amount used, with similar efficacy to control soft rot disease.	(Kim et al. 2023)

Table 2.2

Bacterium	**Phage**	**Outcome**	**Study**
Pectobacterium spp.	ϕMA1, ϕMA1A, ϕMA2, ϕMA5, ϕMA6 and ϕMA7	A cocktail of six phages reduced softness in artificially infected potato tuber significantly. They further tested the phage cocktail under field conditions over three years with divergent application strategies. Phage soil drenching significantly increased the mass and number of harvested potato tubers. Most importantly, they could show significant disease reduction in all years for the phage-treated condition. Also, vacuum infiltration of seed potatoes showed promising results.	(Zaczek-Moczydlowska et al. 2020)
Pectobacterium spp.	PP16, PP47, PP101, Q51 and Possum	The treatment of ware potatoes with a phage cocktail resulted in a 10- to 12-fold reduction of pectolytic bacteria on the tubers.	(Bugaeva et al. 2021)
Pseudomonas syringae pv. actinidiae	PHB09	Kiwifruit leaf-based assays showed that PHB09 could significantly reduce the Psa load over kiwifruit leafs 24–72 h post-infection.	(Liu et al. 2021)
Pseudomonas syringae pv. actinidiae	ϕ6	Using *ex vivo* experiments with artificially contaminated kiwifruit leaf tissue they could show a reduction of cfu/mL by 17.6% for Psa CRA-FRU 12.54 and 29.3% for Psa CRA-FRU 14.10.	(Pinheiro et al. 2019)

Table 2.2

Bacterium	**Phage**	**Outcome**	**Study**
Pseudomonas savastanoi pv. glycinea	P421	Phage treatment of infected soybean leafs and seeds. Showed a two-fold reduction of disease progression 12 days after phage treatment compared to the control. For the infected seed, they showed a decrease of 59.7% (disease incidence) or 55.0% (disease severity).	(Tarakanov et al. 2022)
Pseudomonas syringae pv. porri	KIL3b and KIL5	A cocktail of both phages used for seed decontamination could reduce the bacterial concentration 100-fold.	(Holtappels et al. 2020)
Pseudomonas syringae pv. tomato	47 putative bacteriophages	Phage candidate PH34 showed the most potent disease reduction in tomato seedlings and an increased germination rate.	(Cement et al. 2018)
Ralstonia solanacearum	RsPod1EGY	Complete prevention of wilt symptoms in phage-treated tomato plants nine days after artificial infection in a pot-based greenhouse trial.	(Elhalag et al. 2018)
Ralstonia solanacearum	NJ-P3, NB-P21, NC-P34 and NN-P42	Evaluated the influence of the number of phage players within a phage cocktail and their impact on bacterial diversity within the rhizosphere community. They showed a decreased disease incidence by up to 80% in greenhouse and field experiments. A four-phage combination had the highest biocontrol efficacy, leading to the lowest mean disease index among all the treatments; interestingly also, the rhizosphere diversity was highest in this condition.	(Wang et al. 2019)

Table 2.2

Bacterium	**Phage**	**Outcome**	**Study**
Ralstonia solanacearum	RsoM1USA	The phage significantly reduced the *in vitro* growth of *R. solanacearum* strain RUN302 at MOIs between 0.001 and 10. However, it did not significantly reduce disease symptoms in tomato plants at an MOI of 0.1.	(Addy et al. 2019)
Ralstonia solanacearum	ϕsp1	They showed a substantial reduction of cfu in a tuber assay and a protective effect of phage ϕsp1 in a pot experiment with tomato plants.	(Umrao et al. 2021)
Ralstonia solanacearum	vRsoP-WF2, vRsoP-WM2 and vRsoP-WR2	Tested lyophilization of phages with mixed outcomes. However, it was shown that a phage cocktail reduces disease severity by up to 65% four weeks after treatment in tomato plants.	(Alvarez et al. 2022)
Streptomyces scabies	SscP1EGY	Soaking of mother tubers with phage and bi-/triweekly soil drenching significantly reduced potato common scab incidence and severity.	(Abdelrhim et al. 2021)
Xanthomonas axonopodis pv. allii	ϕ16, ϕ17A and ϕ31	Performed both greenhouse and field trials with Welsh onions and showed that, indeed, both a single phage ϕ31 and a phage cocktail consisting of all three phages are capable of reducing the progression of the disease.	(Nga et al. 2021)
Xanthomonas campestris pv. campestris	FoX2 and FoX4	Studied then influence on microbiome composition in field soil and *in planta* and could show any off-target effects.	(Fortuna et al. 2023)

Table 2.2

Bacterium	**Phage**	**Outcome**	**Study**
Xanthomonas campestris pv. campestris	ϕSeregron	Application of Phage Seregron at an MOI of 5 protects *Arabidopsis thaliana* in a sterile system and restores plant leaf are to control level at 14 dpi. Further differences in defence gene expression were reported	(Erdrich et al. unpublished)
Xanthomonas euvesicatoria pv. euvesicatoria	BsXeu269p/3	In a qPCR-based approach they showed five-fold reduction of the pathogen on the leaf surface after aerosol spraying, and av. 59.7% reduction of the amount of the pathogen in infectious lesions after artificial infection with needles, compared to the untreated control.	(Shopova et al. 2023)
Xanthomonas oryzae pv. oryzae	pXoo2106 and pXoo2107	A cocktail consisting of both phages significantly reduced cfu/mL *in planta* from 6 dpi on up to 14 days post-treatment.	(Liu et al. 2023)
Xanthomonas oryzae pv. oryzae	Nϕ-1 and Nϕ-3	Tested formulation of phages and protection against leaf blight in rice plants. The formulated phage Nϕ-1 lead significantly higher plants and chlorophyll content than infected plants.	(Liu, J. et al. 2022)

Table 2.2

Bacterium	**Phage**	**Outcome**	**Study**
Xanthomonas oryzae pv. oryzae	Phages J2, J3 and E	Showed in the pot experiment that phage treatment can significantly reduce the disease index in rice by varying degrees from 44.0% in single phages up to 64.3% in a phage cocktail. They showed that phage combination treatments restored the impact of pathogen invasion on phyllosphere communities to a certain extent and increased the diversity of bacterial communities, which further reduced the abundance of the pathogen.	(Jiang et al. 2023)
Xanthomonas perforans	ϕXp06-02-1	Formulation of phages for higher UV stability. NAC-ZnS-formulated phages reduced tomato bacterial spot disease severity significantly (16.4%) compared with non-formulated phages.	(Choudhary et al. 2023)

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2.3 Plant pathogen biocontrol with phages

With this project being the first step towards biocontrol of plant pathogenic bacteria in our lab, it was crucial to select economically relevant plant pathogenic bacteria and isolate a diverse set of phages against them. Having a reliable pathosystem is mandatory to properly develop bacteriophage-based strategies as a means for biocontrol, but the best pathosystem is useless without stable phage isolates for systematic assessment of their biocontrol capacity. Thus, our research approach included the isolation and characterisation of our own phage isolates for the plant pathogenic bacterial species *Xanthomonas campestris pv. campestris (Xcc), Xanthomonas translucens pv. translucens (Xtt), Agrobacterium fabum C58 (Atum), Pseudomonas syringae pv. lapsa (Pss)* and *Pseudomonas syringae pv. tomato (Pst).*
In order to use phages for plant biocontrol, it is important to isolate new phages with specific desirable traits, such as a lytic lifestyle, production of sufficent virion progeny, and the ability to bind to a diverse set of bacterial host receptors. By identifying, characterizing, and cataloguing novel phages, we can gain a better understanding of how phages interact with a particular host bacterium and how the host defends against viral attacks. This knowledge is crucial for the development of more efficient phage biocontrol applications.

For *X. translucens (Xtt)* causing leaf streak in wheat and cereal crops, no phages are available in phage collections. Only one paper from 1953 reports the isolation of four phages for different pathovars of the "translucens group" (Katznelson and Sutton, 1953). Unfortunately, none of them were neither sequenced nor morphologically characterised, making the isolation of lytic *Xtt* plaques a prerequisite for the establishment of phage-based biocontrol strategies of this important pathogen. *Xtt* was shown to be also transmitted from leaf to seed, but classical decontamination treatments have been reported not to be always completely successful (Sapkota et al., 2020). Thus, we included *Xtt* in our isolation efforts and were able to isolate and characterise six novel phages (Langgrundblatt1, Langgrundblatt2, Pfeifenkraut, Elanor, Laurilin, Mallos) during this PhD project. All our isolated phages for *Xtt* were showing a siphovirion morphology by transmission electron microscopy (TEM) (Figure 2.3). Further, all of them were predicted to have a lytic lifestyle and showed host suppression during *in vitro* growth curve experiments (Erdrich et al., 2022). Research on this pathogen is important due to its devastating potential. Outbreaks of *Xtt* have been reported from many geographical regions, with the most prevailing in warmer regions so far (Curland et

al., 2018; Sapkota et al., 2020). In the context of climate change and worldwide rising average temperatures, it is likely that the impact of *Xtt* as plant diseases will increase also in Europe.

For *X. campestris (Xcc)* causing black rot of crucifers only a limited amount of phage isolates has been reported in literature, and therefore there is a strong need for further phage isolates applicable for biocontrol. Until recently, only a few phages were described for *Xcc* (Table 2.3). As part of this thesis, we added a further phage, *Xanthomonas* phage Seregon, to the pool of available phages for *Xcc* (Erdrich et al., 2022). Phage Seregon is a virulent phage with a siphovirion morphology (Figure 2.3), belonging to the taxonomic genus of *Salvovirus*. The phage was selected as a promising candidate for *in planta* experiments due to its potential in suppressing bacterial growth *in vitro.*

For *Agrobacterium*, the causal agent of crown gall disease, despite the large range of plants it can infect and its role as a well-studied tool for genetic engineering, only a few phages have been reported (Table 2.3). We have successfully added a new podovirion phage called "Alfirin" to the list. The phage was isolated from a winter wheat rhizosphere sample and showed a lytic lifestyle, which was confirmed in *in vitro* experiments. Based on its characteristics, it was later chosen for *in planta* experiments, where it showed high persistence and stability (Erdrich et al., 2024).

In the case of *P. syringae,* there are more phages available, but due to the fact that phages are highly specific and the *Pseudomonas syringae*-complex is so diverse that over 50 pathogenic variants (pathovar; pv) infect crop plants from tomato to kiwi (Silby et al., 2011; Xin et al., 2018), a high phage diversity is needed to support efficient biocontrol. During this project, we isolated *Pseudomonas* phage Athelas, which is able to infect the *Pseudomonas pv. tomato* as well as *Pseudomonas pv. lapsa* (Erdrich et al., 2024). Phage Athelas is a podovirion (Figure 2.3) belonging to the *Autographiviridae* family.

For species and genus determination of our isolates a genome comparison was conducted. The average nucleotide identity (ANI) of all isolates and closest related phages from NCBI was calculated (Apendix Figure A.1.1). The species threshold was set to 95% and the genus threshold to 70% (Turner et al. 2021). Phage Seregon belongs to the genus *Salvovirus*, closely related to the name giving bacteriophage for the genus phage Salvo. As noted above, for phages Langgrundblatt1, Langgrundblatt2 and Pfeifenkraut, we established the new genus *Shirevirus*. Phages Elanor and Mallos both belong to the subfamily *Bradleyvirinae* (family:

Mesyanzhinovviridae), and for both new genera were proposed *Elanorvirus* and *Mallosvirus,* underpinning the large natural diversity of phages that potentially can be harnessed for plant biocontrol. Phages Athelas and Laurilin belong to the genus of *Ghunavirus* within the *Autographivirdae* family. The recently published *Agrobacterium* phage Alfirin (Erdrich et al. 2024) is closest related to *Agrobacterium* phage Atu_03 and represents a new genus with an ANI of 59% to the former, we suggest "Agrovirus" as genus name.

The massive spread of bacteria resistant to conventional antibiotic treatments triggered a global renaissance of phage biology and phage-based biocontrol (Svircev et al. 2018). In the context of this PhD project, we tested potential application strategies by making use of our phage isolates combined with phage models from a systematic collection of *E. coli* phages (Svircev et al. 2018). As well as transcriptional changes during the tripartite interaction between plant-bacteria and bacteriophages, recent research has explored different strategies for phage biocontrol in planta, which range from spraying phage solutions on the phyllosphere for irrigation water treatment in pot experiments to treating seed tubers and coating leaves with phage formulations (Alvarez et al. 2019; Balogh et al. 2003, 2008; McKenna et al. 2001). Due to the inherent exposure of the phyllosphere to a significant amount of solar radiation, there is a suboptimal impact on the longevity of bacteriophages (Iriarte et al. 2007). The first strategies to improve phage stability in the phyllosphere were nocturnal application as well as the formulation with UV-protectants, e.g. clay, wheat protein, and dry skim milk (Balogh et al. 2003; Jones et al. 2012). Another approach is phage application via irrigation water (Alvarez et al. 2019). However, the outcomes were variable due to the diverse nature of the soil; multiple inherent properties could influence phage stability, accessibility and activity, e.g. pH, particle size, water content, soil pore size and content of free ions (Florent et al. 2022; Williamson et al. 2017). More recent studies focused on treating the generative plant parts with phages before they are sown. They are aiming to block the transmission of pathogenic bacteria by infected seeding material and, at the same time, protecting against potential pathogens in close proximity, potentially present in the field soil (Gazdik et al. 2021). Therefore, we tried in particular to stop the disease transmission cycle at the seed stage, which is more accessible and controllable and provides the advantage of less work for the user in the field.

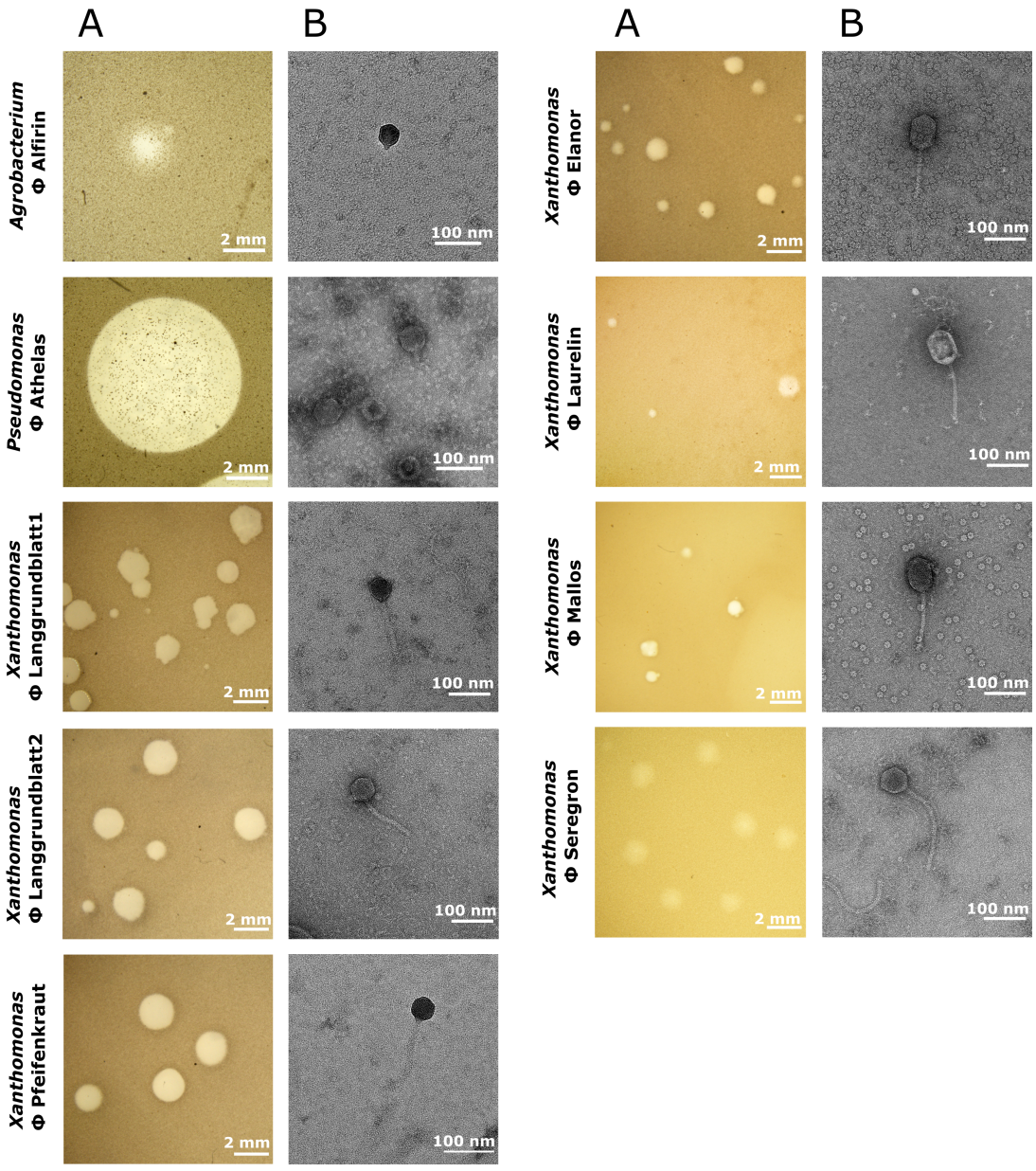

Figure 2.3: **Morphology of the bacteriophage isolates**. Displayed is the plaque and virion morphology of the nine different phages infecting *A. tumefaciens* C58 (Alfirin), *P. syringae pv. syringae* (Athelas), *Xanthomonas translucens pv. translucens* (Langgrundbatt 1, Langgrundbatt 2, Pfeifenkraut, Elanor, Laurelin, and Mallos) and *Xanthomonas campestris pv. campestris* (Seregon). (A) Stereo microscopy of single plaques. Scale bar: 2 mm; (B) Transmission electron microscopy (TEM) images of virion particles. The phage isolates were negative stained with uranyl acetate. Scale Bar: 100nm.

Table 2.3: Phages described for pathogens studied in this work

Pathogen species	**Bacteriophage**	**Reference**
Xanthomonas camestris pv. campestris	ϕ Carpasina	(Weiss et al. 1994)
	ϕ LF.	(Lin and Tseng 1996)
	ϕ L7	(Lee et al. 2009)
	Xcc ϕ 1	(Papaianni et al. 2020)
	ϕ FoX 1-7	(Holtappels, Fortuna, Vallino, Lavigne and Wagemans 2022; Holtappels et al. 2022)
	ϕ Lf2	(Yeh et al. 2023)
	ϕ PPDBI	NCBI Direct Submission (MT210154)
	ϕ XcP1	NCBI Direct Submission (MH191395)
	ϕ PBR31	NCBI Direct Submission (MT119766)
	ϕ Seregon	(Erdrich et al. 2022)
Xanthomonas translucens pv. translucens	ϕ Langgrundblatt1	(Erdrich et al. 2022)
	ϕ Langgrundblatt2	(Erdrich et al., 2022)
	ϕ Pfeifenkraut	(Erdrich et al. 2022)
	ϕ Elanor	(Erdrich et al. 2022)
	ϕ Laurilin	(Erdrich et al. 2022)
	ϕ Mallos	(Erdrich et al. 2022)
Agrobacterium fabrum strain C58	ϕ Atu02 and ϕAtu03	(Attai et al. 2017)
	ϕ 7-7-1	(Gonzalez et al. 2018)
	ϕ Atu07	(Attai et al. 2018)
	ϕ Atu04, ϕAtu08	(Attai and Brown 2019)
	ϕ Pasto	(Boeckman et al. 2022)
	ϕ Alfirin	(Erdrich et al. 2024)
Pseudomonas syringae pv. tomato	ϕ FRS	NCBI Direct Submission (MZ598487)
	ϕ SHL	NCBI Direct Submission (NC048200)
	ϕ Eir4	(Korniienko et al. 2022)
	ϕ Eisa9	(Korniienko et al. 2022)
	ϕ Medea1	(Skliros et al. 2023)
	ϕ Athelas	(Erdrich et al. 2024)
	> over 100 phage-genomes for *Pseudomonas syringae* on NCBI VIRUS	(Brister et al., 2015)

2.4 Binding of phages to seed surfaces

Based on the body of knowledge about phage morphotypes and their specific receptor binding proteins (RBP), we asked if the RBP like tail fibres and tail spikes, which are crucial for recognizing chemical patterns on the host bacterium's surface, might have an even broader function (Taslem Mourosi et al. 2022; Witte et al. 2021). For example, studies in animals showed that phage adhesion to the mucus is relevant for their persistence in mice intestine, where they provide non-host-derived immunity, shown with *E.coli* and a T4 phage (Barr et al. 2013). Other phage receptors bind to sugar moieties of polysaccharides (Bertozzi Silva et al. 2016).

Also, plants produce layers of polysaccharides on certain tissues like the columella of the root cap where mucilaginous exudates are secreted into the soil, fulfilling a diverse set of functions from moistening the soil for better root conduction through the soil to microbe recruitment (Ganesh et al. 2022). One other important plant tissue containing polysaccharide matrix is the seed coat mucilage (SCM). The seed coat mucilage is present in many economically relevant plant families e.g. *Lamiaceae* and *Solanaceae*. Also some members of the Brassicaceae family contain SCM, one prominent example is the model plant *Arabidopsis thaliana* (Western 2012; Yang et al. 2012). The mucilage is a layer of pectin, hemicelluloses, cellulose and proteins produced by the epidermal cells during seed development. It is released after imbibement of the mature seed with water and subsequently starts to swell and to cover the seed with a glycopolymer-matrix. Although the composition can differ amongst ecotypes of the same species, the major sugar-building-blocks are fucose, arabinose, rhamnose, galactose, glucose, mannose, xylose and galacturonic acid (Voiniciuc and Guenl 2016). The mucilage was associated with anchorage to the soil and regulation of water content as well as an advantage in dispersal (Western 2012; Kreitschitz et al. 2021). In this PhD project, we assessed the influence of the seed coat mucilage on phage binding and stability.

Asking the question whether phage binding to polysaccharides also plays a role in plants, we hypothesised that they could bind components of the mucilage. Another important property of the mucilage, possibly relevant for phage binding, is the physical structure of the polymer-matrix, functioning as a mesh with a pore size between 2-50nm (Sanka et al. 2017). Most icosahedral phage capsids (Figure 2.1) range in size from 40 to 180nm in diameter (Lee et al. 2022), which is in most cases larger than the mucilage pore size. These facts led to second hypothesis, namely that the mucilage might also function as a physical entrapment

of phage particles. Further, we asked the question if phages could not only be used for seed decontamination but also could be stored on seeds for prolonged periods, thereby adding an additional layer of pathogen defence from early on.

In this PhD project, we aimed to fight against diseases caused by bacterial pathogens at a very early point, even before the plant germinates, since many studies highlight that seed transmission remains a primary route for plant pathogen transmission (Burdman and Walcott 2012; Darrasse et al. 2018, 2010; Giovanardi et al. 2018; Mansfield et al. 2012; Morris et al. 2007; Johnston-Monje et al. 2021; Shade et al. 2017; Vishunavat et al. 2023). Other phage biocontrol approaches like spraying the phyllosphere or soil drenching. These strategies require the production of large amounts of phages and often depend on external factors like weather conditions. Therefore, breaking the disease transmission cycle at the seed stage, is a meaningful goal, achievable in a more controlled setting. A recent study applied a decontamination procedure with artificially and naturally infected *Xcc* seeds (Holtappels et al. 2022). We chose the model plant *Arabidopsis thaliana* to further investigate the potential of the mucilage, a layer of polysaccharides on the seed coat, to provide a platform for phage binding and storage.

Focusing on the model plant *Arabidopsis thaliana* and selected plant pathogens (e.g. *X. campestris, P. syringae*), we dissected the question if phage binding occurs during seed imbibement. The respective phages for the pathogens, which were isolated in the course of this PhD project (Figure 2.3) were used. We could show that the mucilage is a necessary component for some of our phages. This initial set of phages was further expanded by a systematic *E.coli* phage collection provided by Maffei and colleagues (Maffei et al. 2021) to broaden the scope of our observations. Initial explorations with *Agrobacterium* phage Alfirin, the *Pseudomonas* phage Athelas and the *Xanthomonas* phage Pfeifenkraut showed that all of them bound to *Arabidopsis* seeds (Erdrich et al. 2024). To further test our hypothesis that the mucilage is directly relevant for phage binding, we included wildtype seeds and those from which the mucilage had been artificially removed (Voiniciuc and Guenl 2016). Briefly we removed the mucilage by hydration of the seeds and subsequent shaking at 30 Hz in a ball mill. Those seeds as well as wildtype seeds were co-incubated with phages and placed on plates containing a bacterial lawn (Figure 2.12 **A**). After incubation of the plates we quantified lysis zones around seeds indicating phage binding and activity. Here, we found that some phages displayed significantly reduced binding to seeds with removed mucilage layers (Figure 2.12 **B** & **C**). We hypothesised that phage adhesion to seeds may be facili-

tated by physical properties of the mesh-like polymer structure of mucilage or through direct chemical interactions between phage receptor binding proteins and specific sugar residues. Our experiments revealed that especially *Autographiviridae* phages from our isolates as well as the *Autographiviridae* subset of the BASEL *E. coli* phage collection were highly dependent on the presence of the mucilage. To delve deeper into the mechanism of phage binding to *Arabidopsis* seeds, we tested a selection of *Arabidopsis* SCM mutants being deficient in genes, which are responsible for the formation of specific sugars or structures of the mucilage (Figure 2.12 D). The analysis of the *Arabidopsis* seed coat mutants suggested a particular importance of the diffusible cellulose component of the mucilage for phage Athelas. This finding coincides with the fact that *Pseudomonas* was reported to use cellulose in its biofilm (Perez-Mendoza et al. 2019), hinting at the fact that this might also be the phage target in its natural host. This is further underpinned by many reports of phages recognizing capsule polymers or bacterial biofilms (Born et al. 2014; Gong et al. 2021).

Based on these findings, we were interested in the particular role of phage receptor binding proteins during adhesion to the SCM. By creating phylogenetic trees of the tail fibre proteins as well as in silico structure prediction using Alphafold 2, we showed a high degree of structural similarity as well as phylogenetic proximity for the mucilage-dependent *Autographvirade* phages like the *Pseudomonas* phage Athelas and the set of BASEL phages, which were affected in seed binding. Interestingly, *Agrobacterium* phage Alfirin as well as *Escherichia* phage AlfredRasser strain Bas69, which did not depend on the presence of the mucilage (Erdrich et al., 2024), possessed two distinct tail fibre proteins, while the mucilage-dependent *Autographiviriae* relied on only one type of tail fibre proteins. For phage Bas69, the literature describes enterobacterial common antigen (ECA) as potential primary receptor and an outer membrane porin as terminal receptor (Maffei et al. 2021). Future studies can further investigate the receptors for phage Alfirin and its behaviour within the mucilage. A more general understanding of such structural components, e.g., tail fibres and virion-associated appendages and their interaction with universal chemical building blocks found interkingdom-wide would be extremely useful in phage formulations or protective coating of surfaces with phages. This is not only important during crop production but also holds the potential for produce protection after harvest and distribution to customers.

In order to further foster phage applications in plant treatment, we evaluated the possibility of phage storage on seeds. Therefore, we coated *Arabidopsis thaliana* seeds with three different phages and stored them at 4°C for different numbers of days to study their stability. Subsequently, coated seeds were placed on a double agar containing the respective host bacterium. Upon cultivation, the amount of seeds showing lysis in their surroundings were counted as an indicator of phage presence (Figure 2.5 A). While phage Athelas lost its infectivity within two weeks during storage on seeds, the phages Alfirin and Pfeifenkraut showed high stability for over four weeks on seed surfaces. These different degrees of infectivity highlighted that the stability of phage coating was highly dependent on the individual phage, even under favourable conditions (storage at 4°C).

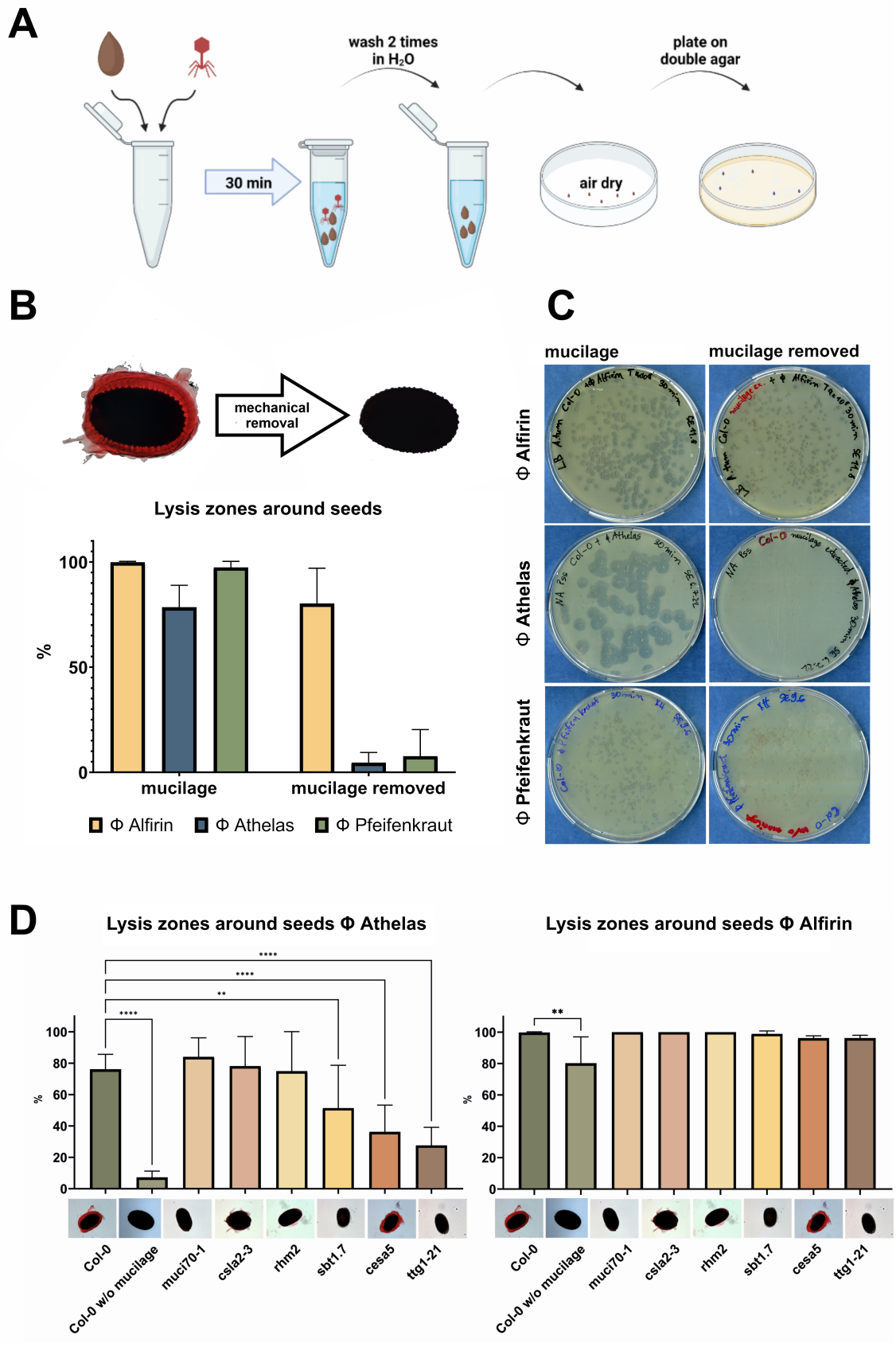

Figure 2.4: Phage binding to seeds and influence of artificially removed seed mucilage.

Figure 2.4: **Phage binding to seeds and influence of artificially removed seed mucilage.** **A)** Seed-coating-workflow; binding of phage particles on seeds of *Arabidopsis thaliana*. **B]** 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. Image represents a wild type Col-0 seed stained with 0.01% ruthenium red solution before and after mechanical removal of the mucilage. **C)** Double agar overlay with phage coated seeds. Host-bacterium was embedded in soft-agar 0.4% on top of an agar plate with the respective medium. Seeds were placed on top of the softagar layer. **D)** Double agar overlay with phage coated seeds od selected Arabidopsis SCM mutants.

Transmission of bacterial pathogens via seeds is a major threat in agriculture (Burdman and Walcott 2012; Darrasse et al. 2010, 2018; Giovanardi et al. 2018; Mansfield et al. 2012; Morris et al. 2007; Shade et al. 2017). Cleaning of seeds with phages is a promising approach to reduce the plant pathogen load, which was explored recently in *Xcc* (Holtappels et al. 2022). By testing cleaning of artificially infected *Xcc* seeds with our *Xanthomonas campestris* phage Seregon, we showed that the phage is also capable of increasing the plant survival rate significantly, even to a level of the non-infected control plant (Figure 2.5 B). Many recent studies came to the consistent conclusion that high MOIs result in best phage biocontrol (Choe et al. 2023; Holtappels et al. 2022; Kim et al. 2022). This brings its own challenges, as high MOIs require large amounts of active phage particles or multiple application rounds. To move the production of phages from the fermenter to the field, we took advantage of the natural propagation ability of phages. For this purpose, we used a non-infectious version of the bacterial host bacterium as an "on-field booster" to multiply the phages in close proximity of the plant without increasing the plant pathogen load. Therefore, we co-inoculated seeds with phage Alfirin and an *Agrobacterium* strain lacking the Ti-Plasmid, which is essential for plant infection (Morton et al. 2014), while the co-inoculation with the non infectious *Agrobacterium* strain increased phage longevity and numbers the plant responded still to the inoculation with this bacterial strain via growth arrest most likely the recognition of microbial molecular patterns, underpinning the importance of plant the defence during phage biocontrol.

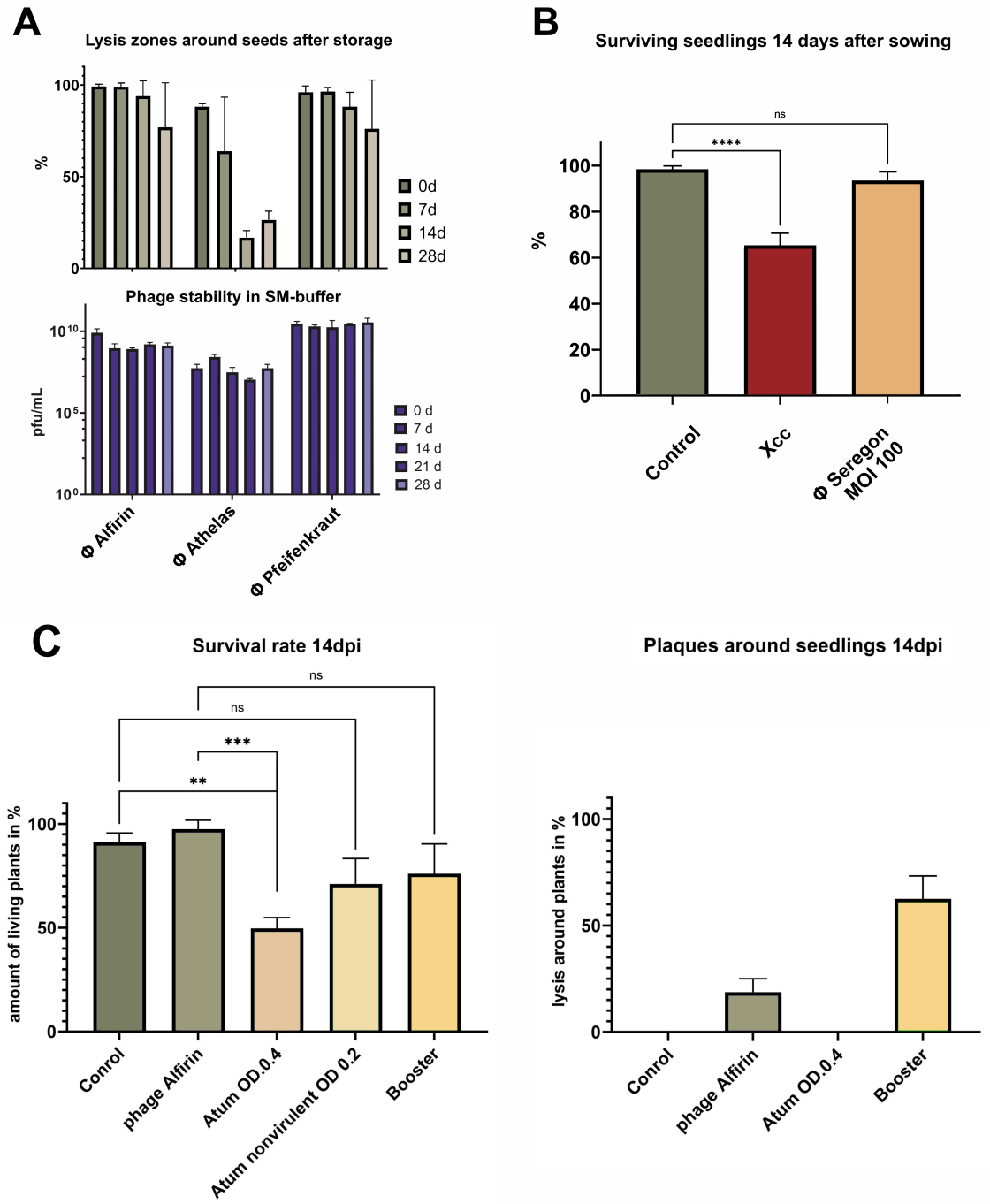

Figure 2.5: **Phage seed application strategies. A)** Prolonged storage of phage-coated seeds at 4°C and activity testing. A portion of the stored seeds was placed on a double-agar containing the host bacterium, at 0, 7, 14 and 28d to test phage activity after storage (upper row. Phage stored in SM buffer over the same time period (bottom row). **B)** Seed cleaning with *Xcc* phage Seregon. Artificially *Xcc* inoculated seeds were cleaned with phage Seregon at a MOI of 100. **C)** Phage "Booster" - amplification of phages in close proximity to the plant using a non-virulent host.

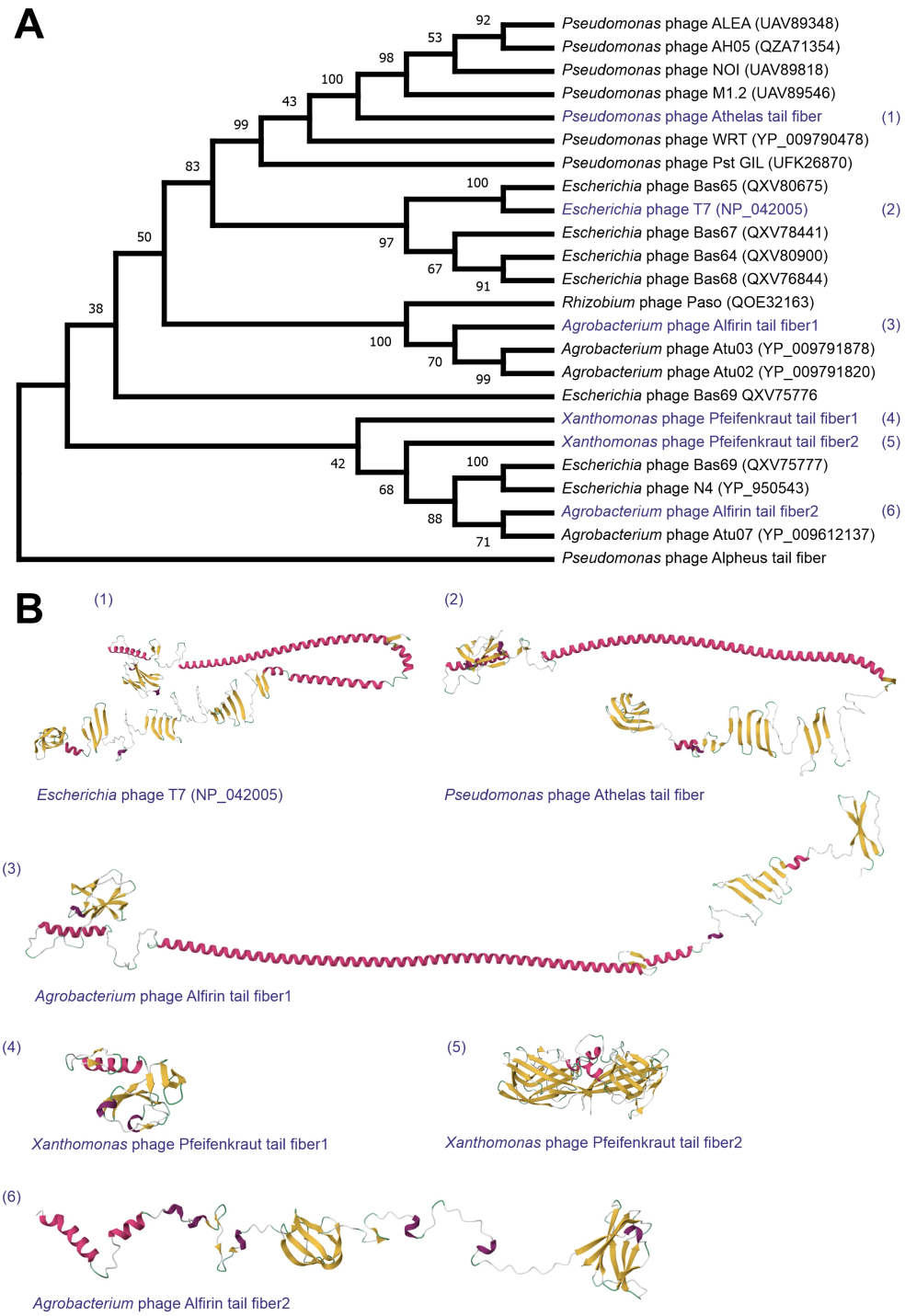

Figure 2.6: **Phylogenetic tree of phage receptor binding proteins and *in silico* folding. A)** Phylogenetic tree of phage receptor binding proteins. Ancestral states were inferred using the Maximum Likelihood method (Nei and Kumar 2000) and JTT matrix-based model (Jones et al. 1992). Evolutionary analyses were conducted in MEGA11 (Tamura et al. 2021). **B)** Protein structures of selected phage receptor binding proteins. 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.

2.5 Phenotypic quantification of the Plant-Bacteria-Phage interaction

To investigate the plant-bacteria-interaction in detail we aimed to study gene regulation in a sterile system in combination with simultaneous non-invasive phenotyping. While phage biocontrol was heavily researched in recent years, only a few studies addressed the topic from the plant perspective. It is primarily important to reduce the bacterial load on the plant to give it a growth advantage, most recent studies have focused on this central aspect (Korniienko et al. 2022; Pinheiro et al. 2019). Within this line are also investigations observing the influence of phage treatment on bacterial communities *in planta* (Morella et al. 2018; Wang et al. 2019). A further important area of phage-based biocontrol is of course phage persistence in the plant system. A better understanding of the microenvironments of phages is also important, but only a few studies addressed transportation of phages *in planta* (Rahimi-Midani et al. 2020).

Another so far mostly overlooked aspect is the recognition of the bacterial pathogens by the plant and the question how phages might influence this perception and the corresponding regulation. Therefore, we aimed to add a puzzle piece to this important question. Molecular plant research heavily benefited from defined model systems, including *Arabidopsis thaliana*. It contributed to our understanding of how plants defend themselves against various diseases during the last decades (Glazebrook 2005; Thatcher et al. 2005; Zhalnina et al. 2018). Nevertheless, the tripartite interaction between a plant, its bacterial pathogen and an active bacteriophage has not been studied so far on a molecular level, motivating us to investigate this tripartite interaction.

By implementing A. *thaliana* as plant, *X. campestris* as bacterial pathogen and phage Seregon, the tripartite interaction was established on a plate-based sterile gnotobiotic system. Briefly surface sterilized *Arabidopsis* seeds were placed on plant agar containing plates and sealed with micropore tape, placed into a phyto-chamber. The design was set up, similar to Korniienko et al. (Korniienko et al. 2022). Seedlings were inoculated with the microorganisms eight days after sowing as described in Figure 2.7 A). Plant growth was monitored via scanning of the plates. The system was proven to be robust (Figure 2.7 B), confirming its qualification for plant phenotyping under different treatment conditions and for the evaluation of the effectiveness of phage treatment.

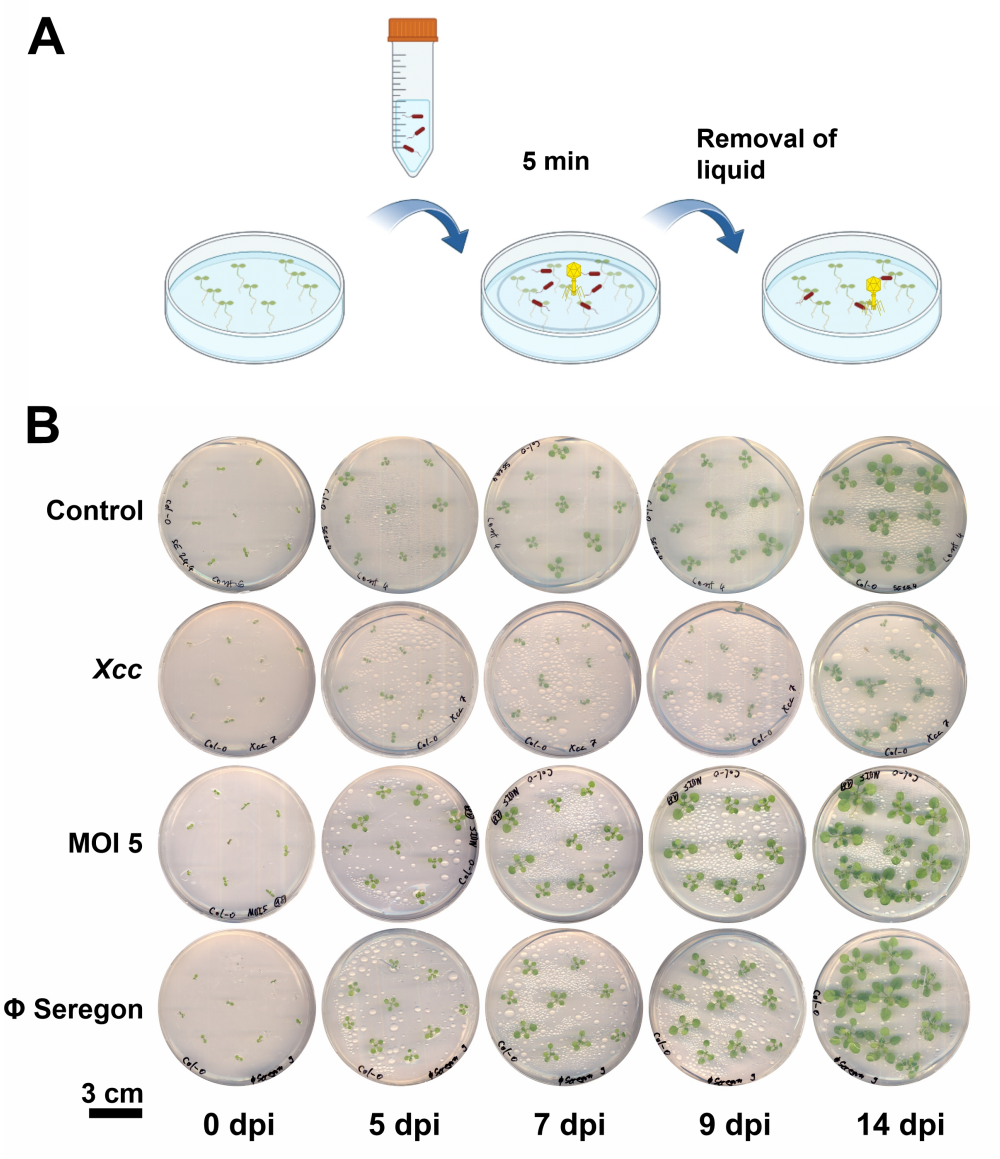

Figure 2.7: **Tripartite interaction in a gnotobiotic system. A)** Inoculation of 8 days old *A. thaliana* seedlings with 50 mL ½ MS media for 5min, excess liquid was removed thereafter. For *Xcc* treatment, 10^9 cfu/mL were added to the ½ MS media. Phage Seregon was added at a MOI of 5, meaning the flooding solution had a phage concentration of $5*10^9$ pfu/mL. The same amount of phage Seregon was used for the phage only control. Control plants received ½ MS media only. **B)** Depiction of non-invasive phenotyping over time. Displayed are control inoculated plants, pathogen inoculated plants (*Xcc*) and phage Seregon treated *Xcc* inoculated plants MOI5.

The quantification of the influence of the pathogen and of possible phage-provided plant protection mechanisms, was achieved by non-invasive monitoring of the leaf area of all plants over time. Plates were scanned at the indicated time points (Figure 2.8 A). And the leaf area per plant was calculated. At the beginning of the experiment, each condition contained 216 plants. The first phenotypic variations, meaning significant differences in leaf area, were noticed after 7 dpi. In the following days this observed phenotypic diversity accelerated further resulting in significant differences in leaf area between the control and the *Xcc* treatment after 12 dpi. After 14 dpi, the phage treated condition (MOI5) had even recovered to control levels (Figure 2.8 B), highlighting that the phage treatment was effective against *Xcc* infection. At the endpoint of the experiment (14 dpi), the fresh weight confirmed the phenotypic observations (Figure 2.8 C). Nevertheless, it is worth mentioning that the fresh weight of the phage only treatment samples was significantly increased compared to the control. One could only hypothesise what causes this differences, higher water content of phage treated plant due to biotic interactions is a potential hypothesis, because literature has shown that interaction with microorganisms can lead to water enriched tissues (Ievinsh 2023). An alternative hypothesis is that the phage triggers alteration of hormonal regulation leading to growth promotion. Both hypotheses are matter to future research.

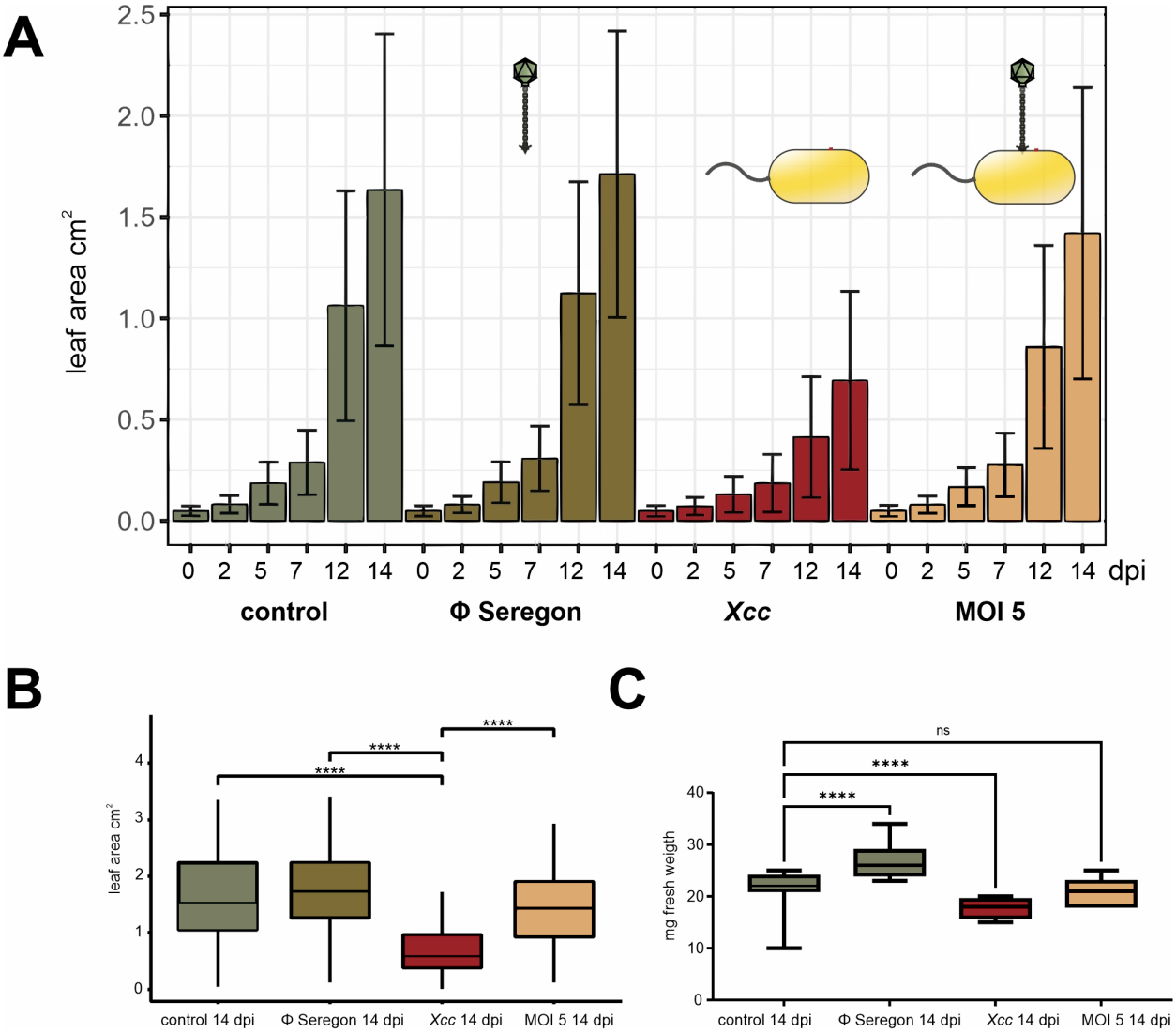

Figure 2.8: **Plant phenotyping. A)** Phenotyping of leaf area over time starting with the inoculation at 0 dpi. Seedlings were inoculated with 50 mL ½ MS media for 5 min. Subsequently, excess liquid was removed. For *Xcc* treatment, 10^9 cfu/mL were added. MOI 5 contained in addition $5*10^9$ pfu/mL. The same amount of phage Seregon was used for the phage-only control. Control plants received ½ MS media only. The plant leaf area per plant was retrieved by scanning at 0, 2, 5, 7, 12, 14 dpi. **B)** Total leaf area per condition 14 days post-inoculation. Displayed are the means of each condition (N > 45) at the endpoint 14 days post-inoculation. ANOVA was performed with RStudio, followed by Tukey HSD [F(3,234)=31,25, p=0.0001]. **C)** Fresh weight per plant at 14 dpi. The fresh weight was measured at the endpoint in a final harvest.

2.6 Transcriptome analysis providesinsights into tripsrtite intersctions between plants-bacteria-phages

To gain a genome wide transcription profile, to see which genes contribute to the observed phenotypic changes we went for a novel meta-transcriptomics approach. Therefore, plants from the gnotobiotic system were sampled at 0, 2, 5, 7 and 14 dpi. A subset of 0, 2, 7 dpi was identified to be most relevant by a RT-qPCR of defence marker genes. For validation of this novel approach, 2 biological replicates of the samples indicated in Table 4.2 were send for a first round of tripartite mRNA sequencing. Samples were subjected to double rRNA depletion for both plant and bacteria. A read depth 100 M reads per sample was achieved by 150 bp Ilumina sequencing. The resulting reads of the conditions control, *Xcc* and MOI5 at 2 and 7 dpi were mapped to each of the three genomes individually using Kallisto as an alignment algorithm (Bray et al. 2016). Identified transcribed genes were subject to a subsequent analysis.

Table 2.4: RNA-Samples used for tripartite sequencing. Number of total as well as already sequenced samples from pooled plants is indicated.

day	**0 dpi**	**2 dpi**	**7 dpi**
Control A. thaliana	**0/3**	**2/3**	**2/3**
A. thaliana + ϕSeregon	**0/3**	**0/3**	**0/3**
A. thaliana + Xcc	**0/3**	**2/3**	**2/3**
A. thaliana + Xcc + ϕSeregon (MOI5)	**0/3**	**2/3**	**2/3**

The molecular plant responses in the phage treatment condition were of special interest for us since no study has addressed the genome-wide plant gene regulation during the tripartite interaction so far. Only one recent study addressed this question partly by studying two plant genes, during phage treatment against *P. syringae* (Skliros et al. 2023).
The obtained mapping reads were used for differential expression analysis with DESeq2 (Love et al. 2014), using the control samples at 2 dpi as reference unless other mentioned. Significant (p-value < 0.05) differentially expressed genes over all conditions were plotted by log2 fold change (Figure 2.12 A). This analysis showed that several genes are differentially expressed compared to the mock inoculated plant control. A principal component analysis (PCA) of the differentially expressed genes revealed that the largest contributor to the variance within the data is the condition, followed by the time point. To dissect the contribution of each condition in more detail, significant genes were plotted group specific as Venn-Diagram (Figure 2.12 C, D). The largest fraction of up-regulated genes as well as down-regulated genes were found in the *Xcc* condition at 2 dpi (97 % up/ 79 % down)of all significant differentially expressed genes at this time point and 7dpi (93 % up/ 90 % down), highlighting the impact of bacterial effectors on plant gene expression during plant takeover and reprogramming (Büttner and Bonas 2010). Interestingly, in the MOI5 condition at 2 dpi, the upregulation of condition-specific genes is much subtler, with 33% of genes up-regulated and 45% of genes down-regulated compared to the control.

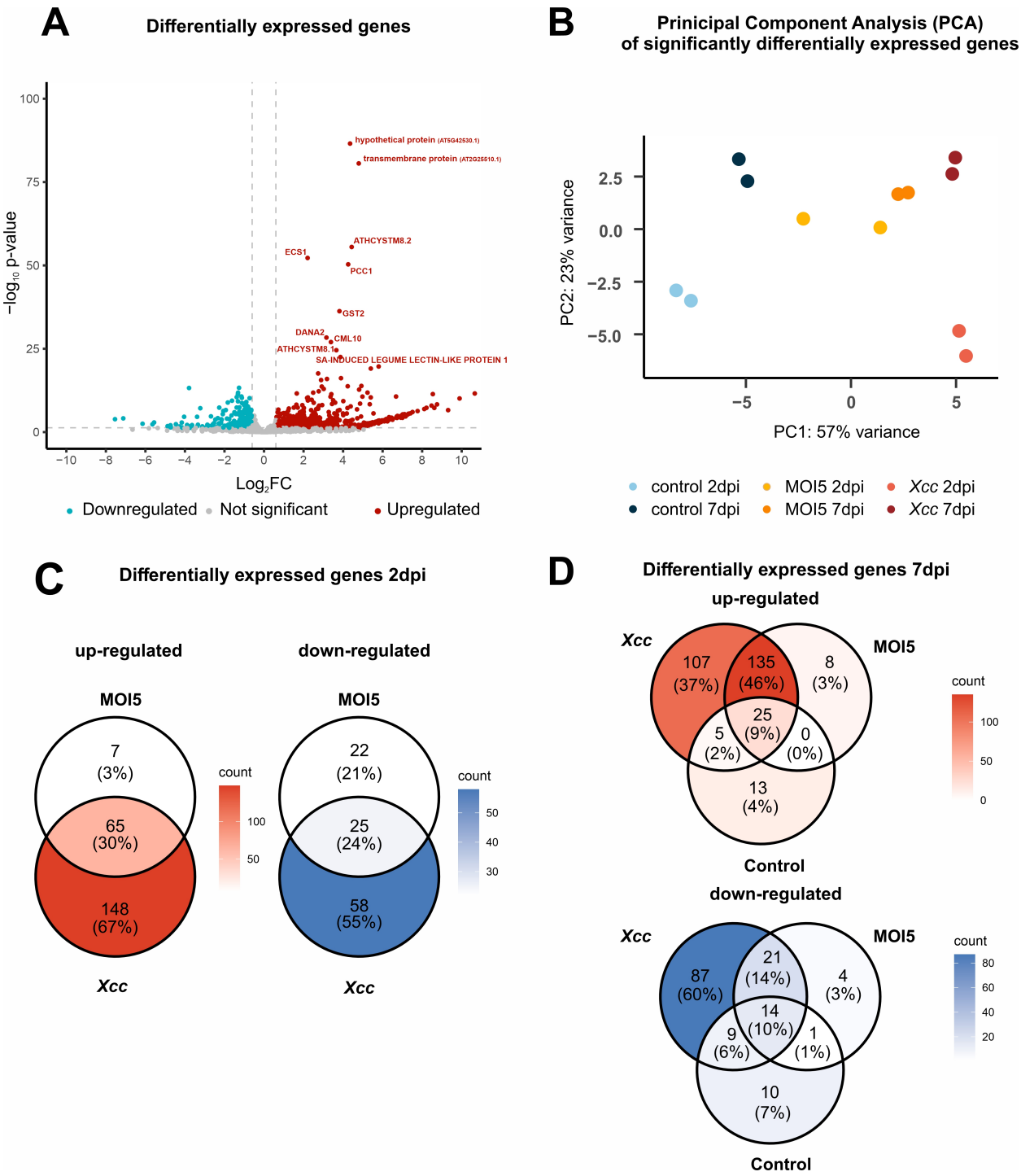

Figure 2.9: Differential expression of plant genes during tripartite interaction.

Figure 2.9: **Differential expression of plant genes during tripartite interaction between *Arabidopsis* - *Xanthomonas* - phage Seregon.** Differentially expressed genes were identified with DESeq2; p-val < 0.05 **A)** Vulcano-plot of differentially expressed genes. Up-regulated genes are shown in red, down-regulated genes are shown in blue **B)** Principal component analysis, samples: control 2 days post inocculation (dpi), control 7 dpi, MOI5 2 dpi, MOI5 7 dpi, *Xcc* 2 dpi and *Xcc* 7 dpi **C)** Venn-Diagram of differentially expressed genes at 2 dpi in the presence of phages (MOI5), the control treatment 2 dpi was used as reference. Up-regulated genes are shown in red and down-regulated genes are shown in blue. **D)** Venn-Diagram of differentially expressed genes at 7 dpi, the control treatment 7 dpi was used as reference. Up-regulated genes are shown in red and down-regulated genes are shown in blue.

2.7 Plant defence regulation and phage influence

In the context of this PhD thesis, we report on the impact of a plant-bacteria-phage interactions during plant biocontrol with the important plant pathogen *Xanthomonas* utilizing a gnotobiotic system. This novel tripartite transcriptomics approach gives insights into genome-wide expression profile during the interaction with the pathogen as well as during phage mediated biocontrol. Showing successfully that a MOI of five of the phage Seregon is sufficient to protect *Arabidopsis thaliana* seedlings against *Xcc* infection. Moreover, phage Seregon restored the the leaf area to non-infected control levels at 14 days post-inoculation. This demonstrates again the great potential of phage biocontrol with bacteriophages as an excellent alternative to classical plant control strategies, providing immunity against pathogens as well as curing infections.

Typically, *Xcc* infects its host plants through natural openings such as hydathodes, stomates, cracks or wounds (Williams 1980). When the bacterium successfully enters the vascular system, it spreads systematically and causes blackening of the veins in crop plants. For *Arabidopsis*, three main ways of defence against bacterial pathogens have been identified by now: First, early sensing of the bacterium, e.g. by detection of lipopolysaccharides located in the bacterial membrane via receptor kinases at the plant plasma membrane (Hussan et al. 2020; Silipo et al. 2008), second, detection of bacterial effectors and changes within the defence signalling (Guo-Feng Jiang 2012; Rong et al. 2010; Yan et al. 2019) and third, up-regulation of genes involved in the growth suppression of the pathogen, for example, by triggering the production of reactive oxygen species, flavonoids and pathogenesis-related proteins (Lu et al.

2021; Sels et al. 2008; Sun et al. 2022).

In an attempt to get a more condition-specific picture of the function of differentially expressed genes, the 50 most significantly affected genes were used to generate a heatmap with gene names (Figure 2.10). Further, the genes were assigned to categories by the description found on The Arabidopsis Information Resource (TAIR)(Rhee 2003) and colour-coded, respectively. In the heatmap, a condition-specific pattern is easily noticeable. Interestingly, MOI5 at 2 dpi had an expression pattern close to the phage- and bacteria-free control. In contrast, significant differences in gene expression between the 2 and 7 dpi time points became apparent. For the plants inoculated with *Xcc*, many genes involved in salicylic acid (SA) signalling were highly up-regulated (SA-induced legume lectin-like protein 1, WAK1, WYRY70, DMR6, PROSCOOP4, NDR1) after two days. Remarkably, these genes were up-regulated to a significantly lower extent in the phage-treated plants (Figure 2.10). Most interestingly, three genes were up-regulated in the phage and bacteria treated MOI5 sample, while they were down-regulated in the pathogen-only condition: glycine-rich protein 3 (GRP3.1, GRP3.2) and a hypothetical protein at 2 dpi. A schematic model is included in Figure 2.10.

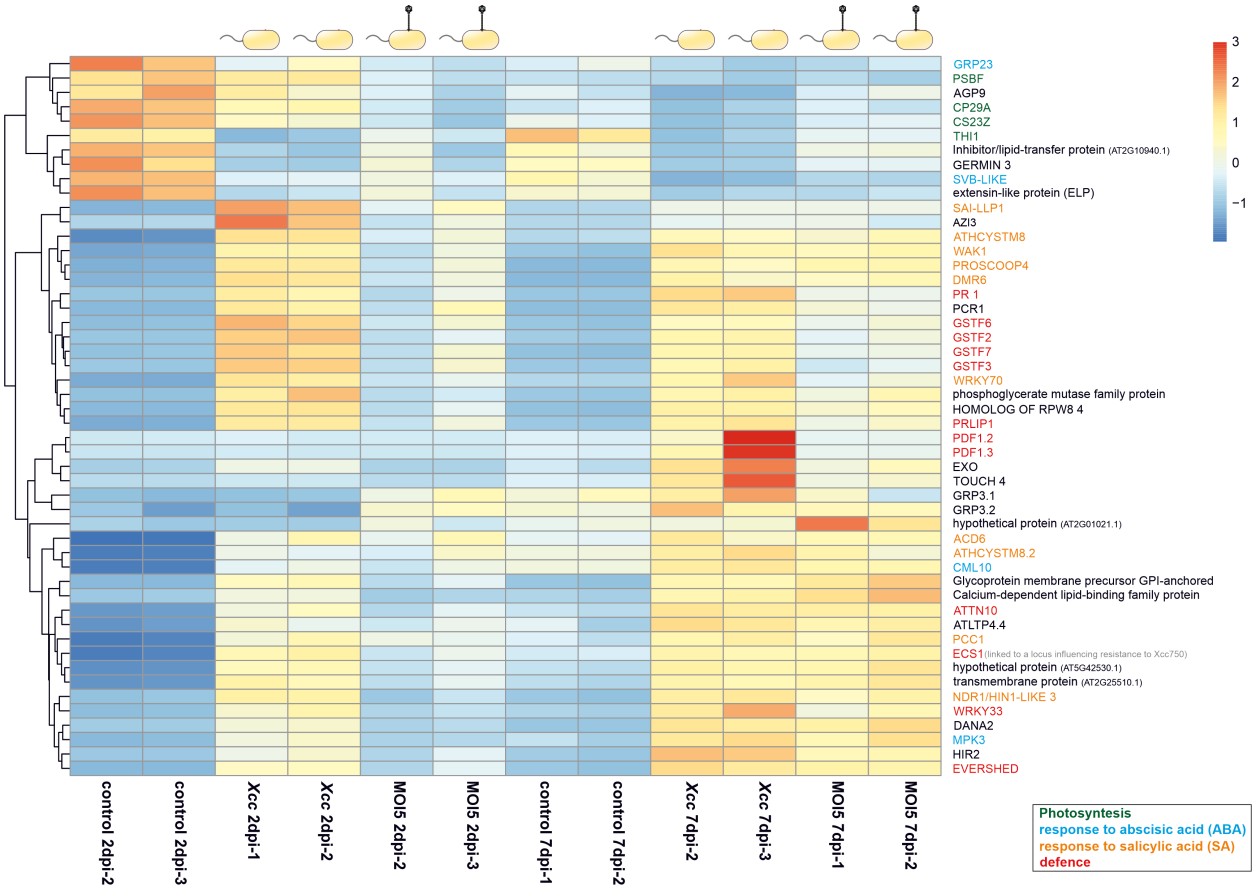

Figure 2.10: Global transcriptome analysis during the tripartite interaction. Heat map of the 50 most significantly differentially expressed genes, the values have been transformed to Z-scores which indicate relative expression to the mean of all samples. Gene identifiers were replaced with gene names based on TAIR entries and colour-coding of the gene names was applied based on the biological function described for the genes. Shown are z-scores ranging from -2 (dark blue) to 3 (dark red) and data for two independent biological replicates ('control', '*Xcc*', 'MOI5' at 2 or 7 dpi)

One important messenger in early plant-bacteria interactions is Calcium (Ca^{2+}) (Negi et al. 2023). Calcium fluxes are triggered upon detection of a bacterial pathogen from early on; in our transcriptome analysis, we found up-regulated plant genes involved in calcium signalling in the *Xcc* condition at 2 dpi. Interestingly, those genes were not induced in the sample combining bacterial pathogen and phage (MOI5) at 2 dpi, but at the later 7 dpi time point, these genes were expressed in both conditions. Calcium (Ca^{2+}) acts as an important second messenger in plant cells, and elevation of its concentration is essential during plant defence responses (Aldon et al. 2018; Lecourieux et al. 2006; Zhang et al. 2014). One of these genes is calmodulin-like protein 10 (CML10) induced by oxidative and other stress (Yu et al. 2022). DANA2/ TOUCH 3 is a calmodulin-like protein recently shown to amplify pathogen-associated molecular patterns (PAMPs) together with a calcium-dependent protein kinase Sun et al. (2022). To this group further belongs a calcium-dependent lipid-binding family protein (AT1G48090.1), whose function is still elusive.
The next block of differentially expressed genes is linked to SA signalling, which is strongest up-regulated upon *Xcc* inoculation at 2 dpi, with a Log2FC of 5.1 compared to the control. One of those genes is SA-induced legume lectin-like protein 1, an early SA-activated gene in *Arabidopsis*. This is in line with previous literature, suggesting an effector-triggered SA-mediated defence response (Armijo et al. 2013). In the MOI5 condition containing *Xcc* and the respective phage, this gene was also up-regulated, but to a lower extent with a Log2FC of 3.9 indicating that the expression in presence of the phage is only half as high as with the pathogen alone.

Furthermore, NDR1/HIN1-LIKE 3 was up-regulated, which recently was reported to be necessary to integrate SA signalling with microbe-associated molecular patterns (MAMP) signalling from membrane receptors (Tee et al. 2023). Also, WAK-1, a signalling receptor of extracellular matrix linked to SA responsiveness, was up-regulated (He et al. 1999). Furthermore, the SA signalling-dependent transcription regulator WRKY70, which mediates the choice of hormonal defence signal in favour of SA, was found to be up-regulated but only in the Xcc-treated plants. Two variants of cysteine-rich transmembrane module 8 (ATHCYSTM8) were found among the differentially expressed genes. ATHCYSTM8 encodes an SA response gene that was also up-regulated from early on in the *Xcc* condition, which was in line with earlier reports (Pereira Mendes et al. 2021). The proteins encoded by these genes are described as membrane-bound and salicylic acid (SA) responsive. In a recent study, they were reportedly involved in defence through an impact on developmental processes affecting pathogen performance without leading to the expression of classical defence proteins (Pereira Mendes et al. 2021). A further highly up-regulated gene with a

product acting at the plant cell wall is ECS1. It was up-regulated at 7 dpi in the MOI5 and *Xcc* condition but only found in *Xcc* at 2 dpi. Another family of unregulated genes encoded glutathione S-transferases, namely GSTF2, GSTF3, GSTF6 and GSTF7, a protein family that is known to respond to stress stimuli, and involved in the synthesis of the antimicrobial compound camalexin. Here, the induction of some members is triggered by H_2O_2 signalling, while others are activated by SA (Sappl et al. 2009). For example, GSTF2 was linked to SA signalling and lipooligosaccharide (LOS) triggered resistance in previous studies (Lieberherr et al. 2003; Proietti et al. 2014). GSTFs were strongly expressed upon infection with *Xcc*. HR4, a protein also belonging to this block, codes for a resistance protein involved in autoimmunity and cell death, thereby providing a broad-spectrum resistance (Li et al. 2020). The last member of this block of defence-related genes induced by the bacterial pathogen *Xcc* in our study is the pathogenesis-related lipase 1 (PRLIP1), which also responded to SA signalling in previous studies (Szalontai and Jakab 2010). Also involved in defence signalling and induced in Xcc treatment is the WRKY33 transcription factor. Remarkably, the SA-induced genes were consistently expressed weaker in the samples where *Xcc* and phages were present. This suggested either a reduction in bacterial load or crosstalk with other pathways inhibiting SA signalling.

In line with this is the expression profile of the gene coding for pathogenesis-related protein 1 (PR1). A vast body of literature states its importance in defence responses against pathogens (van Loon et al. 2006; Van Loon and Van Strien 1999), with recent studies presenting proof of extracellular secretion (Pecenkova et al. 2022) and defence amplification due to proteolytic activation, which is regulated by dimerisation of PR1 (Lu et al. 2013). The mode of action as an antimicrobial of PR-1 proteins depends on sterol sequestration, resulting in sterol deprivation of the pathogen and cellular leakage (Gamir et al. 2017). Expression of this gene was observed in the *Xcc* conditions at 2 and 7 dpi but not in all other conditions. This suggests that only in the presence of the pathogen alone is a full activation of the salicylic acid pathway accomplished since the release of its negative regulator is SA signalling dependent (Seyfferth and Tsuda 2014). Past studies have shown that the type 3 secretion system-delivered effector protein AvrXccC induces PR1 expression in *Arabidopsis thaliana* Col-0 (Rong et al. 2010).

Another hormone whose signalling targets have been found differentially expressed upon pathogen treatment at 2dpi was abscisic acid (ABA). The first differentially expressed target we found was a hypothetical protein (AT5G42530.1), which has a MYC2 recognition site and showed responsiveness to singling ABA in a previous study (Abe et al. 2003). The same was observed for a transmembrane protein that was not further specified (AT2G25510.1). In-

terestingly, it shared 49% sequence identity with the former one, indicating that both might be involved in the same biological functional loop. The transmembrane protein AT2G25510.1 was also found to be up-regulated in a study identifying the role of Receptor-like kinase1 (RPK1) as a key player in early ABA signalling in *Arabidopsis* (Osakabe et al. 2005). Notably, none of these genes showed increased expression in the other conditions at that time point, indicating ABA signalling as an early response during *Xcc* infection (Figure 2.10). This is in line with a recent study that showed that calcium-dependent ABA signalling is involved in stomata closure during *Xcc* infection in *Brassica napa* (Mamun et al. 2023). Another up-regulated gene also in the ABA pathways was GRP23, encoding a glycine-rich protein responsive to abscisic acid (ABA) and salicylic acid (SA). Previous studies have shown that this protein interacts with RNA polymerase II, suggesting it function as a transcriptional regulator (Ding et al. 2006). Another differentially expressed gene responsive to ABA is Arabinogalactan protein 9 (APG9), belonging to a group of membrane-bound glycosylated proteins involved in a diverse set of cell functions (Lin et al. 2022; Pereira et al. 2014). In contrast, the Pathogen and Circadian Controlled 1 (PCC1) was also highly up-regulated is SA responsive and was shown to have a negative regulatory effect on ABA signalling. The up-regulation of PCC1 upon *Xcc* infection aligns with prior studies in *A. thaliana* (Tan et al. 2015).

The third group of hormone signalling activated genes were linked to Jasmonate (JA) signalling, a major player in regulation of the growth-defence trade-off (Li et al. 2022). The jasmonate-responsive plant defensins PDF1.2 and PDF1.3 were expressed (Penninckx et al. 1998; Sels et al. 2008), only in the pathogen treated sample at 7 dpi.

To better understand the influence of the phage Seregon in the phage treated Xcc condition, we focussed on up-regulated genes in the phage treatment samples, compared to the control. Thereby, we identified GRP3.1, GRP3.2 and a hypothetical protein, which were up-regulated at 2 dpi only if the bacterial pathogen was present in combination with the phage Seregon at MOI5 and not in the presence of the bacterium alone. Interestingly, GRP3 was proposed to regulate the activity of the wall-associated kinase1 (Wak1) negatively (Behnami and Bonetta 2021; Gramegna et al. 2016). In literature, further function linked to GRP3 are root size determination and stress tolerance. When looking at the z-score GEP3 is up-regulated in the phage treatment at 2 dpi, while being down-regulated in in comparison to the mean of all other conditions at this time point. This could indicate that the damage associated molecular pattern (DAMP) signalling is tuned down in the presence of the phage. Never the less it is worth mentioning, that one study proposed a contrasting model for the GEP3-WAK interaction as modulator of defence responses (Mangeon et al. 2017). All this points to

differential regulation of the SA-Pathway in the MOI5 condition or even a SA independent pathway being activated.

The observed differences in the MOI5 treatment have to be investigated in future studies. Also, the phage only control which remains to be analysed is of great interest. This may shed light on the question of whether the bacterial reduction by the phage primarily leads to the increased fitness of the plant or if a yet not fully understood defence pathway is triggered in the presence of the phage. A model of the gene activation occuring during the tripartite interaction is displayed in Figure 2.11.

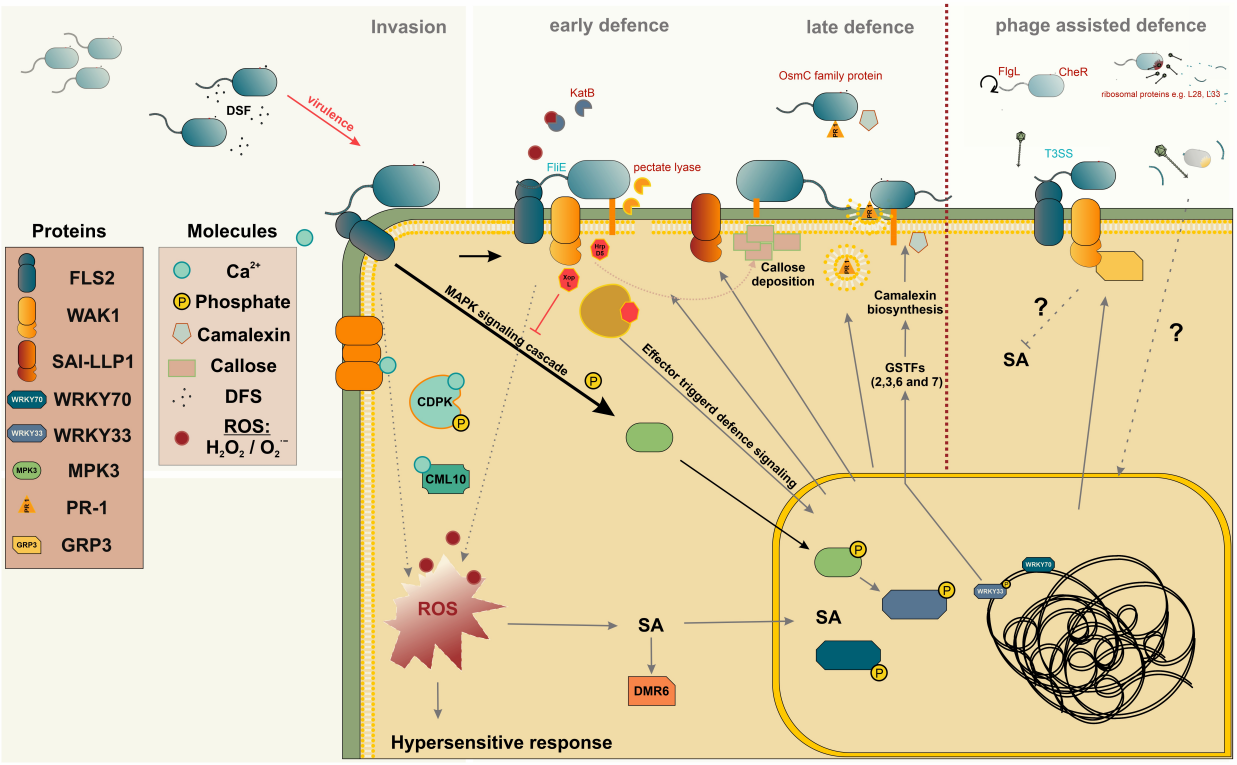

Figure 2.11: **Tripartite regulation model.** Simplified model of the potential defence regulation in our system. Depicted are the genes we found to be regulated upon pathogen attack as well as in combination with the phage. Further major pathways they are involved were deduced from literature. Preliminary.

2.8 Bacterial regulation under phage predation *in planta*

Excited by the regulatory changes found in the plant during the tripartite interaction, we were curious about the changes in the bacterial transcripts. Not only did we find differentially

expressed genes when comparing *Xcc* and *Xcc* + phage and *vice versa* (Figure 2.12 A). Further a PCA showed that there is a difference expression of bacterial genes between the two conditions (Figure 2.12 B). At 2 dpi the half of the of significantly expressed can be found in both conditions. Interestingly a LysR-like transcription regulator is up-regulated in the *Xcc* condition compared to the phage treated bacterium at MOI5. But deeper analysis of the data is needed and a plant free bacterial transcriptomic reference, would be helpful to distinguish between the response to the plant and response to the phage.

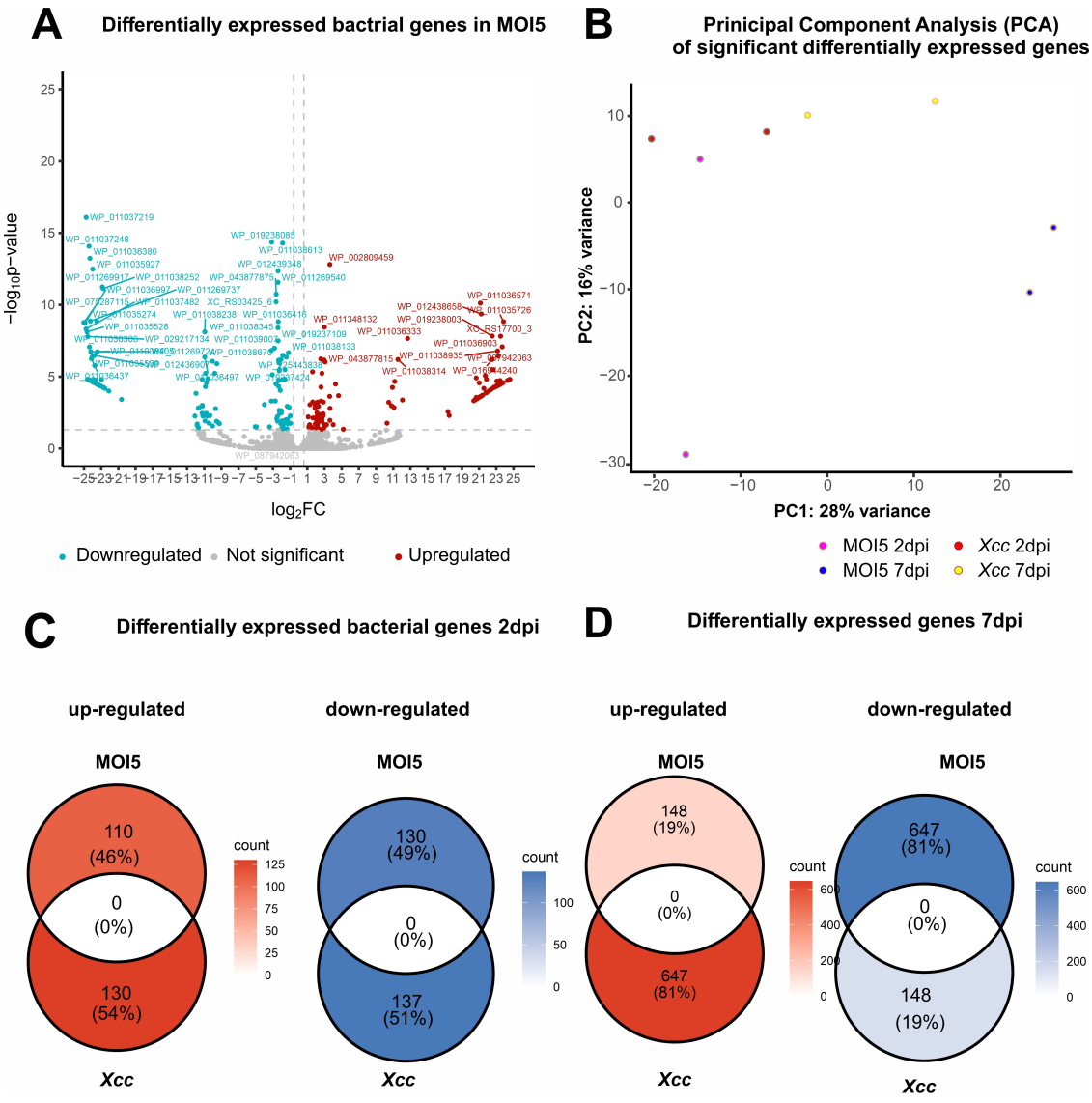

Figure 2.12: Differentially expressed bacterial genes during tripartite interaction.

Figure 2.12: **Bacterial transcripts during tripartite interaction.** Significant genes were identified with DESeq2; p-val < 0.05 **A)** Vulcano-plot of differentially expressed genes. Up-regulated genes are shown in red, down-regulated genes are shown in blue. **B)** Principal component analysis, samples: MOI5 2 dpi, MOI5 7 dpi, *Xcc* 2 dpi and *Xcc* 7 dpi **C)** Venn-Diagram of differential expressed genes at 2 dpi in the presence of phages (MOI5), *Xcc* inocculated 2 dpi was used as reference. Up-regulated genes are shown in red and down-regulated genes are shown in blue. **D)** Venn-Diagram of differentially expressed genes at 7 dpi, the Itreatment 7 dpi was used as reference. Up-regulated genes are shown in red and down-regulated genes are shown in blue.

2.9 Phage gene expression during the tripartite interaction

Also for the phage we obtained transcripts during the RNA sequencing (Figure 2.13). While the coverage of structural components like the major capsid protein is at the same level at 2 and 7 dpi. Some hypothetical phage proteins like gene 47 generate higher transcript number at 7 dpi. Future research could focus on the initial hours of the infection to get a more nuanced expression profile for the prokaryotes.

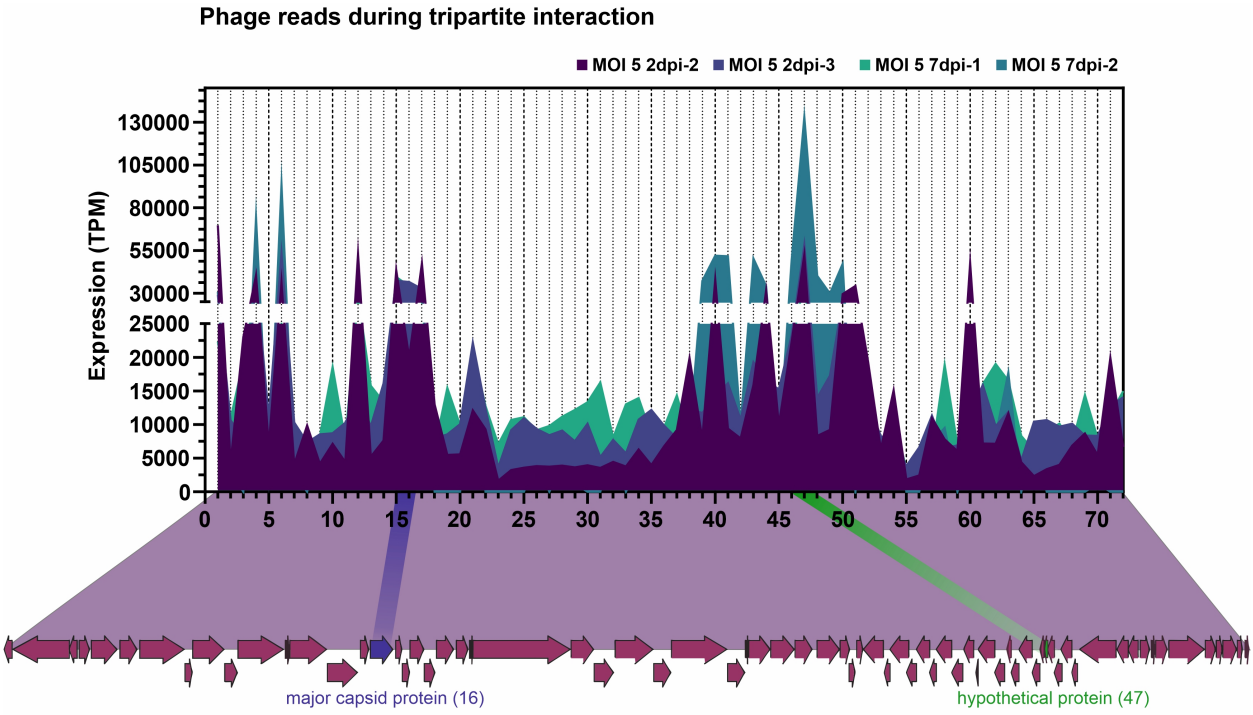

Figure 2.13: **Phage regulation during the tripartite interaction.** Phage transcripts obtained during transcriptomics of the tripartite. Shown is the transcripts per million count of aligned reads for each gene in the genome of phage Seregon.

3 Conclusion and perspectives

The increased need for higher leverage in food production, resulting from a growing population, a limited area with fertile conditions for farming and a reduced efficiency of classical pest control strategies due to resistance emergence, has led to a renaissance in phage biocontrol (Svircev et al. 2018). The high abundance of phages (Dion et al. 2020) and specificity to a limited number of bacterial strains make them a sustainable and precise tool for plant protection.

With the isolation and characterisation of the novel phages Alfirin, Athelas, Langgrundblatt1, Langgrundblatt2, Pfeifenkraut, Elanor, Laurilin, Mallos and Seregon, this doctoral thesis adds new isolates for several important plant pathogens *(A. tumefaciens, P. syringae, X. translucens* and *X. campestris*) to the publicly available pool of phages for biocontrol. Exploring the genetic diversity of the isolates, this work has led to the identification of multiple new phage genera (Shirevirus, Mallovirus, Elanovirus and Agrovirus). Further, we could demonstrate usable properties for biocontrol and explored their application for plant protection (Erdrich et al. 2022, 2024).

This doctoral thesis provides novel insights into the mechanism by which phages interact with the seed coat mucilage of mucilaginous plants. This led to our first observation that the seed coat mucilage is relevant for phage binding and that phages of the *Autographiviridae* are primarily dependent on the polysaccharide matrix for the interaction (Erdrich et al. 2024). Given the fact that podovirions were reported to interact with bacterial polysaccharides in the past (Casjens and Molineux 2012). We addressed the question of which sugar is relevant for the phage in the context of seed binding *in planta*. *A. thaliana* seed coat mutants were tested for this purpose, suggesting that the *Autographiviridae* phages tested bind to diffusible cellulose, which is lacking in the *cea5* mutant. Although the exact underlying mechanism of action remains to be elucidated, our study represents a cornerstone for further integration of phage interaction with seeds and young plant parts. The identification of mechanistic details and other receptor-binding proteins present in phages will expand our knowledge about this previously overlooked facet of phage-plant interaction. The identification of the mechanisms and further eukaryotic transient adsorption targets may also fuel new surface coating strategies as measures to protect crop plants. In addition to that, artificial seed-coatings

making use of polysaccharide matrices similar to the mucilage would improve phage binding, stability and could be used to store phage protectants or nutrients to improve plant growth.

To gain an in-depth understanding of the plant transcriptome during the tripartite interaction we performed a deep sequencing during phage treatment against Xcc in *A. thaliana.* This novel approach allowed us to identify regulatory changes occurring during phage biocontrol and is a cornerstone for a more detailed molecular understanding of the plant response to phage treatment. This could pave the way to an even more effective application of bacteriophages. By showing the differential regulation of genes *in planta* during the tripartite interaction, we point to the complexity of the multispecies interaction even under defined experimental conditions (Erdrich et al. 2024, in preparation) Such differential expression and activation of so far uncharacterised pathways could have implications for defence modulation *in planta*, leading to resistance in the presence of pathogenic bacteria. We also showed an alternation of the salycilic acid pathway in presence of the phage, for example PR1 an end product of this pathway was not up-regulated when treated with the phage. This gives unprecedented insights into the effectiveness of phage biocontrol at the molecular level. Subsequent studies have the potential to delve into the molecular mechanisms in greater detail by uncovering the underlying causes of differential regulation during the tripartite interaction. This could be achieved through the verification and characterization of alternative pathways using knock-out mutants. Basic molecular understanding of phage biocontrol in plants holds great potential for future discoveries.

In addition to gaining a more detailed molecular understanding, we were interested in the physical plant-phage interaction, as a measure of plant protection by using the phages as an additional external defence layer for the plants. To counteract bacterial infections in agricultural crop plants, a detailed understanding of the mechanism of action of the phage *in planta*, as well as the development of effective deployment strategies, is a prerequisite. Past studies used the phyllosphere as a delivery target (Balogh et al., 2003), which is apparent when one aims to fight the pathogen where the symptom occur. We utilized a naturally occurring polysaccharide matrix of mucilaginous plants as storage for bacteriophages to protect the plants from early on. Future study's could test the addition of phage protectants or nutrients to improve plant growth. In the same direction goes the idea of using artificial seed-coatings as microbial engineering tools, by adding phages as well as further beneficial microbes to increase the plant performance.

In the last decades, phage biocontrol in plants was mainly studied in the form of a two-way interactions by either focusing on the phage-bacteria interactions or the plant-bacteria interactions. Integrating the knowledge gained from this bipartite interaction studies into complex multi-species networks could lead to a more holistic view. The regulatory responses of all players interacting with each other provide the basis for future studies, which could be addressed via meta-transcriptomics or reverse genetics in a multipartite system approach. Another exciting aspect for future studies is the spatiotemporal visualisation of bacteriophages and their host-bacteria within the plant system. Since the first attempts to study phage translocation within plants have been made on a rough scale (Kolozsvarine Nagy et al. 2015; Rahimi-Midani and Choi 2020), it might be of particular interest to investigate the mode of translocation non-invasively by fluorescent labelling of the phage and the impact of those phages on co-labelled bacteria *in planta*. Another interesting aspect worth tackling for future studies is deciphering the functional units of phage structural components, e.g., tail fibres and virion-associated appendages, and their interaction with universal chemical building blocks found interkingdom-wide. Since it is known that phages can adhere to the human mucus, establishing persistence within the niche (Barr et al. 2013; Chin et al. 2022), one could imagine that a similar interaction could also occur within the plant. Also bacteria have been observed to transport phages for their competitors to new habitats using fungal hyphae as fast tracks (You et al. 2022). Going one step further in this direction, an *in-silico* prediction tool using the deciphered interacting structures could be a valuable asset for the composition of phage cocktails in medicine and agriculture. One other exciting question is if plants can modulate phage activity by root exudates, for example releasing divalent ions, which could improve phage binding to bacteria or even “weaponize” them by sensitising the bacterium for infection like it was suggested within bacterial communities (Zang et al. 2024).

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4 Publications

Contributions of the listed authors to the manuscripts were attributed using the "Contributor Roles Taxonomy (CRediT)" (CRediT - Contributor Roles Taxonomy (niso.org)):

Table 4.1: Contributor Roles Taxonomy

Conceptualization	Ideas; formulation or evolution of overarching research goals and aims
Data curation	Management activities to annotate (produce metadata), scrub data and maintain research data (including software code, where it is necessary for interpreting the data itself) for initial use and later re-use.
Formal analysis	Application of statistical, mathematical, computational, or other formal techniques to analyse or synthesize study data.
Investigation	Conducting a research and investigation process, specifically performing the experiments, or data/evidence collection.
Methodology	Development or design of methodology; creation of models.
Project administration	Management and coordination responsibility for the research activity planning and execution.
Resources	Provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation, computing resources, or other analysis tools
Software	Programming, software development; designing computer programs; implementation of the computer code and supporting algorithms; testing of existing code components.
Supervision	Oversight and leadership responsibility for the research activity planning and execution, including mentorship external to the core team.
Validation	Verification, whether as a part of the activity or separate, of the overall replication/reproducibility of results/experiments and other research outputs.
Visualization	Preparation, creation and/or presentation of the published work, specifically visualization/data presentation.
Writing - original draft	Preparation, creation and/or presentation of the published work, specifically writing the initial draft (including substantive translation).
Writing - review and editing	Preparation, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision - including pre- or post-publication stages.

4.1 Isolation of Novel *Xanthomonas* Phages Infecting the Plant Pathogens *X. translucens* and *X. campestris*

Erdrich, S.H., Schurr, U., Arsova B. and Frunzke, J. (2022).

Submitted to, Viruses 2022

Table 4.2: Isolation of Novel *Xanthomonas* Phages Infecting the Plant Pathogens *X. translucens* and *X. campestris*

Contributor role	**Contributor**
Conceptualization	SE (50%), JF (40%), BA (10%)
Data curation	SE (100%)
Formal analysis	SE (100%)
Funding acquisition	JF (50%), US (50%)
Investigation	SE (100%)
Methodology	SE (60%), JF (30%), VS (10%)
Project administration	SE (50%), JF (20%), BA (20%), US (10%)
Resources	-
Software	SE (50%), VS (50%)
Supervision	JF (60%), SE (30%), BA (10%)
Validation	SE (60%), JF (20%), BA (20%)
Visualization	SE (90%), VS (5%)
Writing - original draft	SE (70%), JF (20%), BA (10%)
Writing - review and editing	SE (45%), JF (40%), BA (5%), US (5%), VS (5%)

Overall contribution: 80 %

viruses

MDPI

Article

Isolation of Novel *Xanthomonas* Phages Infecting the Plant Pathogens *X. translucens* and *X. campestris*

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Abstract: The genus of *Xanthomonas* contains many well-known plant pathogens with the ability to infect some of the most important crop plants, thereby causing significant economic damage. Unfortunately, classical pest-control strategies are neither particularly efficient nor sustainable and we are, therefore, in demand of alternatives. Here, we present the isolation and characterization of seven novel phages infecting the plant-pathogenic species *Xanthomonas translucens* and *Xanthomonas campestris*. Transmission electron microscopy revealed that all phages show a siphovirion morphology. The analysis of genome sequences and plaque morphologies are in agreement with a lytic lifestyle of the phages making them suitable candidates for biocontrol. Moreover, three of the isolated phages form the new genus "*Shirevirus*". All seven phages belong to four distinct clusters underpinning their phylogenetic diversity. Altogether, this study presents the first characterized isolates for the plant pathogen *X. translucens* and expands the number of available phages for plant biocontrol.

Keywords: phage isolation; phage genomics; *Xanthomonas campestris*; *Xanthomonas translucens*; *Siphoviridae*; phage biocontrol

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1. Introduction

Pathogenic microbes represent a major factor hampering current food production and account for an annual loss of 10% of the global production [1]. Phage-based biocontrol for the treatment of bacterial infections was already assessed in trials by Mallman and Hemsworth in 1924 to treat black rot of cabbage shortly after the discovery of phages in general [2]. However, due to their high specificity, phages were replaced by broad-range antibiotics and cheaper copper treatments in the beginning of the last century. Nowadays, the massive spread of antibiotic-resistant bacteria has fostered a global surge in the reinvestigation of phage biology and phage-based biocontrol as an alternative to antibiotic treatments [3–6].

An overview of the economically most relevant plant pathogens is given in Mansfield et al. 2012 [7]. In recent years, several attempts have been made to isolate phages infecting plant pathogens, including *Ralstonia solanacearum*, *Erwinia amylovora*, *Pseudomonas syringe* spp, *Xylella fastidiosa*, and *Xanthomonas* spp. Multiple studies were conducted on phage biocontrol of *Ralstonia solanacearum* showing suppression of plant wilting on potato and tomato [8–13]. *Erwinia amylovora*, which developed resistance to streptomycin, has led to the evaluation of phage biocontrol with promising outcomes in some cases [14–16]. Due to the broad spectrum of plants infected by *Pseudomonas syringae* spp., multiple biocontrol trails have been conducted in the past, but with a special effort on citrus canker disease [17–23]. *Xylella fastidiosa* is a major threat to olive trees in Europe, where first phages have been isolated and tested [24–26].

The genus of *Xanthomonas* contains multiple gram-negative plant pathogens causing devastating losses in food production in a broad variety of important food crops, from the *Poaceae* family (including rice, sugar cane, and wheat) to the *Brassicaceae* family (including cabbages, broccoli, and oil seed). In this study, we focus on two important *Xanthomonas* pathogens: (1) *Xanthomonas translucens* pv. *translucens* (Xtt), causing bacterial leaf streak in cereals, and (2) *Xanthomonas campestris* pv. *campestris* (Xcc), the major cause of black rot disease in crucifers.

X. translucens (Xtt) infects cereals such as barley or wheat and, thereby, poses a threat to crop production, with annual losses reported from 10% reaching up to 40% in severe cases [27]. For other *Xanthomonas* species [6], first phages have been sequenced and morphologically characterized, but in the case of Xtt no phages are available in phage collections. There is only one report from 1953 on the isolation of four phages for different *X. translucens* pathovars [28], which were not sequenced nor further characterized so far. Therefore, the isolation of lytic phages for Xtt is required for the establishment of phage-based biocontrol strategies of this important pathogen. Xtt has been shown to be transmitted from leaf to seed, emphasizing that seed decontamination treatments are not always completely successful [27]. Outbreaks have been reported from many geographical regions. Nevertheless, they prevail in warmer regions. In the context of climate-change and worldwide rising average temperatures it is likely that the impact of these plant diseases will increase.

As a second host for phage isolation, we chose *Xanthomonas campestris* pv. *campestris* (Xcc), a vascular pathogen causing black rot of crucifers. The rod-shaped uniflagellar yellow colony forming bacteria causes V-shaped lesions in many *Brassicaceae* plants, of which *B. oleracea* (cabbage, cauliflower, and broccoli) is the economically most relevant [29,30]. Its main route of transmission is via the seeds, but it also was shown to be persistent in field soil [31]. For Xcc, phage isolates have been reported in the past (Caudovirales: FoX 1–7 [32]; phage PPDBI, phage PBR31, ϕL7, Phage Carpasina [33], XC1, Xccφ1 [34], Tubuvirales: ϕLf2, ϕLF), but there is still a great need for further phage isolates applicable for biocontrol. Of note, some of the previously isolated phages belong to the order of Tubuvirales, which are known to cause chronic infections of their bacterial host. Chronic infections typically have a fitness cost for the bacterium, but they do not kill the bacterial cell and are, therefore, not the ideal scenario for biocontrol.

One of the most important aspects of phage biocontrol is the isolation of phages with desirable traits, including a lytic lifestyle, the absence of virulence genes, and reasonable repression of host growth. Comprehensive characterization, genome analysis, and the analysis of the host range of newly isolated phages allow us to better understand how phages target their host bacterium. Altogether, these efforts are important to expand the "toolbox" for sustainable and targeted control of plant pathogens and allows researchers to select the phages with the most desirable traits.

Here we present the isolation, characterization, and genome analysis of seven novel *Xanthomonas* phages. Six were isolated using *Xtt* as host bacterium (Langgrundbatt 1, Langgrundbatt 2, Pfeifenkraut, Elanor, Laurilin and Mallos), and one of them (Seregon) was isolated using *Xcc*. Transmission electron microscopy and genome sequencing revealed that all seven phages show a siphovirion morphology and have a lytic lifestyle, making them suitable candidates for phage biocontrol.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

Xanthomonas translucens pv. *translucens* (DSM 18974) [27] and *Xanthomonas campestris* pv. *Campestris* [29,30,35] were used as host strains for phage isolation in this study. Cultures were grown on a nutrient agar plate and inoculated from single colonies in liquid media for overnight cultures. Nutrient broth (NB) or agar was used for culturing the bacterial strains at 30 °C.

2.2. Phage Isolation and Propagation

Phages were isolated from wastewater samples donated by the Forschungszentrum Jülich wastewater plant (50.902547168169825, 6.404891888790708—Jülich, Germany) as well as from soil samples (50.754354003126345, 6.366620681310555 within a 5 km radius—Eifel, Germany).

Virus particles within the samples were solubilized using 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 $MgCl_2$) and incubated for 3 h on a rocking shaker (Heidolph Polymax 1040, Heidolph Instruments GmbH and Co. KG, Schwabach, Germany) at 30 rpm. Afterwards, the samples were centrifuged at 5000 *g* for 15 min to remove solid particles within the samples. The supernatants were filtered through 0.2 µM pore size membrane filters (Sarstedt; Filtropur S, PES). For each sample, an aliquot of 1 mL was mixed with 0.4% NB soft agar and 100 µL of a densely-grown overnight culture (OD600 of 1) of the host and directly plated on an NB agar plate according to a modified version of the double agar overlay [36]. Plates were stored at 30 °C for incubation overnight.

Phage enrichment was performed in 15 mL NB. Filtered supernatant solution (1 mL) and 1 mL of an overnight culture of the host was added. To adjust the enrichment culture to 1-fold NB, appropriate amounts of 5-fold concentrated NB were added to the sample. The culture was incubated at 30 °C and at 150 rpm overnight. Afterwards, the enrichment cultures were centrifuged at 5000× *g* for 25 min to collect the supernatant, which was subsequently filtered with 0.2 µM pore size membrane filters, to remove residual bacteria. Serial dilutions of the enrichment supernatant were spotted on double agar overlay plates containing the host bacterium. Plaques were typically visible after overnight incubation.

Purification of the phage samples was carried out by restreaking single plaques with an inoculation loop on a fresh double agar overlay at least three times. When a stable plaque morphology was observed after three restreakings, a sample was considered as a single phage isolate [36].

Harvesting of purified phage particles was performed after overnight incubation of a double agar overlay containing the purified phage. The top agar was solubilized by adding 5 mL SM buffer (100 mM NaCl, 8 mM $MgSO_4$, and 100 mM Tris-HCl) and 2 h incubation on a rocking shaker. The solution was, subsequently, transferred into a falcon tube and centrifuged at 5000× *g* for 25 min to remove the residual amounts of top agar. The supernatant was filtered through 0.2 µM syringe filters and stored at 4 °C. For titer determination, a dilution series was spotted on overlay agar and the visible plaques at the highest dilution were counted.

Phage particles were either amplified on plates or in liquid cultures. For plate amplification, a double agar overlay was performed using 100 µL phage solution with a high titer (>108 Pfu/mL) added to the top agar and harvested as described above. For liquid propagation 50 mL medium was inoculated with 1 mL host overnight culture and 100 µL phage solution and incubated at 30 °C 150 rpm overnight. The cleared lysate cultures were centrifuged at 5000× *g* for 25 min to collect the supernatant, which was subsequently filtered with 0.2 µM pore size membrane filters. Subsequently, a 10% PEG enrichment according to [37] was performed to obtain very high titers.

2.3. Electron Microscopy Observation 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.

2.4. Phage Infection Curves

Infection was performed in microtiter plates using the BioLector® microcultivation system (Beckmann Coulter GmbH, Krefeld, Germany). For cultivation, biological triplicates

were conducted in 48-well FlowerPlates (Beckmann Coulter GmbH, Krefeld, Germany) at 30 °C and a constant shaking frequency of 1200 rpm. Backscatter was measured by excitation with light of a wavelength of 620 nm (filter module: λEx/λEm: 620 nm/620 nm, gain: 40) in 15 min intervals. Each well contained 1 mL host culture adjusted to an OD_{600} of 0.2 in NB and an addition of 2 mm $MgCl_2$. Phages were added at a multiplicity of infection (MOI) of 1 or 0.1, respectively, and incubated for 15 min at room temperature without shaking to promote phage adsorption. Sampling was performed at the indicated time points. Subsequently, 3 µL of dilutions were spotted on NB double agar plates containing the isolation host.

2.5. Host Range Determination

The host range of the phages was determined on the following strains: *Xanthomonas translucens* pv. *translucens* (DSM 18974) [27] and *Xanthomonas campestris* pv. *campestris* [29,30], *Pseudomonas syringae* (DSM 50274) [38], *Pseudomoas syringae* pv *tomato* DC3000 (Pst) [39,40], *Pseudomonas fluorescence*, *Azospirillum brasilense* sp245 grown on NB and *Sinorhizobium meliloti* 1021, *Herbaspirillum seropedicae, Pseudomonas koreensis* (DSM 16610), and *Bacillus subtilis* grown on Lysogeny Broth (LB).

The host range was determined by spotting dilution series of the phage solution on bacterial lawns prepared as double agar overlays in triplicates. A species was considered as part of the host spectrum of the phage if single plaques were visible. The efficiency of plating (EOP) was calculated relative to the isolation host.

2.6. DNA Isolation

For isolation of phage DNA, 2 mL of phage solution was treated with 1 U/µL DNAse (Invitrogen, Carlsbad, CA, USA) to remove free DNA from the solution and incubated for 30 min at 37 °C. Afterwards, EDTA and proteinase K were added to the mixture at final concentrations of 50 mM. SDS was added to a final concentration of 1% (*w*/*v*) to remove structural proteins. The mixture was incubated at 56 °C for 1 h. Subsequently, phage DNA was separated by adding 250 µL of phenol:chloroform:isopropanol (25:24:1; *v*/*v*). The mixed solution was centrifuged at 16,000× *g* for 4 min and the upper phase containing the DNA was carefully transferred to new microcentrifuge tube. Afterwards two volumes of chilled 100% ethanol were added as well as sodium acetate to a final concentration of 0.3 M. The samples were stored for at least 1 h at −20 °C and centrifuged afterwards for 10 min at 16,000× *g*. The supernatant was discarded, and the pellet was washed with 70% ethanol. The pellet was air-dried and finally resuspended in 50 µL DNAse free water. The purified DNA was stored at 4 °C until further usage for sequencing [41].

2.7. DNA Sequencing and Genome Assembly

The DNA library was prepared 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 were sampled, and a de novo assembly was performed with CLC genomics workbench 20.0.4 (QIAGEN, Hilden, Germany). Finally, contigs were manually curated and checked for coverage.

2.8. Gene Prediction and Functional Annotation

Open reading frames (ORFs) were predicted with PHANOTATE v.1.5.0 [42] and annotated against the custom databases (NCBI viral proteins, NCBI Refseq proteins, Kyoto Encyclopedia of Genes and Genomes (KEGG) [43], Phage Annotation Tools and Methods (PhAnToMe) (www.phantome.org), phage Virus Orthologous Groups (pVOG)) using the multiPhATE v.1.0 pipeline [44]. Additionally, all identified sequences were later curated 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 cutoff of 0.01.

The annotated genomes were deposited on NCBI via BankIt portal under the following accession numbers: ON189042 (Langgrundblatt1), ON189043 (Langgrundblatt2), ON189044 (Pfeifenkraut), ON189045 (Elanor), ON189046 (Laurilin), ON189047 (Mallos), and ON189048 (Seregon).

Genome termini classes were determined using Phage Term [47] parameters were set by default. Phage lifestyle was predicted by the machine-learning-based program PhageAI [48] using default parameters and further confirmed by the absence of integrase genes inside the genomes.

2.9. Genome Comparison and Classification

Genome maps were created using the R package gggenes (version 0.4.1) with fixed length parameters.

Novel phages were classified based on complete nucleotide sequences by comparing them against known sequenced phages infecting *Xanthomonas*, including closely related members recovered from the NCBI nucleotide blast searches, resulting in a total of 97 phage genomes. The 90 known genomes were downloaded from NCBI by their unique identifier. The average nucleotide identities (ANI) were calculated by pairwise comparison of the seven novel phages to the 90 reference genomes using the Perl program ClusterGenomes (https://github.com/simroux/ClusterGenomes) with default settings (80% coverage and 95% ANI). Further, we performed a clustering analysis using the VIRIDIC tool [49]. Heatmap clustering were displayed using the R package "pheatmap v.1.0.12". In addition, more than 2000 proteobacteria-specific complete genomic sequences based on Virus–Host DB [50] information list of accession numbers were downloaded using the python program NCBI-genome-download (https://github.com/kblin/ncbi-genome-download). This set of sequences was clustered at 95% identity into 725 clusters using ClusterGenomes (https://github.com/simroux/ClusterGenomes) with default settings (80% coverage, 95% ANI). Lastly, a representative sequence from each 725 clusters, including seven novel phages with related phages obtained from blast-based searches, was analysed using k-mer clustering phylogeny (https://bioinformaticshome.com/bioinformatics_tutorials/R/phylogeny_estimation.html).

3. Results

3.1. Phage Isolation and Virion Morphology

Seven novel phages infecting *Xanthomonas* species were isolated from wastewater samples taken at Forschungszentrum Jülich (Germany) or from soil samples from the Eifel (Germany). The phages Langgrundbatt 1, Langgrundbatt 2, Pfeifenkraut, Elanor, Laurelin, and Mallos were isolated on *Xanthomonas translucens* pv. *translucens* (DSM 18974) (Figure 1A). Langgrundblatt 1 and 2 formed clear plaques with a mean diameter of 0.6 and 0.9 mm, respectively, but with considerable variation in plaque sizes. Phage Pfeifenkraut formed homogeneous round and clear plaques with a diameter of 1.3 mm. Phages Elanor, Laurilin, and Mallos formed clear irregular plaques with average diameter of 0.3 mm, 0.45 mm, and 0.65 mm, respectively (Figure 1B). The phage Seregon was isolated using *Xanthomonas campestris* pv. *campestris* as host. Sergon's plaques had a diameter of 0.9 mm and were turbid in appearance. They were visible only after two days of incubation (Figure 1B).

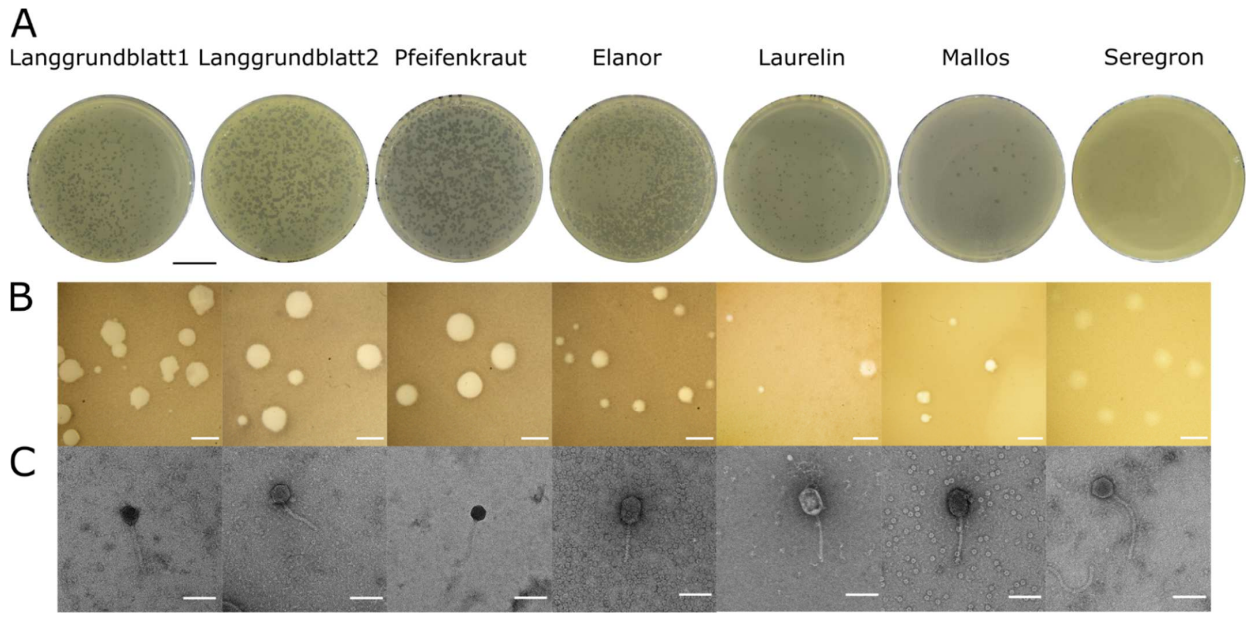

Figure 1. Phage morphology of novel *Xanthomonas* phage isolates. (**A**) Plaque morphologies of the seven different phages infecting *Xanthomonas translucens* pv. *translucens* (DSM 18974) (Langgrundbatt 1, Langgrundbatt 2, Pfeifenkraut, Elanor, Laurelin, and Mallos) and *Xanthomonas campestris* pv. *campestris* (Seregon). 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.

TEM analysis of purified phage particles showed that all seven phages have an icosahedral capsid with sizes ranging 55–82 nm (Table S1) and a non-contractile tail 134–225 nm (Figure 1C). Therefore, based on their morphology the phages were classified as siphovirion phages.

3.2. Infection Curves and Host Range Determination

All isolated phages suppressed growth of the host culture when applied at a multiplicity of infection (MOI) of 0.1 or 1 (Figure 2). Interestingly, in some cases, the lower MOI had a stronger inhibitory effect on the host culture. Quantification of the phage titer by spotting dilution series on a bacterial lawn allowed us to visualize the amplification dynamics of the phages over the course of the experiment (Figure 2 right panel). Amplification was in several cases more pronounced at an MOI of 0.1.

In the context of plant biocontrol, the host range of phages represents an important parameter, since phages should not target plant growth promoting bacteria. Therefore, we assessed the host-range of the seven phages by spotting them on bacterial lawns of different *Xanthomonas* species (Xtt; Xcc), plant growth promoting bacteria (*Pseudomonas fluorescence*, *Azospirillum brasilense*, *Sinorhizobium meliloti* 1021, *Herbaspirillum seropedicae*, *Pseudomonas koreenis*, and *Bacillus subtilis*) and other plant pathogenic bacteria (*Pseudomonas syringae* pv *lapsa*, *Pseudomonas syringae* pv. *tomato*, and *Agrobacterium tumefaciens*). *Xanthomonas campestris* showed a slight susceptibility to the phages Langgrundblatt1, Langgrundblatt2, Pfeifenkraut, and Mallos. Vice versa, the phage Seregon isolated on *Xcc* showed lytic activity on *X. translucens*, but also at low levels. The EOPs are listed in Table S2. Interestingly, phage Mallos showed additional lytic activity on the plant pathogenic *P. syringae* strain DC3000, but also infected the plant growth-promoting *P. fluorescens*. This is making phage Mallos an omnilytic phage infecting distinct species and the phage with the broadest host range of the phages isolated in this study.

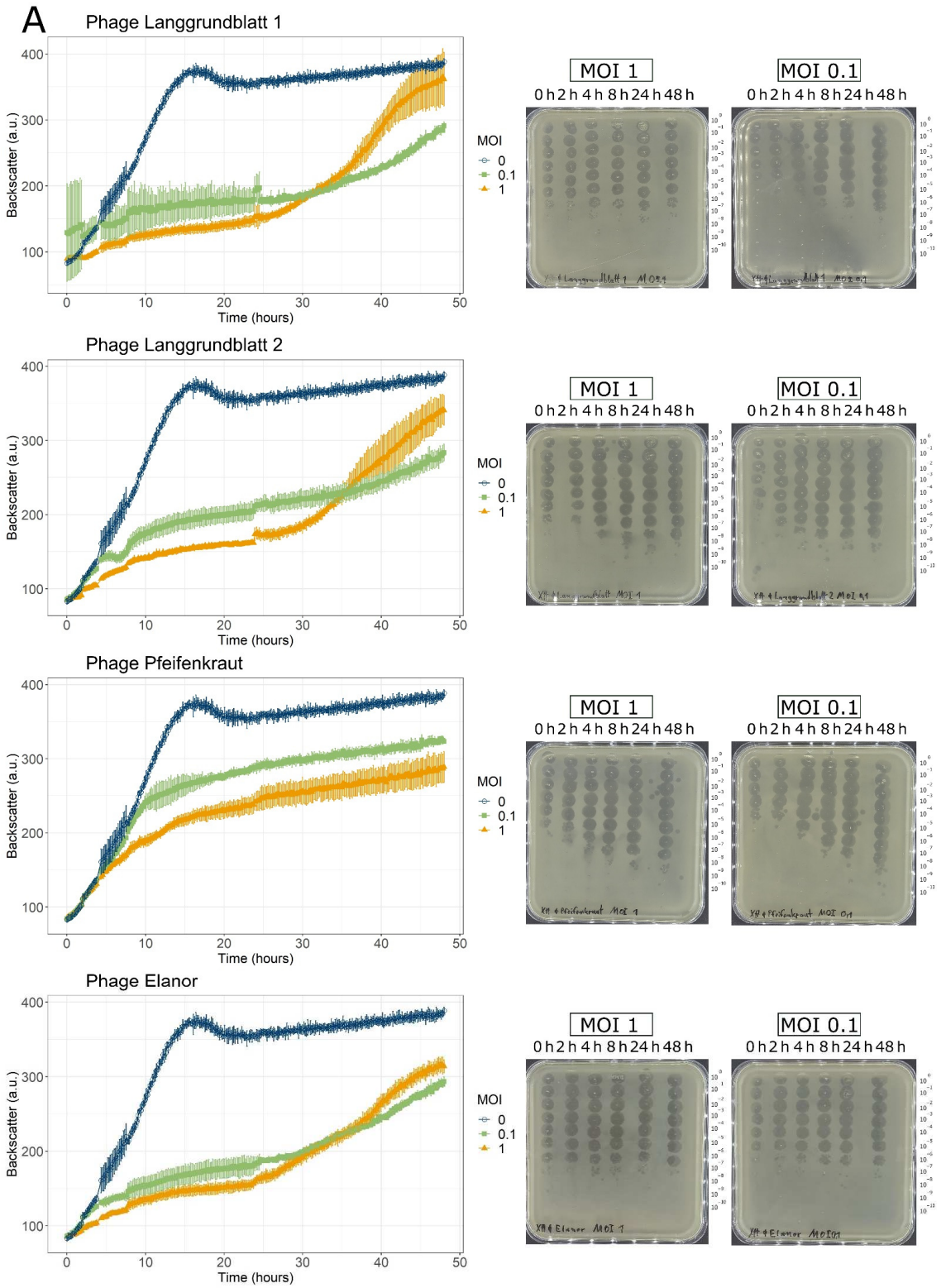

Figure 2. *Cont.*

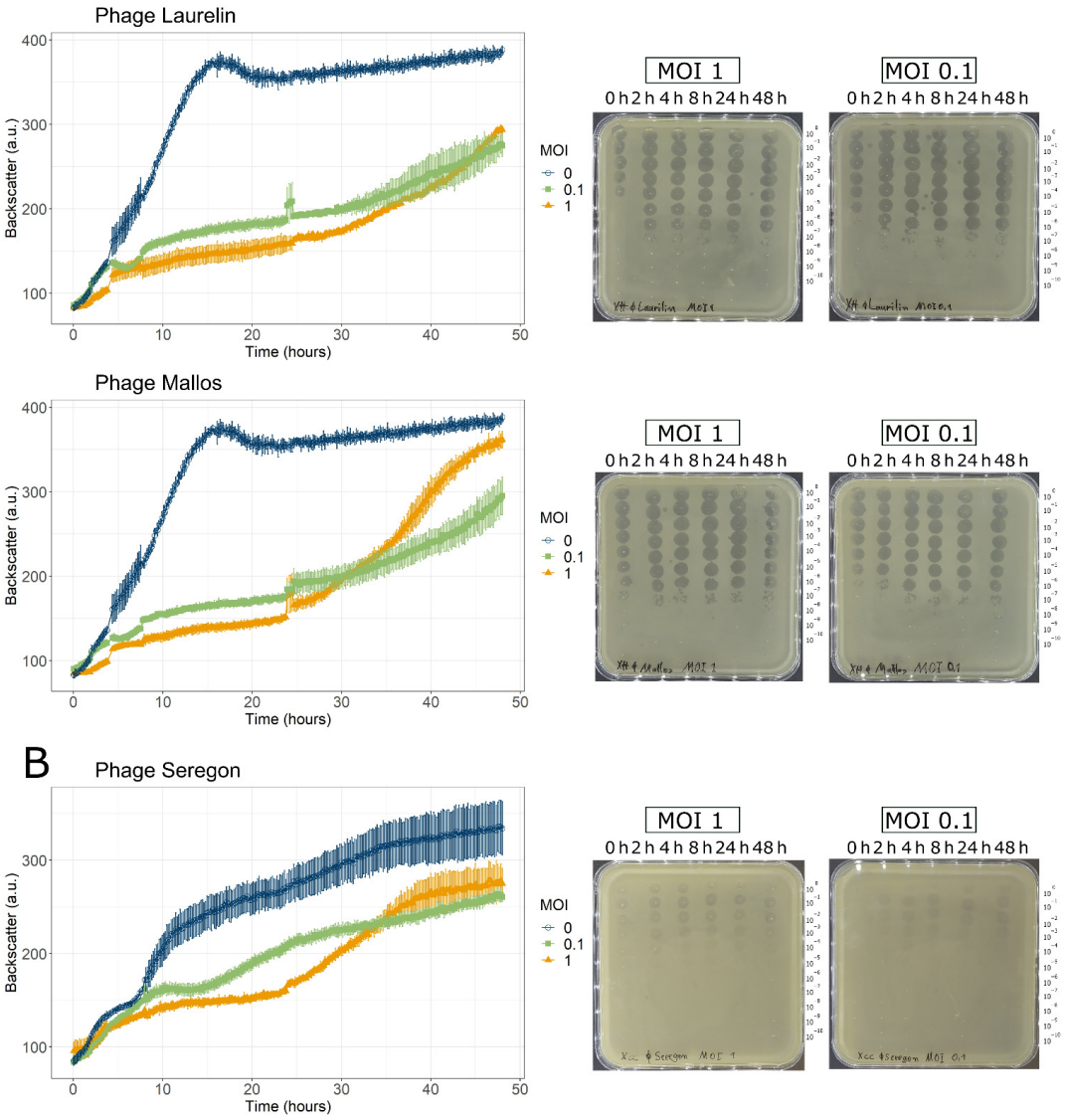

Figure 2. Infection curves of seven phages infecting Xtt (**A**) and Xcc (**B**). Full-grown overnight cultures of the host were adjusted to an OD_{600} of 0.2 and phages at the corresponding multiplicity of infection (MOI) were added. The infection was performed as biological triplicates. Backscatter was measured over time (left panels), as well as phage titers (right panels). Plaques of phage Seregon have a turbid appearance and are, therefore, hardly visible (see Figure 1B).

3.3. Genome Sequencing and Genome Features

All isolated phages were sequenced using Illumina Mi-Seq. short-read technology. Genomic features of the seven isolated phages are summarized in Table 1. Briefly, they show genome sizes from 40 to 62 kb, a GC content varies in the range of 53 to 64% and contain 56–89 annotated ORFs.

Table 1. Basic genomic features of the seven novel phages.

Phage Name	Accession Number	Reference Host	Genome Size (Bp)	GC Content (%)	ORF Number [a]	Genome Termini Class [b]	Lifestyle Prediction [c]
Langgrundblatt 1	ON189042	*Xanthomonas translucens* DSM 18974	44.239	53.3	67	Headful (pac)	virulent
Langgrundblatt 2	ON189043	*Xanthomonas translucens* DSM 18974	44.768	53.4	68	Headful (pac)	virulent
Pfeifenkraut	ON189044	*Xanthomonas translucens* DSM 18974	43.791	53.3	72	Headful (pac)	virulent
Elanor	ON189045	*Xanthomonas translucens* DSM 18974	62.341	64.5	86	Headful (pac)	virulent
Laurelin	ON189046	*Xanthomonas translucens* DSM 18974	40.498	57.4	56	DTR (short)	virulent
Mallos	ON189047	*Xanthomonas translucens* DSM 18974	59.242	61.8	88	Headful (pac)	virulent
Seregon	ON189048	*Xanthomonas campestris*	55.527	63.2	72	COS (5′)	virulent

[a] Open reading frames (ORFs) were predicted using multiPhate [44] with Phanotate [42] and, subsequently, annotated against different customized databases (NCBI viral proteins, NCBI Refseq proteins, Kyoto Encyclopedia of Genes and Genomes (KEGG) [43], Phage Annotation Tools and Methods (PhAnToMe) (www.phantome.org), and phage Virus Orthologous Groups (pVOG)). Additionally, manually curated using NCBI Blastp. Encoded Protein Domains were further predicted by using the batch function of NCBI Conserved Domain Database (CDD). [b] Genome termini classes were determined using PhageTerm [47]. [c] Phage lifestyle was predicted by the machine-learning-based program PhageAI [48] and, further, confirmed by absence of intergrade genes inside the genomes.

Phages Pfeifenkraut, Langgrundbatt 1, and Langgrundbatt 2 exhibit a remarkably low GC contend (53%) in contrast to their host *Xtt* (68%). Phage Laurelin differs from the other phages, in that its genome is the smallest, containing only 56 genes. Furthermore, it is the only one of the isolated phages containing 218 bp directed terminal repeats (DTRs). The genomic ends were determined using Phage Term [47]. In contrast, phages Langgrundbatt 1, Langgrundbatt 2, Pfeifenkraut, Elanor, and Mallos show a headful packing mechanism were the genome is translocated to the capsid at dedicated *pac* sites [51] resulting in variable genome ends. For the phage Seregon, cohesive ends with a length of 12 bp (5′ GGGGGCGCTGAC) were predicted. Since for plant biocontrol the phage lifestyle is of special importance, the lifestyle was predicted using PhageAI, a machine-learning tool which compares the genomes to over 20,000 publicly available phages [48]. All isolated phages were classified as virulent. This is further supported by the absence of integrase genes within the genomes.

The genome architecture for our isolated phages was very typical, in the sense that genes involved in the same function are clustered together featuring the typical modularity of phage genomes (Figure 3). These include units involved in DNA replication and repair, regulation, virion structure, and assembly (capsid, tail, and tail fibres), DNA packaging, and lysis. Further inspection of the phage genomes revealed that Langgrundblatt1, Langgrundblatt2, and Pfeifenkraut encode MazG nucleotide pyrophosphohydrolases [52], which, potentially, interfere with the hosts' programmed cell death. The mechanism of abortive infection is widespread among bacteria and represents a typical antiviral strategy protecting the entire population by sacrificing a single cell [53,54]. Interestingly, phage Pfeifenkraut carries a group one Intron which have a limited distribution among bacteria but have been reported only in a few cases for phages infecting gram-negative bacteria [55,56].

With an average nucleotide identity of 97% Langgrundblatt1 and Langgrundblatt2 are members of the same species [57]. The genomes of the phages Elanor and Mallos share 75% sequence identity. Both phages contain a relatively high fraction of ORF encoding proteins of unknown functions (hypothetical proteins/CDS; 40/86 for Elanor and for Mallos 46/89) reflecting once more the significant amount of 'dark matter' harboured in phage genomes.

An important family of nucleoid-associated proteins involved in the silencing of foreign (e.g., phage) DNA are H-NS proteins [58]. Interestingly, phage Laurilin encodes an H-NS-binding protein which could function as inhibitor or of the host-encoded xenogeneic silencer protein [59]. NCBI protein blast revealed a broad distribution of homolog H-NS-binding proteins among phages of gram-negative bacteria. CDD blast revealed this protein as a hypothetical protein conserved in T7-like phages (cl10202).

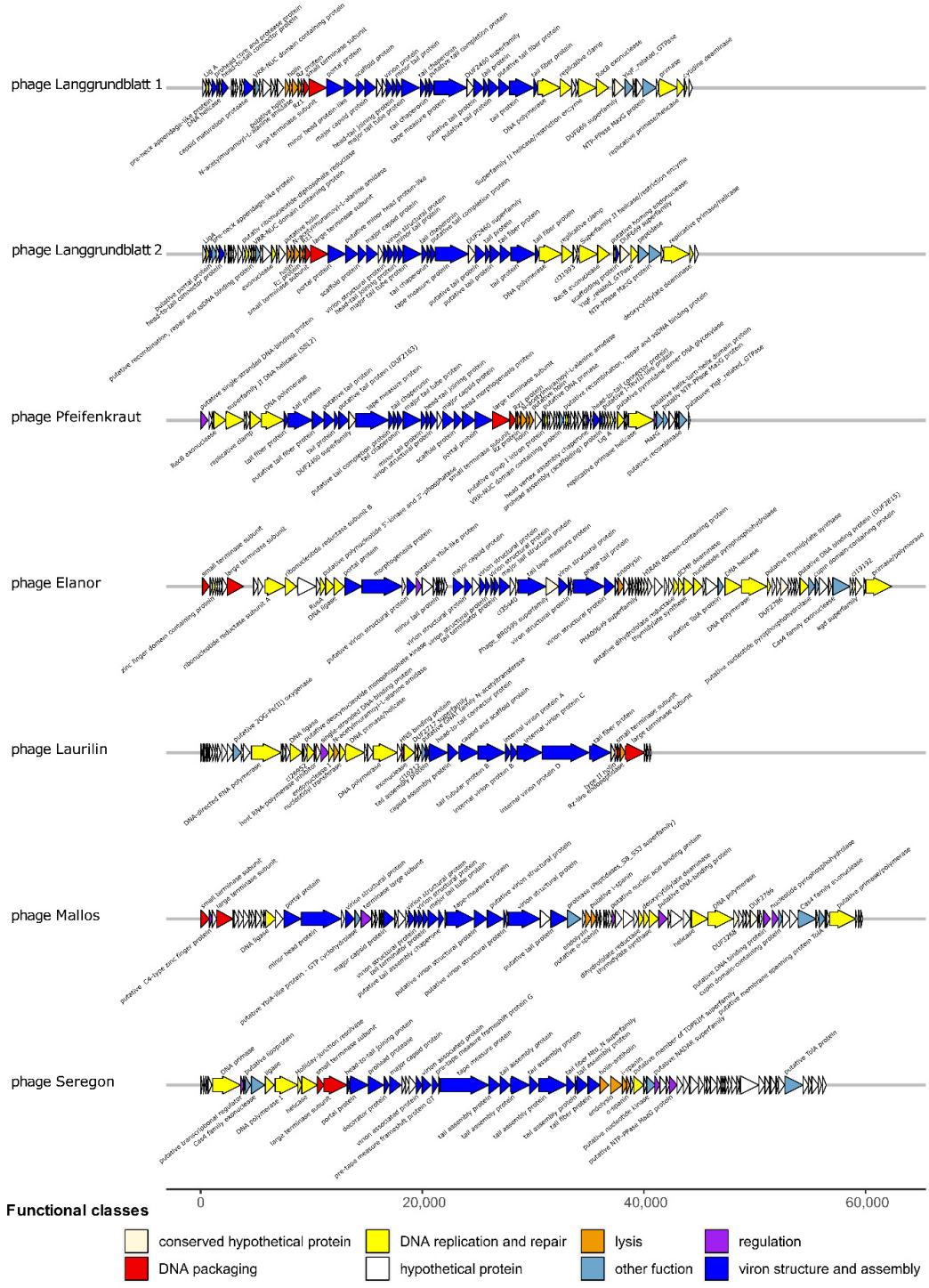

Figure 3. Genome map of the six *Xanthomonas* phages. Open reading frames (ORFs) were predicted using multiPhate [44], with Phanotate [42] and were later curated manually using NCBI Blast. Encoded protein domains were further predicted by using the batch function of NCBI Conserved Domain Database (CDD). Genome maps were created using the R package gggenes using fixed-length parameters.

3.4. Average Nucleotide Identity (ANI) Analysis

To analyze the phylogenetic relationship of our sequenced phages, we performed a comparison based on genome wide pairwise identity. Due to the high variability within phages genomes, traditional approaches often use single-gene phylogenies for the classification; however, no single gene is shared by all seven isolated phages. Only the large terminase shares sequence identity for five of the seven phages (dark purple arrow, Figure 4). Therefore, we selected all available genomic sequences for phages infecting *Xanthomonas* reviewed by Nakayinga et al. (2021) [6] and from literature [24]. The set was further expanded by unique entries of VirusHost DB [50], as well as by our phages and their closest relatives according to NCBI nucleotide blast, resulting in a total of 97 genomes. With those genomes we performed two independent clustering analysis (using VIRIDIC and ClusterGenomes), based on average nucleotide identity (ANI). The results of the clustering dendrogram created with VIRIDIC [49] show that our isolated phages fall into four distinct clusters within the *Xanthomonas* phages (Figure 5). The ANI-based clustering analysis showed that phages Langgrundblatt1, Langgrundblatt2, and Pfeifenkraut form a distinct cluster, resulting in the novel genus "*Shirevirus*". Phage Laurilin clusters with three phages known to infect bacterial species of the genus *Pseudomonas*. Phage Elanor clusters together with the phages Bosa, Xp12, and Xoo-sp2. Interestingly, all members of this cluster infect *Xanthomonas* species that are pathogens of plants belonging to the *Poaceae* family. Seregon clusters together with phages infecting *Xanthomonas* and *Xyllea* [24]. This is supported by both clustering approaches (Table S1). Phage Mallos is a special case, while in VIRIDIC it clusters with the Elanor group and phage PaMx28 as a neighbour, in the Perl-based clustering it forms a group of its own. Altogether, our isolated phages display a broad diversity and cluster broadly among the known phages for *Xanthomonas*.

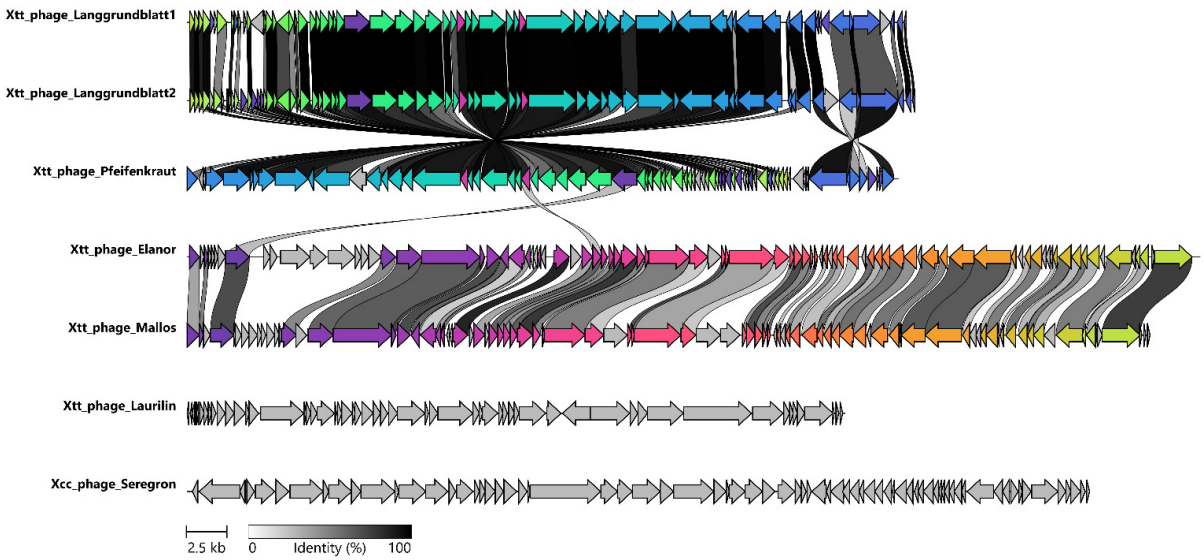

Figure 4. Genome comparison on the level of coding sequences. The coding sequences (CDS) of the isolated phages were compared using the clinker pipeline [60] to cluster them in groups by similarity (each colour represents one group) and per cent identity of the member of one group is indicated by grey values. The circular genomes are represented linearly and the direction of the arrows is in line with transcription direction of each CDS.

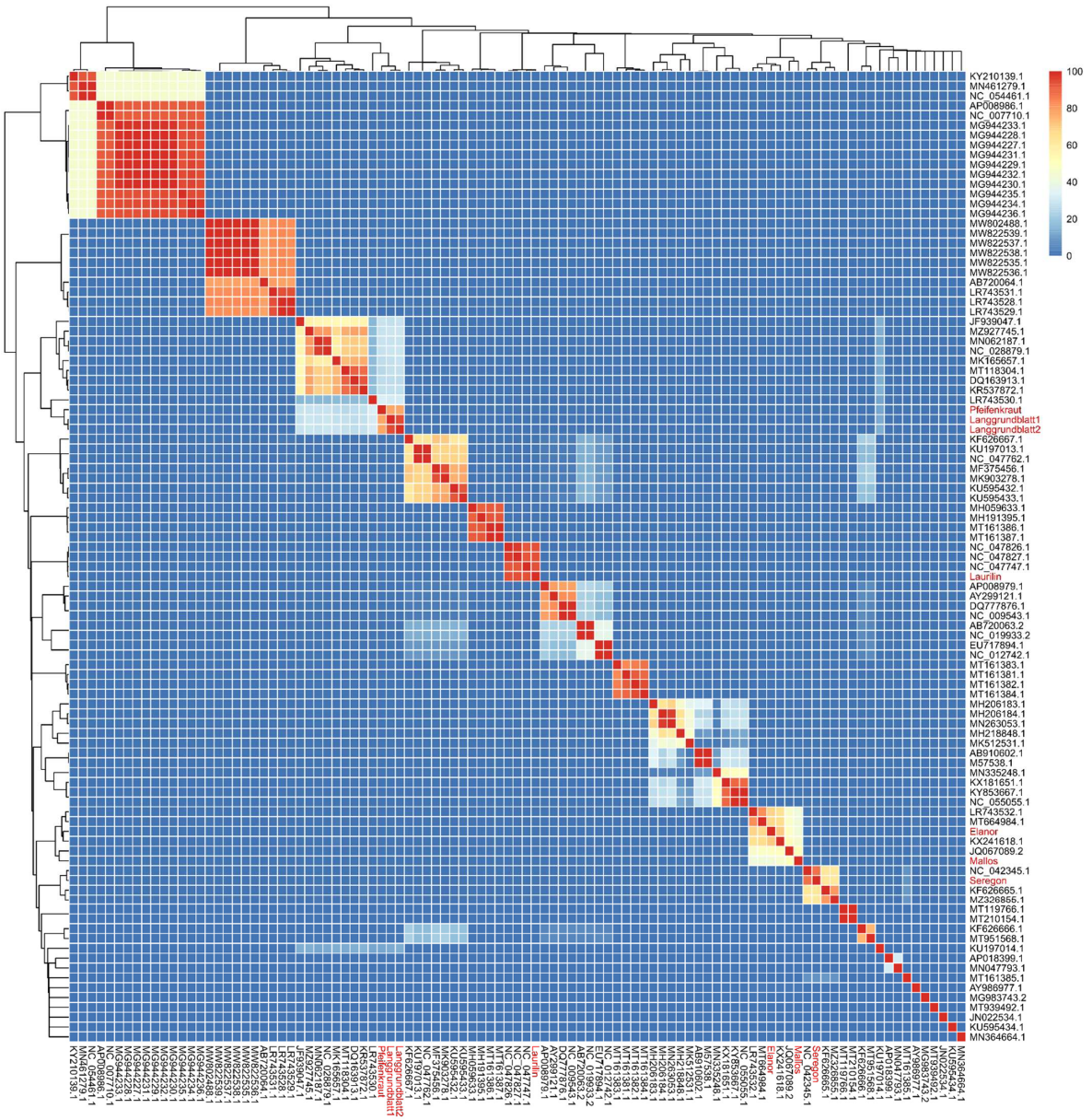

Figure 5. Average nucleotide-based dendrogram analysis using 97 phage genomes of phages infecting *Xanthomonas* species. Genomes are derived from [6] or Virus Host DB (https://www.genome.jp/virushostdb/, accessed on 19 January 2022). Furthermore, 11 genomes were acquired from NCBI based on similarity to our seven novel phages.

This diversity within our isolates is further supported by an additional clustering performed against 700 representative phages infecting proteobacteria retrieved from VirusHost DB [50] (supplementary Figure S1).

4. Discussion

Phage-based products bear a great potential for the sustainable and targeted treatment of bacterial infections in agriculture. However, to the best of our knowledge, no phage-based product was registered as plant protection product or biopesticide by the European

Food Safety Authority until now [61]. Some of the main challenges to move bacteriophages towards application are: gaining an comprehensive understanding of the bacteria–phage–plant interaction on a molecular level, the development of standard operation procedures for the evaluation of novel phages as pest-control agents, and reliable 'on-field' application strategies [61].

While many of the early phage-isolation studies [2] were limited by the technical possibilities of their time. Out of 176 known phages infecting bacteria of the genus of *Xanthomonas* only roughly 100 are sequenced. Additionally, morphological characterization has become more feasible in recent years, with more access to high-resolution imaging devices. This information provides an important basis to potentially link phage morphological traits and binding preferences known to occur in certain virion morphotypes with biocontrol possibilities [62]. Last, but not least, phages for *Xanthomonas* are highly under-sampled given the fact that the number of known phages has almost doubled in the last decade, reaching over 14,244 complete sequenced phage genomes by the beginning of 2021 [63–65].

Here we report the isolation and characterization of seven novel *Xanthomonas* phages. Genomic analysis revealed typical arrangement of genes into clusters linked to functional units, but also a significant amount of 'dark matter' harboured in phage genomes. The phages Elanor, Mallos, and Seregon encode a Cas4 family exonuclease which normally plays a role in acquiring functional spacers in bacterial CRISPR immunity [66]. Nevertheless, cases have been reported were phage-derived Cas4-like proteins led to host spacer acquisition and, subsequently, autoimmunity of the bacterial host [67]. This way, the phage uses the bacterial genome as a decoy for its own immune system, thereby gaining time for the production of phage progeny.

An essential trait for phages used in biocontrol is a lytic lifestyle. Since temperate phages could equip their bacterial host with further virulence traits [68]. All our phages were predicted to have a lytic lifestyle by the machine-learning-based phage lifestyle determination tool PhageAI [48]. This was further underpinned by the absence of integrase genes within their genomes. These results are in line with genomic clustering analysis where they cluster with virulent phages. It has, however, to be noted, that phage Seregon forms turbid plaques on lawns (Figure 1). Therefore, further analysis is required to determine the lifestyle of this phage.

Characterization of the host range assay showed that phages Langgrundblat1, Langgrundblat2, Pfeifenkraut, Elanor, and Seregon are highly specific, only infecting their isolation host and the other plant-pathogenic Xanthomonads but featuring a significantly lower efficiency of plating. Nevertheless, these assays confirmed that they do not infect the majority of the here-tested plant growth-promoting bacteria (PGPB). This shows the advantage of phages as targeted plant biocontrol argent in contrast to other antimicrobials, which also broadly affect the beneficial part of the plant microbiome. Phage Laurilin displayed the highest specificity, only infecting its isolation host *X. translucens*. In contrast, phage Mallos displayed the broadest host range and also infected the plant pathogenic bacterium *Pseudomonas syringae* DC3000, making it an omnylitic phage infecting species of two different alphaproteobacterial genera. This makes sense in the context that *Xanthomonas* and *Pseudomonas* are among the most abundant genera in the plant phyllosphere [69]. In addition, a recent study of two phages infecting *Pseudomonas syringae* pv. *tomato* DC3000 also finds lytic activity on two Xanthomonas species [70].

ANI-based comparison of our isolates to phages from the literature [6,24] revealed that the phages are very diverse. Phages Pfeifenkraut, Langgrundblatt1, and Laggrundblatt2 cluster with each other but show very little homology with other described phages, therefore forming the new genus of "*Shirevirus*". This highlights that there is a vast number of uncharacterized phages out there that can potentially be harnessed for biocontrol. This is also in line with a recent phage metagenomic study of wheat phyllosphere where *Xanthomonas* and *Pseudomonas* were the most abundant bacterial genera in the plant phyllosphere and 96.8% of the generated viral taxonomic units did represent phages which have not been isolated so far [69].

Our results suggest that our phages are suitable candidates for an application in planta and, thereby, will add up to the many phage biocontrol trials performed against a broad spectrum of different plant pathogenic bacteria in the recent years [4–6,61]. A comprehensive overview over the current phage isolates for *Xanthomonas* species is given by Nakayinga et al. (2021) [6]. Further the application of Xcc phages (FoX6 and FoX2) at different stages of plant lifecycle (seed, seedling, and field condition) was accessed recently, showing reduction of disease severity at all stages demonstrating the potential benefits of phages as biocontrol agents [32].

Recent trials aim at circumventing problems caused by classical chemical treatments against Xtt, for example, by using supernatants of lactic acid bacteria, *Bacillus* strains, or plant natural products [71]. The main downside of all these substances is that they—unlike phages—cannot multiply themselves in the presence of the pathogen. Combinatorial approaches using phages and plant-growth-promoting bacteria producing those chemically active compounds could overcome this hurdle and could be part of future integrated pest control strategies to maximize crop yield and protection. Here, the phages isolated and characterized in this study add suitable candidates to be benchmarked in future biocontrol experiments for the treatment of Xtt and Xcc infections in planta.

Additionally, by way of an example from personalized medicine, the treatment of bacterial infections in humans or animals is routinely based on the isolation of bacterial strains from the patient and isolation of specific phages for the respective pathogen [72–74]. This approach has shown promising results and demands for the regular update of phage cocktails in the clinic. It is a strong advantage of phage-based biocontrol, that this natural diversity can be harnessed. This is very likely also required for sustainably successful agricultural applications and would require phage isolation from plants growing exposed to pathogenic bacteria in field conditions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v14071449/s1, Figure S1: K-mer clustering dendrogram of Proteobacteria phages; Figure S2: Host range assay; Table S1: Phage particle size. Measurements of virion particles analyzed by Transmission electron microscopy (TEM); Table S2: Strains /Phages used in this study.

Author Contributions: Conceptualization, S.H.E., B.A. and J.F.; methodology, all; validation, all; formal analysis, all; investigation, S.H.E.; resources, B.A., U.S. and J.F.; data curation, S.H.E. and V.S.; writing—original draft preparation, S.H.E. and J.F.; writing—review and editing, all; visualization, S.H.E. and V.S.; supervision, B.A., U.S. and J.F.; project administration, J.F.; funding acquisition, B.A., U.S. and J.F. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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

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Table 4.3: Seed coating with phages for sustainable plant biocontrol of plant pathogens and influence of the seed coat mucilage

Contributor role	**Contributor**
Conceptualization	SE (40%), JF (15%), BA (15%), US (30%)
Data curation	SE (100%)
Formal analysis	SE (100%)
Funding acquisition	JF (50%), US (50%)
Investigation	SE (100%)
Methodology	SE (60%), JF (20%), BA (20%)
Project administration	SE (50%), JF (20%), BA (20%), US (10%)
Resources	-
Software	SE (100%)
Supervision	BA (50%), JF (30%), SE (20%)
Validation	SE (60%), JF (20%), BA (20%)
Visualization	SE (100%)
Writing - original draft	SE (70%), JF (20%), BA (10%)
Writing - review and editing	SE (30%), JF (30%), BA (30%), US (10%)

Overall contribution: 80 %

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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

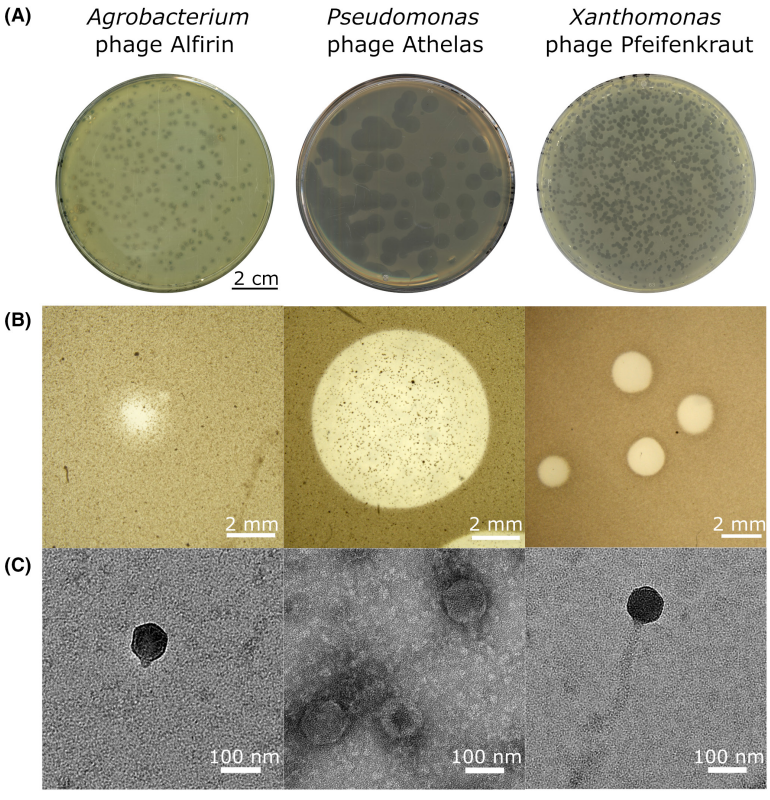

FIGURE 1 Phage morphology of the novel *Agrobacterium* phage Alfirin, and the *Pseudomonas* phage Athelas as well as *Xanthomonas* phage Pfeifenkraut, which was previously described (Erdrich et al., 2022). (A) Plaque morphologies of phages on 0.4% soft agar. Scale Bar: 2 cm; (B) Stereo microscopy of 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,

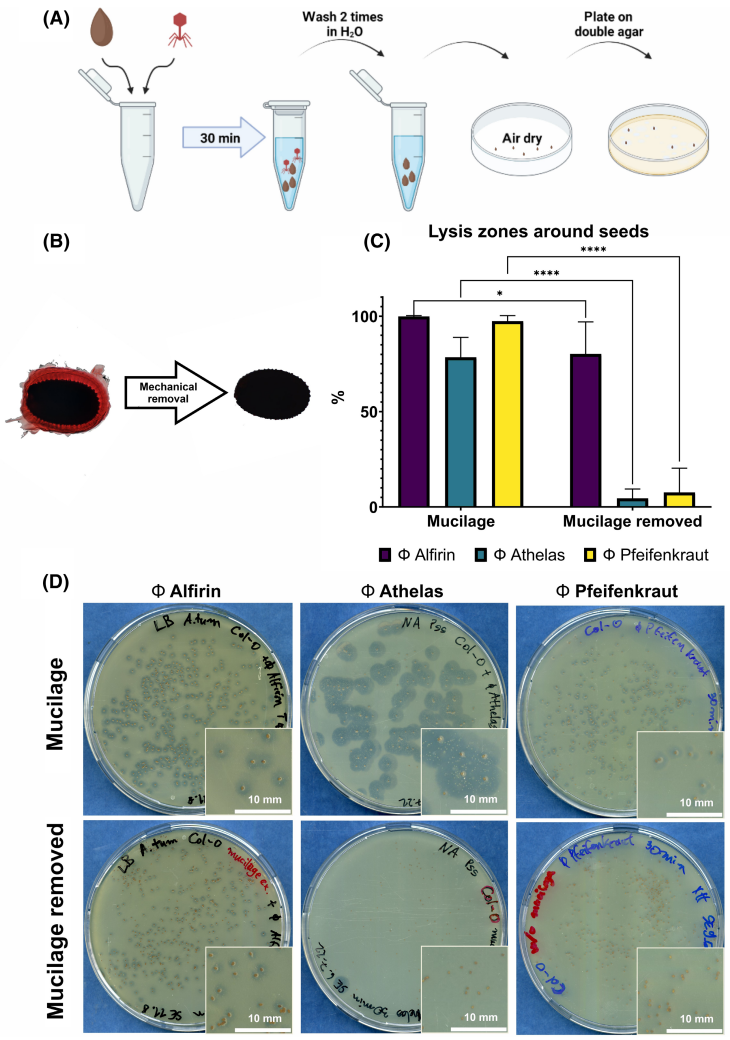

FIGURE 2 Phage binding to seeds and influence of artificial removal of the seed mucilage. (A) Seed-coating-workflow; binding of phage particles on seeds of *Arabidopsis thaliana*. (B) Wild-type Col-0 seed stained with 0.01% ruthenium red solution before and after mechanical removal of the mucilage. (C) 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

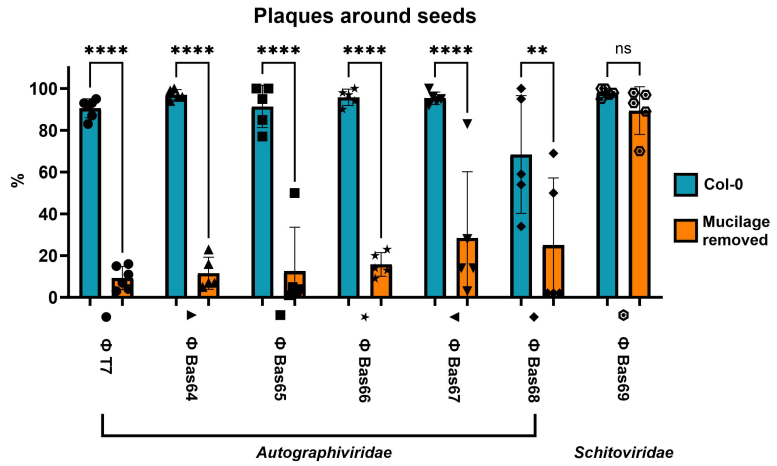

FIGURE 3 Binding of podovirions to seeds and influence of artificial removal of the seed mucilage. All BASEL featuring podovirus morphology were selected, as well as model phage T7. Percentage of plaques around seeds with and without mucilage. At least three independent experiments were performed for 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.

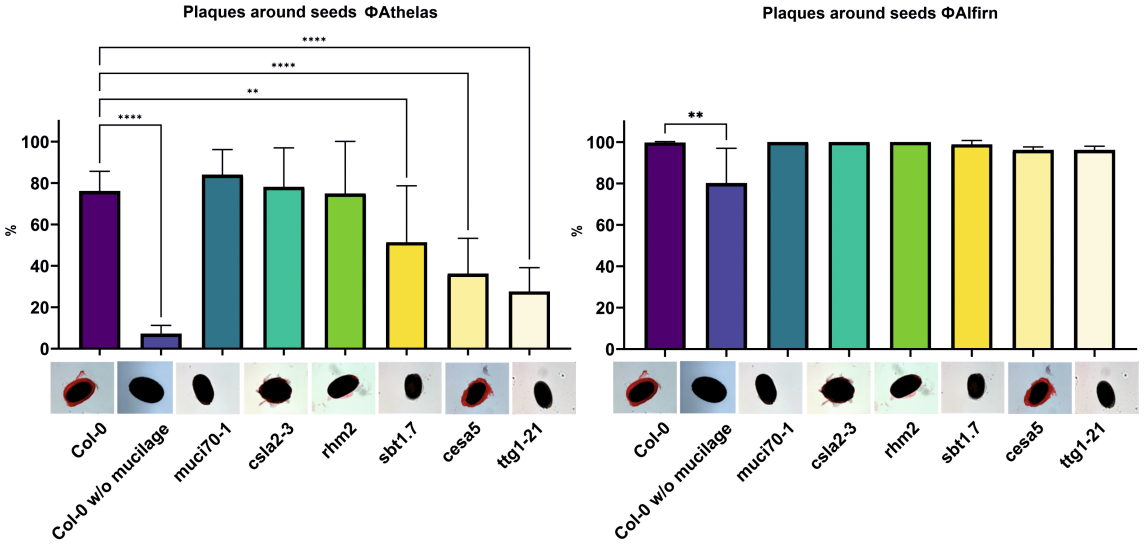

FIGURE 4 Phage seed coating on different *Arabidopsis* mutants. Left panel phage Athelas; right panel phage Alfirin. Shown is the amount (%) of plaques detected on a double agar overlay post seed coating of the *Arabidopsis* mutants: *muci70-1, csla2-3, rhm2, sbt1.7, cesa5* as well as the wild type *A. 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)

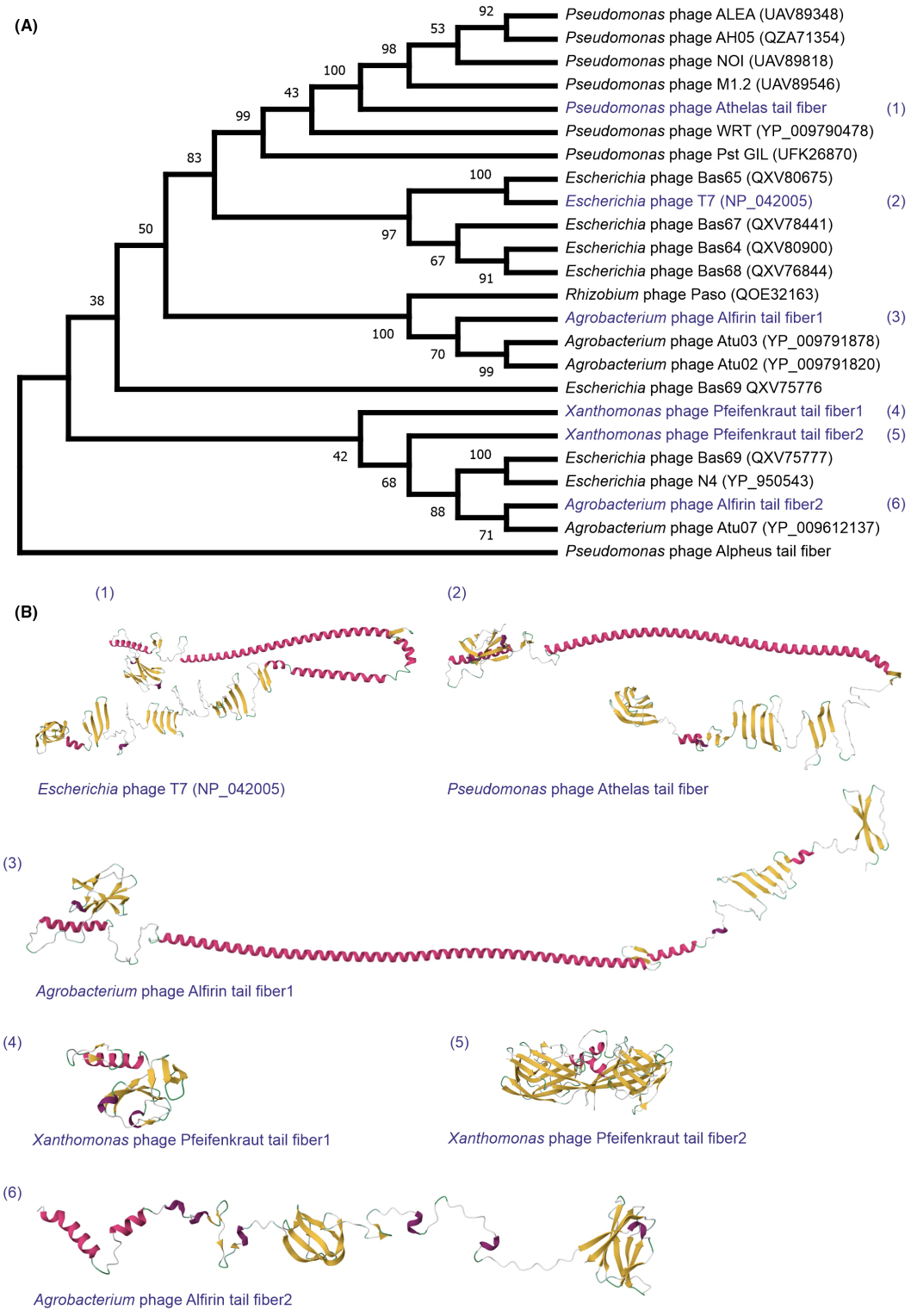

FIGURE 5 Phylogenetic tree of phage receptor binding proteins (RBPs) and in silico folding. (A) Phylogenetic tree of phage RBPs. Ancestral states were inferred using the Maximum Likelihood method (Nei & Kumar, 2000) and the JTT matrix-based model (Jones et al., 1992). Evolutionary analyses were conducted 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.

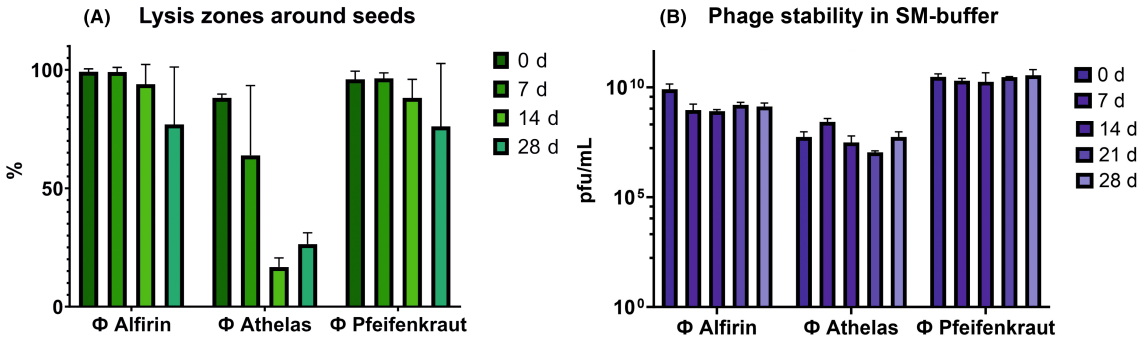

FIGURE 6 Stability of phage on *Arabidopsis* seeds stored at 4°C. (A) Shown is the percentage of seeds showing plaques when plated on a double agar lawn containing the host bacterium (*A. tumefaciens* for phage Alfirin, *P. syringae* for phage Athelas, and *X. translucens* for phage Pfeifenkraut). Portions 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

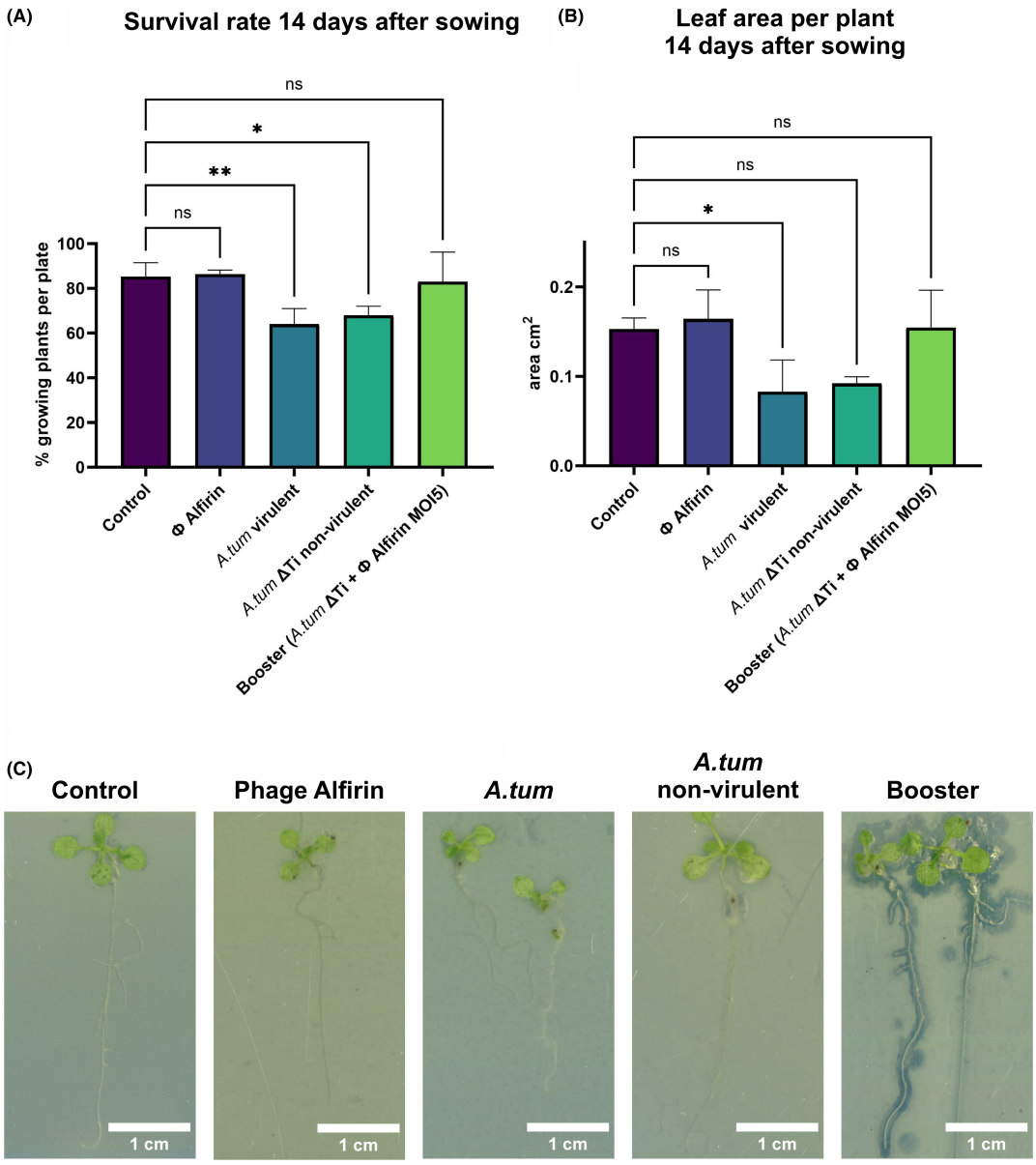

FIGURE 7 Local phage amplification acceleration with a non-virulent bacterial host. (A) Leaf area after 14 days ($N = 25$) the experiment was performed in triplicates. ANOVA; $F(4, 23) = 5734$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns = not significant. (B) Amount of plants with active 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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4.3 Molecular responses in phage biocontrol and conclusions on tripartite interactions *in planta*

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Table 4.4: Molecular responses in phage biocontrol and conclusions on tripartite interactions *in planta*

Contributor role	**Contributor**
Conceptualization	SE (50%), JF (25%), BA (25%)
Data curation	SE (100%)
Formal analysis	SE (100%)
Funding acquisition	JF (50%), US (50%)
Investigation	SE (100%)
Methodology	SE (60%), JF (20%), BA (20%)
Project administration	SE (50%), JF (25%), BA (25%)
Resources	-
Software	SE (100%)
Supervision	JF (60%), SE (30%)
Validation	SE (60%), JF (20%), BA (20%)
Visualization	SE (100%)
Writing - original draft	SE (65%), JF (15%), BA (20%)
Writing - review and editing	SE (30%), JF (30%), BA (30%), US (10%)

Overall contribution: 85%

“Molecular responses in phage biocontrol and conclusions on tripartite interactions in planta”

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Abstract

Bacteriophages have long been recognised for their potential in controlling plant diseases like crucifers' black rot, with research spanning a century from initial conceptualisation to the recent renaissance of phage-based plant biocontrol. Here, we present the first gnotobiotic study of a plant-bacteria-phage interaction involving the plant pathogen *Xanthomonas campestris*, providing novel insights into plant expression profiles during this interaction. Our findings demonstrate that a multiplicity of infection (MOI) of five with the phage Seregon effectively protected *Arabidopsis thaliana* seedlings against *Xanthomonas campestris* pv. *campestris* (*Xcc*) infection, restoring leaf area to control levels within 14 days post-inoculation. We elucidate the mechanisms of phage-based plant resistance to *Xcc* in *Arabidopsis*. We highlight resistance responses such as early bacterial recognition, detection of effectors, and genes involved in pathogen growth suppression. Our results indicate a modulation of salicylic acid (SA) signalling pathways in the presence of phage. Additionally, we identify upregulated genes associated with cell wall defence and stress tolerance, suggesting a complex interplay between phage treatment and plant defence mechanisms. Notably, in the plant-bacteria-phage condition, differential regulation of SA-dependent and -independent pathways is observed, implicating potential crosstalk in the plant defence activation.

1. Introduction

The escalation of bacterial plant pathogens (Mansfield et al., 2012), coupled with the diminishing effectiveness of traditional antibacterial treatments due to overuse and shifting climatic conditions, threatens our food supply (McManus et al., 2002; Miller et al., 2022). The increasing prevalence of antibiotic-resistant pathogens adds to the concern. Bacteriophages (in short, phages), as specialised viruses against bacteria, hold great potential to overcome this challenge. In a recent study, we isolated and characterised Xanthomonas phage Seregon, a lytic siphovirus, showing promising results in controlling the growth of Xanthomonas campestris pv. campestris (Xcc) in in vitro (Erdrich et al., 2022). Molecular studies in recent years have heavily contributed to our understanding of phage-bacteria interactions during infection (Gao and Feng, 2023; Rostøl and Marraffini, 2019; Tal and Sorek, 2022). The same applies to molecular plant research, adding valuable puzzle pieces to our understanding of how plants defend themselves against various diseases, with Arabidopsis thaliana as a model plant for genetic studies (Glazebrook, 2005; Thatcher et al., 2005; Zhang et al., 2018). Nevertheless, the tripartite interactions between a plant, its pathogen and an active bacteriophage have not been studied on a molecular level so far.

The causal agent of black rot disease in *Brassicaceae X. campestris* is a seed-transmitted bacterium with an epiphytic and endophytic vascular life phase (Vicente and Holub, 2013). When the conditions are favourable, and the population density is high enough, they enter the plant through natural openings such as hydathodes, stomates, cracks or wounds (Cerutti et al., 2017; Williams, 1980). This collective invasion is coordinated by diffusible molecules and recognition systems, also known as quorum sensing systems (He et al., 2009). Once inside the plant, *Xcc* builds a biofilm and feeds off the xylem sap. By clogging vascular bundles with biofilms and oversteering plant defence responses, *Xcc* causes the typical V-shaped necrotic lesions in important crop plants like *Brassica oleracea.*

Plants, as sessile organisms, face a multitude of challenges from their environment. Besides competition for resources, e.g. light, water and nutrients, they also have to fight off predators like herbivores or pathogenic microbes (Fujita et al., 2006; Hasanuzzaman et al., 2020; Jan et al., 2021; Sharma et al., 2020; Zhang et al., 2020). Since they can't avoid these problems through spatial relocation, they have developed complex sensing and regulation networks, allowing them to fine-tune their organism for the particular challenge they face at any given moment. For plant defence, Jones and Dangel 2006 established the classic ZIG-ZAG model of plant immunity (Jones and Dangl, 2006). This model outlines a defence mechanism with multiple layers that sequentially activate to protect the plant. If one layer fails, the activation of a subsequent layer can still provide resistance when successfully triggered or susceptibility if the pathogen prevails. The first layer of defence against pathogenic bacteria by the plant consists of the detection of microbe-associated molecular patterns (MAMPs) with membrane receptors, also known as pattern recognition receptors (PRR). These primary receptors respond to common bacterial features like bacterial cell wall components (Abdul Malik et al., 2020; Hussan et al., 2020; Offor et al., 2022) Or the flagellum (Aslam et al., 2009; Gimenez-Ibanez et al., 2009; Ma et al., 2020; Murakami et al., 2022). One prominent example is the recognition of the highly conserved N-terminal region of the bacterial flagellin Flg22 amongst multiple bacterial pathogens. However, some *Xcc* strains evade this detection by expression of altered versions of the flg22 (Lü et al., 2022). This first detection of pathogen components by the PRR gets transduced by a cascade of mitogen-activated kinases (MAP-kinases)28–30, finally leading to transcriptional activation of defence genes and the so-called microbe-triggered immunity (MTI). Pathogenic bacteria often use type 3 secretion systems (T3SS) to deliver small proteins into the plant cell, the so-called effectors (Deslandes and Rivas, 2012; Kazan and Lyons, 2014; Ryan et al., 2011; White et al., 2009; Zhang et al., 2022), to interfere with the MTI signalling cascade, counteracting plant defence activation. *X. campestris* is no exception to this. With its well-researched T3SS, it delivers a vast array of effectors

into the plant cell (Büttner, 2016). The *Xcc* strain 8004 used in this study was shown to suppress the flg22-induced MAP-kinase signalling via one of its secreted effectors (Huang et al., 2020).

The second layer of plant defence is tuned to detect these effectors, either directly or indirectly (bait and guard model)(Collier and Moffett, 2009; Dangl and Jones, 2001), and to activate heavy defence responses rapidly, e.g. production of reactive oxygen species. If successful, this leads to effector-triggered-immunity (ETI)(Thordal-Christensen, 2020). Past studies have shown that ETI also plays a crucial role in the interaction between *Xcc* and *Arabidopsis* (Rong et al., 2010).

This local activation of plant defence on the level of single cells is translated to systemic responses by plant hormones, which are produced after activation of defence cascades in single cells and transported across the plant tissue. Their detection leads to transcriptional activation of defence genes far apart from the infection site, preparing the plants for further encounters with the pathogen and leading to induced systemic resistance or systemic acquired resistance (Kamle et al., 2020). The most prominent plant hormones involved in defence signalling are salicylic acid (SA)(Benjamin et al., 2022), jasmonate (JA)(Okada et al., 2015; Ruan et al., 2019), ethylene (ET)(Binder, 2020) and abscisic acid (ABA)(Chen et al., 2020). Nevertheless, all defence responses are energetically costly for the plant and result in a growth defence trade-off. Plants have one last trick up their sleeve: close monitoring of their environment and detection of bacterial quorum sensing molecules like N-acetyl-homoserine lactones by a yet not fully understood mechanism (Schenk et al., 2014; Schikora et al., 2016), leading to low-level defence activation across the whole plant, which enables rapid defence activation when the plant gets under attack by a pathogen.

On the other hand, bacteria themselves are susceptible to a special group of viruses - bacteriophages. The name bacteriophages comes from Greek for bacteria eater, describing their ability to lyse a bacterial lawn and clearing it, leading to the observation of visible plaques where they have “eaten away” the bacteria. In nature, phages outnumber bacteria tenfold and thrive in various environments (Dion et al., 2020). They are naturally found in the phyllosphere of crop plants (Forero-Junco et al., 2022) and in agricultural soils (Pratama et al., 2020; Williamson et al., 2017). Bacteria and phages fight an ongoing battle spanning millions of years, making dynamic defence systems a necessity (Labrie et al., 2010; Rostøl and Marraffini, 2019). To combat these challenges, the bacterial community employs defence mechanisms across various levels. At the level of the cellular surface, this includes masking binding sites for the bacteriophages, e.g. altering the lipopolysaccharide pattern of the cell envelope, masking receptors, or downregulating receptor gene expression (Rostøl and Marraffini, 2019). Acting in the same direction is the switch from a motile lifestyle to a biofilm, where the majority of bacteria are less accessible for the virus (Visnapuu et al., 2022). *Xanthomonas* is also a vivid biofilm producer (Crossman and Dow, 2004), but phages have been shown to degrade *Xanthomonas* biofilms (Nakayinga et al., 2021; Papaianni et al., 2020). Suppose the inevitable has happened, and the phage genetic material has entered the bacterial cell. In that case, the bacterium can use nucleic targeting defences like restriction-modification systems or CRISPR-Cas systems to render the foreign genetic material useless (Blumenthal and Cheng, 2002; Deveau et al., 2010; Hampton et al., 2020). Besides these more well-known systems, the recent advances in sequence technology and bioinformatics unravelled a myriad of bacterial defence systems against phages (Georjon and Bernheim, 2023), ranging from a cell-wide translation arrest over energy depletion (Wang et al., 2023) to the production of secondary metabolites with antiphase functions (Kever et al., 2022). If all these attempts remain fruitless, the bacterium has one last option: activating a self-destruction system to prevent the assembly of new phages and to protect the community of its clonal copies by following the principle of abortive infection (Lopatina et al., 2020).

Interactions with eukaryotes are considered non-existent since phages are prokaryote-specific. And investigations in eukaryotes proved them to be harmless for humans and other animals. Nevertheless, some studies suggest a subtler indirect interaction between phages and eukaryotes. For example, Barr et al. 2013 showed that phages that infect gut bacteria have specialised receptors for binding to the mucus (Barr et al., 2013; Chatterjee and Duerkop, 2018). Thereby, the phages increase persistence in the niche where they encounter their target bacteria in high numbers, which in turn leads to shaping the host microbiome by establishing an equilibrium below the critical threshold for some species. There is even less literature on plant phage interaction, and the field remains heavily understudied. Our Lab recently showed that phages can interact with seed surfaces in a protective manner (Erdrich et al., 2024), but there is still much to discover. In the past two decades, phage biocontrol had a renaissance; phages were explored as a treatment option for many plant pathogens, primarily members of the genera *Pseudomonas, Agrobacterium, Xanthomonas, Erwinia, Ralstonia* and *Pectobacterium*. An excellent review of the outcomes until 2017 was presented by Buttimer et al. 2017 (Buttimer et al., 2017). Subsequently, more than 130 new studies on the isolation and application of phages for plant pathogens have been published, with only 38 per cent conducting experiments involving plants. In this study, we advance prior understanding of the efficacy of phages in combating Xanthomonas, with a specific focus on the newly discovered phage, Seregon. We aim to elucidate the dynamic interplay among plants, bacteria, and phages over time, shedding light on the process of plant recovery and response to bacterial invasion. The Xanthomonas phage Seregon showed significant plant protection at a multiplicity of infection (MOI) of five. After two weeks, the phage treatment allowed the plants to reach a leaf area and fresh weight similar to the control. Further, we could show condition-specific expressional differences 48 hours post-infection. Most prominent was the difference in the regulation of defence-related genes in the presence of the phage, tipping the balance in the direction of immunity.

2. Material and Methods

2.1 Plant propagation

Arabidopsis thaliana Col-0 plants were propagated on ½ Murashige and Skoog-Agar (Murashige and Skoog, 1962). Individual surface sterilised seeds were placed in a defined pattern under a laminar flow bench. Plates were sealed with micropore tape and put into a climate chamber with a 12 h light–12 h dark regime at 22 °C day and 18°C night, with 100 µE illumination at a relative humidity of 60%.

2.2 Bacterial growth conditions

Xanthomonas campestris pv. campestris str. 8004 (obtained from Prof. Naberhaus Lab; RUB)(An et al., 2020; Vicente and Holub, 2013) was used in this study. Cultures were grown on plates and inoculated from single colonies in liquid media for overnight cultures; bacteria were grown on nutrient agar (5.0 g peptone, 3.0 g yeast extract and 15.0 g Agar in 1,000 ml of dH_2O). Unless mentioned otherwise, bacterial cultivation was performed in shake flasks at 30°C and 150 rpm.

2.3 Phage isolation and amplification

The *Xanthomonas* phage Seregon was isolated and described previously (Erdrich et al., 2022). Phages were either amplified on plates or in liquid cultures. For plate amplification, a double agar overlay was performed using 100 µL phage solution with a titer 10^8 Pfu/mL added to 3,5 mL 0.4%-top agar containing the bacterium at a final OD of 0.2. Harvesting of purified phage particles was performed after overnight incubation. The top agar was solubilised by adding 3 mL ½ MS-Buffer and two h incubation on a rock shaker. The solution was subsequently transferred into a falcon tube and centrifuged at 5000 g for 25 min to remove residual amounts of agar and bacterial cell fragments. The supernatant was filtered twice through 0.2 µM syringe filters and stored at 4°C. For titer determination, a dilution series was spotted on overlay agar, and the visible plaques at the highest dilution were counted to determine the plaque-forming units (pfu) per mL.

2.4 Plant inoculation

Inoculation of plants was performed by flood inoculation, similar to Korniienko et al. 2021 (Korniienko et al., 2022). Eight-day-old seedlings were submerged within 50 mL ½ Murashige and Skoog medium (½ MS) liquid medium containing 10^9 cfu/mL *Xcc* for the pathogen condition. Both phage and bacterium were used in a small volume of a higher concentrated stock. For the phage treatment condition, phages were added in the respective multiplicity of infection (MOI) to the solution before flooding. The phage-only control was flooded with 50 mL ½ MS containing the same number of phages as the bacteria and phage (MOI 5) treatment condition. In contrast, the plant-only control was flooded with ½ MS. After 5 minutes, the liquid was removed carefully, and the plates were again sealed with micropore tape, and the plates were incubated in the climate chamber. Plant growth in the different conditions was consistent over several independent rounds; the data presented here are obtained from a single round, and all biological samples were generated in triplicates.

2.5 Non-invasive plant phenotyping

The non-invasive plant phenotyping was performed at 0, 2, 5, 7, and 14 days post-infection (dpi). Plates were placed onto an Epson 10000XL WinRhizo scanner, and images were acquired using the manufacturer's scanner interface. After covering the top of the plates with white cardboard for better contrast. Tiff images were retrieved by scanning from below, with a resolution of 1200 dpi. The plant leaf area was calculated within Image J (Version 1.54f), and green pixels were isolated by HSV thresholding. Single plant leaf area was determined using the analyse particle function.

2.6 RNA extraction

For RNA extraction, multiple plants with a weight of 45 mg were harvested from each group and pooled in a microcentrifuge tube. The obtained tissue was immediately frozen in liquid nitrogen and stored at −80 °C. Sampling occurred in triplicates at 0, 2, 7 and 14 dpi. For nucleic acid extraction, the frozen tissue was ground in a ball mill (Retsch MM200, Retsch, Germany) with a 4-5mm stainless steel beat. Total RNA extraction from the resulting tissue powder was performed using the Rneasy® Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To remove genomic DNA contamination, total RNA was incubated with DNAse I (Thermo Fisher Scientific, AM1906) for 15 min.

2.7 RNA-Seq

RNA-Seq for samples (currently sequenced conditions: control, phage only, Xcc and MOI5 at 2 and 7 dpi, each in two biological replicates) was performed with rRNA depletion strand-specific RNA library preparation at Azenta Life Science, followed by RNA-Seq on a Novaseq 6,000 with a sequencing depth of 100M read pairs per sample (2x 150 bp). Raw read sequences were archived at NCBI (Bioproject NC_Placeholder). The read quality was assessed with the FastQC software v.0.11.9 (http:// www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequencing Adapters and low-quality sequences were removed using the Trimmomatic tool v. 0.39 (Bolger et al., 2014). The obtained reads were mapped against the Arabidopsis thaliana genome (TAIR 10.0)(Rhee, 2003) using Kallisto (Bray et al., 2016). Differential expression analysis was performed using DESeg2 (Love et al., 2014). The significance intervals were defined as follows, *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Further, a threshold of log2fold change of 1 was set for differentially expressed genes.

2.8 RT-qPCR

For quantitative real-time PCR, cDNA was synthesised using 1000 ng of total RNA. First-strand cDNA was synthesised using SuperScript IV reverse transcriptase (Thermo Fisher Scientific, cat. no. 18090050). For quantitative real-time PCR, 50 ng first-strand cDNA in 2µl was mixed with 0.6 µl of 10 µM solution of the primer pair of interest, together with ten µl iQ SYBR Green Supermix (Bio-Rad) and water to a total volume of 20 µl. The PCR was performed using a qPCR Bio-Rad CFX Connect system employing the following program: 95 °C for 2 min, followed by 35 cycles of 95 °C for 10 s, 55 °C for 10 s, and 72 °C for 20 s. The primers used for the genes of interest are listed in Supplementary Table S1. The ΔCq and ΔΔCq values were calculated as described in Rao et al. (2013). The housekeeping gene for Arabidopsis qPCR was Ef1a (Supplementary Table S1).

3. Results and Discussion

3.1 Gnotobiotic tripartite interaction and Leaf area phenotyping over time

To study the tripartite interaction of *A. thaliana, X. campestris* and phage Seregon (Erdrich et al., 2022), a plate-based sterile gnotobiotic system was set up, similar to Korniienko et al., 2022. After eight days the seedlings were inoculated as described in Figure 1 A). Plant growth in all conditions was monitored over time (Figure 1 B)

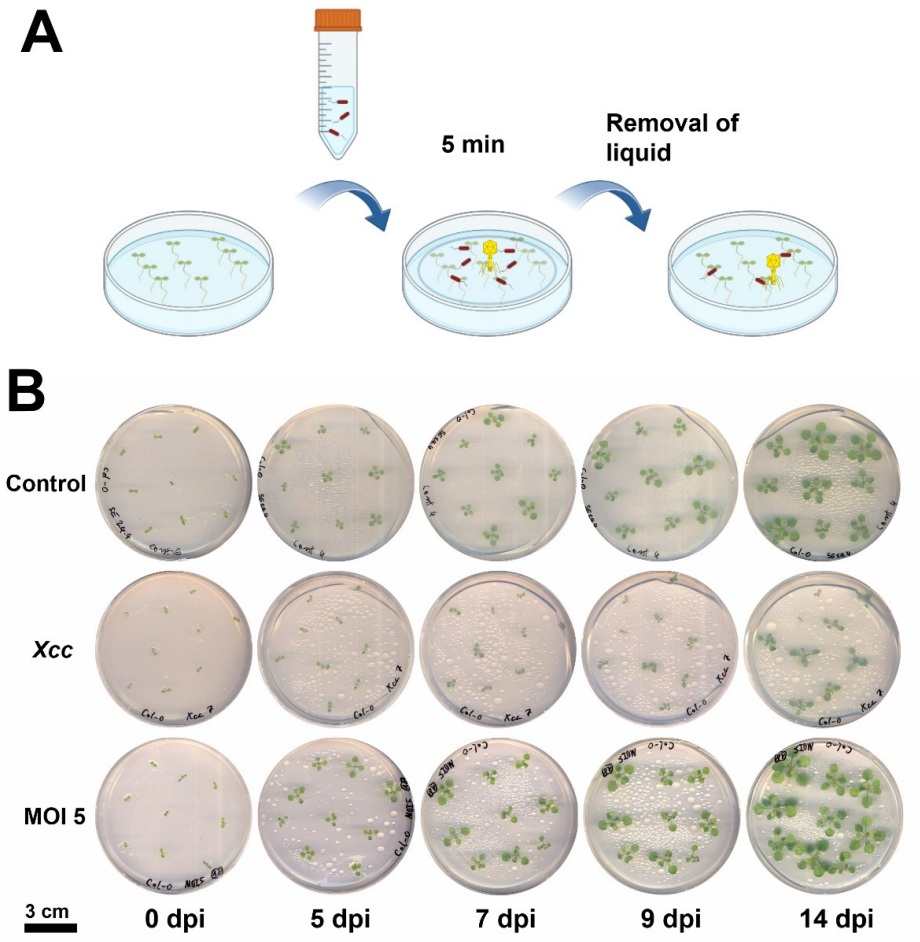


Figure 1. Tripartite interaction in a gnotobiotic system **A)** Schematic of the inoculation procedure. Seedlings were flood inoculated, with either bacterium or phage only or a combination of both (MOI5). **B)** Depiction of the plants over time. Top row control-inoculated plants (½ MS media only). Middle row *X. campestris* inoculated plants. Bottom bacterium plus phage inoculated plants (MOI5).

Co-inoculation with the phage strongly reduced the adversary effect of *Xcc* on *A. thaliana.* The phage Seregon was capable of restoring plant growth to control levels. To quantify our initial observations, we performed non-invasive phenotyping in subsequent repetition of the experiment.

To determine the influence of the pathogen *Xcc*, the plant leaf area was monitored non-invasively over time for all conditions. Scanning the plates at the indicated timepoints (Figure2 A), and colour segmentation on green pixels with ImageJ allowed the calculation of the leaf area of the individual plants. At the start of the experiment, each condition contained 216 plants; the plants for RNA extraction were harvested at 0, 2 and 7 days post-inoculation (dpi) over the course of the experiment. First, phenotypic differences were noticed after 7 dpi. Those differences were more pronounced the following days with a significant difference in the leaf area between the control and the *Xcc* treatment after 12 dpi (Figure 2 A). This disparity continued to widen until 14 dpi. Beyond this point, the phage-treated of *Xcc* (MOI5) exhibited a recovery, restoring nearly the phenotype observed in the uninfected control, indicating the efficiency of the phage treatment against *Xcc*.

At the endpoint measurement 14 days post-infection, there was no significant difference between the control, the phage-only condition and the phage treatment against Xcc and at a MOI5. Only the leaf area of the pathogens-treated plants was significantly lower (Figure 2 B). This is also reflected in the endpoint harvest. Fresh weight measurements were performed at the end of the experiment to gather information on the plant's biomass accumulation (Figure 2 C). As already indicated by the leaf area data, the control and the phage-treated pathogen conditions showed no significant difference. Interestingly, the fresh weight for the phage-only condition was significantly increased despite the observation that the leaf area appeared to be similar.

To gain more detailed knowledge about the molecular processes that led to the recovery of Arabidopsis in the presence of the pathogen when phage Seregon was added, we performed multi-species transcriptomics.

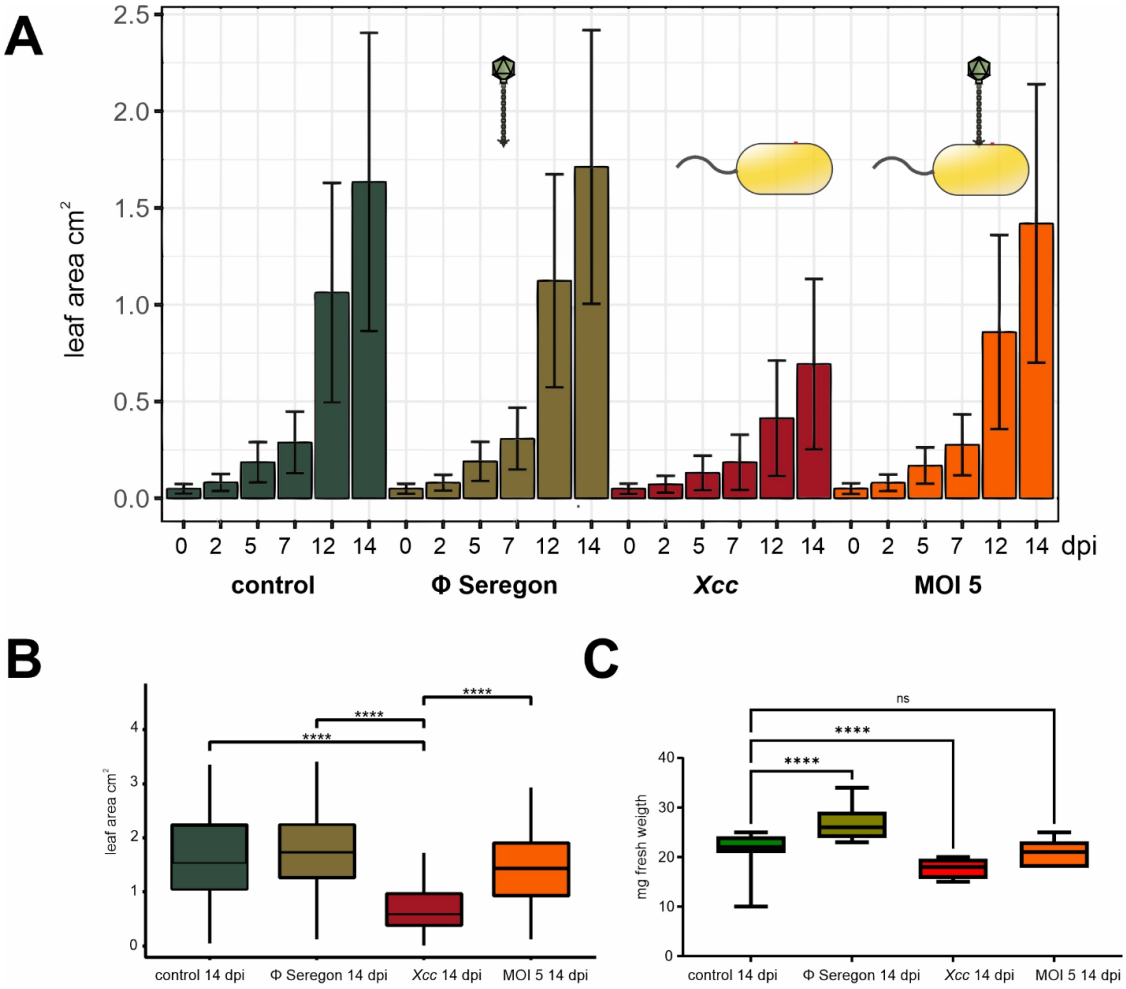


Figure 2. Plant phenotyping **A)** Non-invasive measurement of Leaf area over time. The plant leaf area per plant was retrieved by scanning at 0, 2, 5, 7, 12, 14dpi. **B)** Total leaf area per condition 14 days post-inoculation. Displayed are the means of each condition (N > 45 plants) at the endpoint 14 days post-inoculation. ANOVA was performed with RStudio, followed by Tukey HSD F(3,234)=31,25, p=0.0001. **C)** Fresh weight per plant at 14 dpi. The fresh weight was measured at the endpoint in a final harvest.

3.2 Results of the tripartite transcriptomic regulations

To gain in depth genome wide expression data a RNA-Seq was performed at a high depth including double RNA depletion for plant and prokaryotes, prior to sequencing. After data QC and trimming high quality reads on average 102M reads per sample were obtained. Genome mapping against the all three species in subsequent round using Kallisto-Pseudoalignments were performed. For Arabidopsis across all conditions 82,98% of all genes were detected during alignment. For the pathogen Xanthomonas at the dpi 2 and 7 as well as in the phage treated condition 79.69% of all bacterial genes were detected during alignment. In the untreated control mapping bacterial reads were below 1%. Phage reads in the MOI 5 condition at 2 and 7 dpi were mapped to with 86.68%.

3.3 Regulatory changes over time during tripartite interaction

To gain deeper insights into the response of the plant to the presence of pathogen and phage, a differential expression analysis was performed. The analysis was performed using the DESeq2 algorithm. To get a fist overview over the expression landscape, significant differential expressed genes (p-value <0.05 and Log2 Foldchange > 1) relative to the control condition at 2 dpi, were plotted by log2 fold change (Figure 3), showing that there is a significant difference between the treatment conditions.

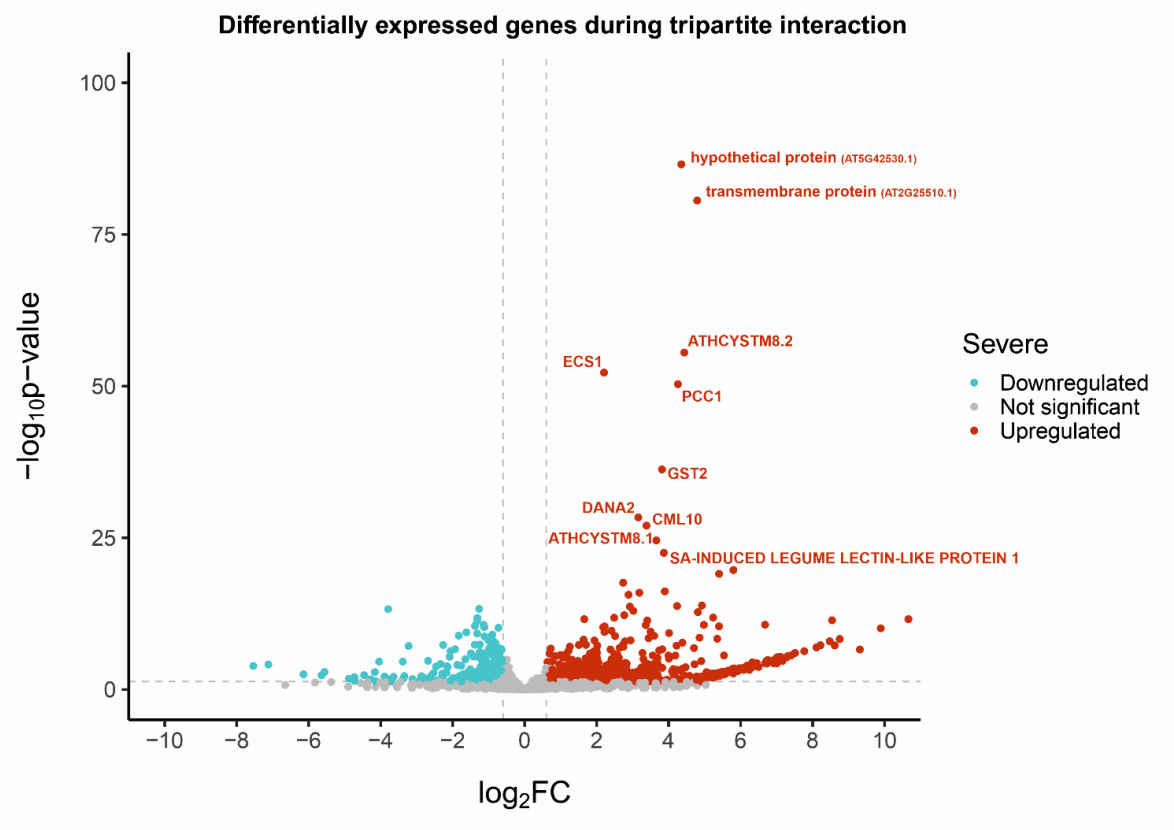


Figure 3. Vulcano plot of differentially expressed genes during tripartite interaction. RNA of the samples control, *Xcc* and MOI5 at 2 and 7dpi was sequenced 100M reads paired-end using Illumina short-read technology—afterwards, a mapping with Kallisto against the *Arabidopsis* genome. Significant differentially expressed genes were identified using the DESeq2 algorithm (Love et al., 2014). Downregulated genes are displayed in blue, whereas upregulated genes are shown in red. Dotted gray lines indicate the significance threshold (p-value <0.05 and Log2 Foldchange > 0.6). The ten most significant differentially expressed are displayed with gene names, the remaining genes are represented by dots.

Looking at the proportions each condition contributed to differential expression by creating a Venn-Diagram revealed that the largest fraction of upregulated genes as well as downregulated genes were found in the *Xcc* condition (Figure 4), highlighting the impact of bacterial effectors on expression during plant takeover (Büttner and Bonas, 2010). Interestingly in the MOI5 condition at 2 dpi the upregulation of genes is much subtler with 3% of genes upregulated and there are 21% of genes downregulated compared to the *Xcc* treatment.

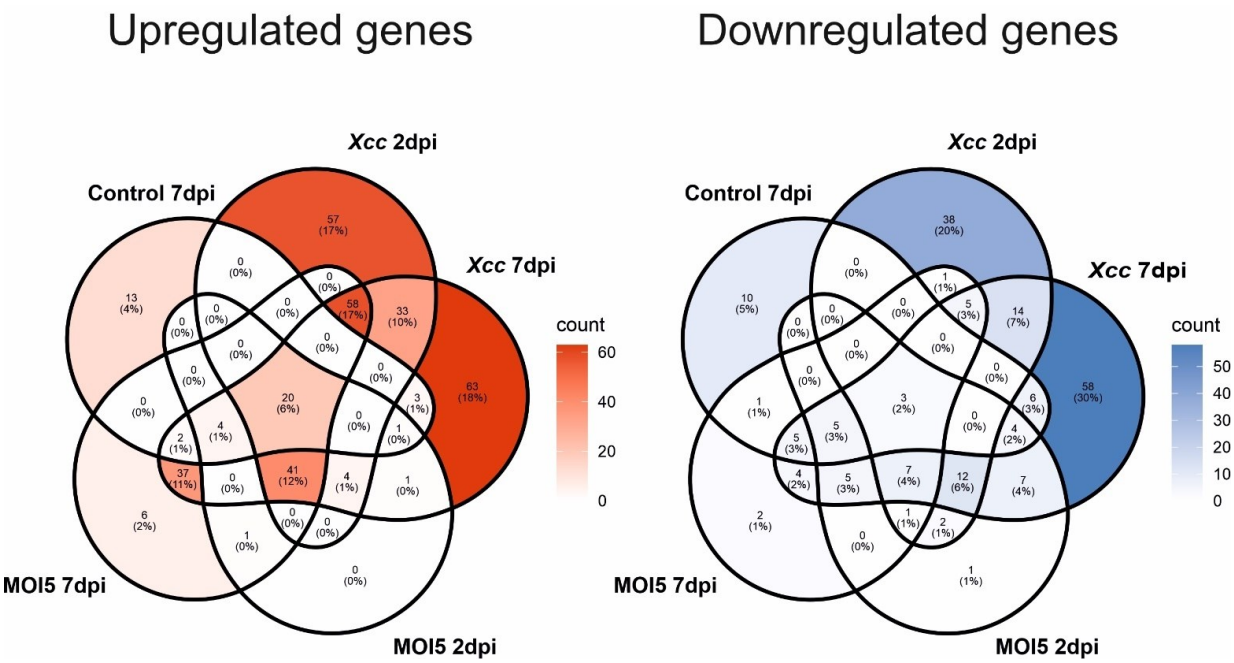


Figure 4. Venn plot of differentially expressed genes during tripartite interaction. Upregulated genes are displayed in red, downregulated genes are displayed in blue. Each condition was tested against the control at 2dpi which was used as reference (p-value <0.05 and Log2 Foldchange > 1). The resulting condition specific differentially expressed genes were then compared to every other condition to see it there is an overlap.

To get a more condition-specific picture and search for district patterns, each sample's 250 most significant differentially expressed genes were transformed to Z-scores and used to generate a heatmap containing the TAIR identifiers as labels (Figure 5). The heatmap shows condition-specific differences. Interestingly, MOI5 at two dpi has an expression pattern that is control-like. Further, an expressional difference exists between the two dpi and seven dpi timepoints. To zoom in on single involved genes in each condition, the 50 most significant differentially expressed genes were plotted in Figure 6 and labelled with the gene name. Further, these genes were assigned to categories by the description found on TAIR and respectively colour-coded.

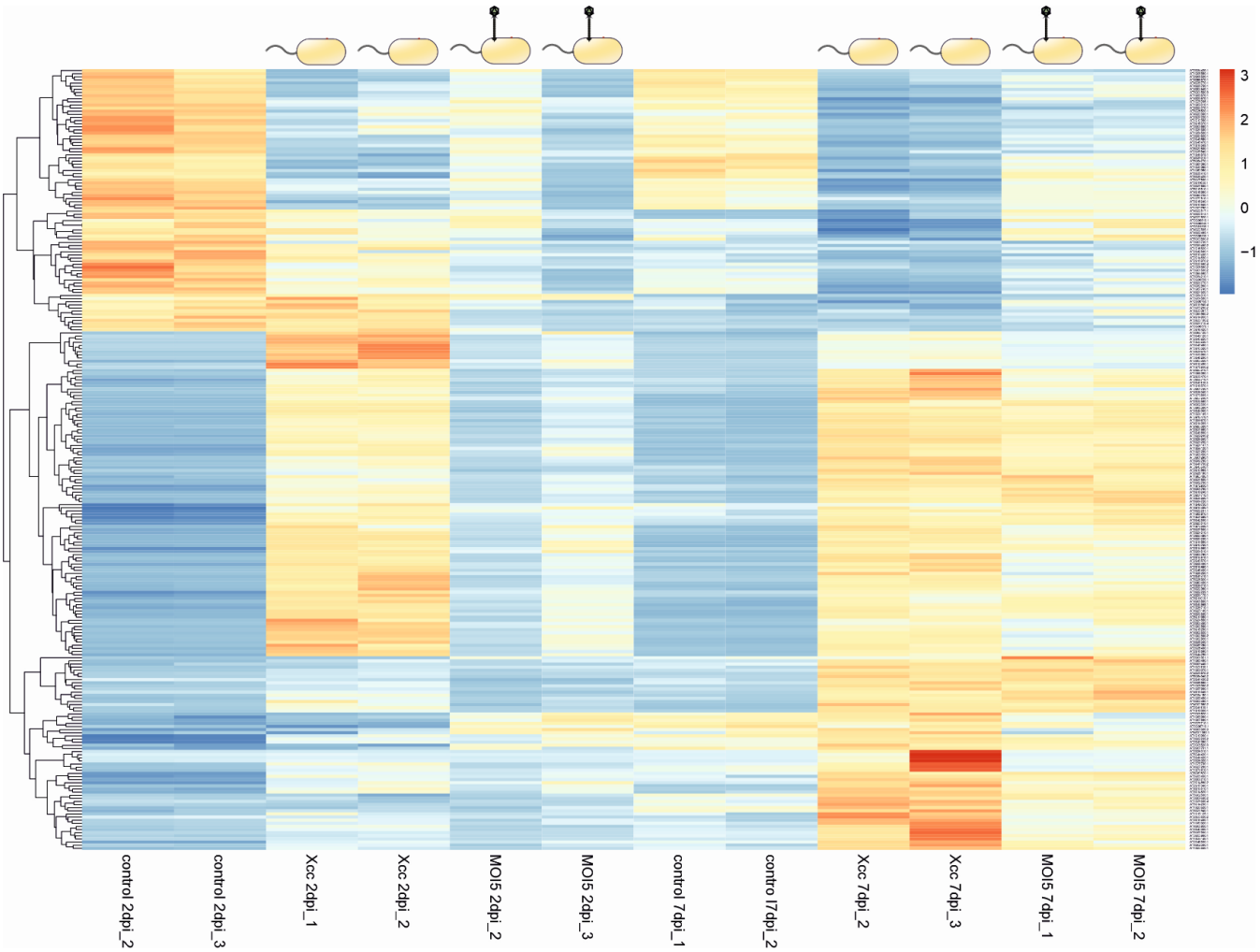


Figure 5. Global transcriptome analysis during the tripartite interaction. Heat map of the 250 most significant differential expressed genes. Shown are z-scores ranging from -2 (dark blue) to 3 (dark red) and data for two independent biological replicates ('control',' *Xcc*', 'MOI5' at 2 or 7 dpi).

Directing the plant transcriptome data we found that Calcium (Ca2+) an important messenger in early plant-bacteria interactions is activated upon *Xcc* inoculation (Negi et al., 2023). Calcium fluxes are triggered upon detection of a bacterial pathogens by PRR; in our transcriptome analysis, we found genes involved in calcium signalling upregulated in the Xcc condition at 2 dpi. Interestingly, those genes were not induced in the sample combining bacterial pathogen and phage (MOI5) at 2 dpi, but at the later 7dpi time point, these genes were expressed in both conditions. Calcium (Ca2+) acts as an important second messenger in plant cells, and elevation of its concentration is essential during plant defence responses (Aldon et al., 2018; Lecourieux et al., 2006; Zhang et al., 2014). One of these genes is calmodulin-like protein 10 (CML10) induced by oxidative and other stress (Yu et al., 2022). DANA2/ TOUCH 3 is a calmodulin-like protein recently shown to amplify pathogen-associated molecular patterns (PAMPs) together with a calcium-dependent protein kinase (Sun et al., 2022). To this group further belongs a calcium-dependent lipid-binding family protein (AT1G48090.1), whose function is still elusive.

The next block of differentially expressed genes is linked to SA signalling, which are strongest upregulated compared to the control upon *Xcc* inoculation at 2 dpi. One of those genes is SA-induced legume lectin-like protein 1, it was upregulated by a Log2FC of 5,1. As one early SA-activated gene in *Arabidopsis*, this indicates salicylic acid (SA) signalling upon *Xcc* inoculation. This is in line with previous literature, suggesting an effector-triggered SA-mediated defence response (Armijo et al., 2013). In the MOI5 condition containing *Xcc* and the respective phage Seregon, this gene was also upregulated, but to a lower extent with a Log2FC of 3.9 indicating that the expression in presence of the phage is only half as high as with the pathogen alone. In addition, NDR1/HIN1-LIKE 3 was upregulated, which recently was reported to be necessary to integrate SA signalling with microbe-associated molecular patterns (MAMP) signalling from membrane receptors (Tee et al., 2023). Also, WAK-1, a signalling receptor of extracellular matrix linked to SA responsiveness, was upregulated (He et al., 1999). Furthermore, the SA signalling-dependent transcription regulator WRKY70, which mediates the choice of hormonal defence signal in in favour of SA, was found to be upregulated twice as high Xcc-treated plants with a Log2FC of 5,4 compared phage treated pathogen with a Log2FC of 4,1. Two variants of cysteine-rich transmembrane module 8 (ATHCYSTM8) were found among the differentially expressed genes. ATHCYSTM8 encodes an SA response gene that was also upregulated from early on in the Xcc condition, which was in line with earlier reports (Pereira Mendes et al., 2021). The proteins encoded by these genes are described as membrane-bound and salicylic acid (SA) responsive. In a recent study, they were reportedly involved in defence through an impact on developmental processes affecting pathogen performance without leading to the expression of classical defence proteins (Pereira Mendes et al., 2021). A further highly upregulated gene with a product acting at the plant cell wall is ECS1. It was upregulated at 7 dpi in the MOI5 and Xcc condition but only found in Xcc at 2 dpi. Another family of unregulated genes encoded glutathione S-transferases, namely GSTF2, GSTF3, GSTF6 and GSTF7, a protein family that is known to respond to stress stimuli. Here, the induction of some members is triggered by H_2O_2 signalling, while others are activated by SA (Sappl et al., 2009). For example, GSTF2 was linked to SA signalling and lipooligosaccharide (LOS) triggered resistance in previous studies (Lieberherr et al., 2003; Proietti et al., 2014). GSTFs were strongly expressed upon infection with *Xcc*. HR4, a protein also belonging to this block, codes for a resistance protein involved in autoimmunity and cell death, thereby providing a broad-spectrum resistance (Li et al., 2020). The last member of this block of defence-related genes induced by the bacterial pathogen Xcc in our study is the pathogenesis-related lipase 1 (PRLIP1), which also responded to SA signalling in previous studies (Szalontai and Jakab, 2010). Also involved in defence signalling and induced in Xcc treatment is the WRKY33 transcription factor. Remarkably, the SA-induced genes were consistently expressed weaker only half as high in the samples where Xcc and phages were present. This suggested either a reduction in bacterial load or crosstalk with other pathways inhibiting SA signalling.

In line with this is the expression profile of the gene coding for Pathogenesis -related protein 1 (PR1). A vast body of literature states its importance in defence responses against pathogens (van Loon et al., 2006; Van Loon and Van Strien, 1999), with recent studies presenting proof of extracellular secretion (Pečenková et al., 2022) and defence amplification due to proteolytic activation, which is regulated by dimerisation of PR1 (Lu et al., 2013). The mode of action as an antimicrobial of PR-1 proteins depends on sterol sequestration, resulting in sterol deprivation of the pathogen and cellular leakage (Gamir et al., 2017). Expression of this gene was observed in the Xcc conditions at 2 and 7 dpi but not in all other conditions. This suggests that only in the presence of the pathogen alone is a full activation of the salicylic acid pathway accomplished since the release of its negative regulator is SA signalling dependent (Seyfferth and Tsuda, 2014). Past studies have shown that the type 3 secretion system-delivered effector protein AvrXccC induces PR1 expression in *Arabidopsis thaliana* Col-0 (Rong et al., 2010). Also, glutathione S-transferases (GSTs) 2 and 6 are upregulated strongly in the

bacterial condition; they are involved in reactive oxygen species production (Gullner et al., 2018) and were linked to SA and ethylene signalling in the past (Lieberherr et al., 2003). Further upregulated genes from this family under the Xcc treatment are GSTF7 and GSTF3.

Another hormone whose signalling targets have been found differentially expressed upon pathogen treatment at 2dpi was abscisic acid (ABA). The first differentially expressed target tied to this pathway we found was a hypothetical protein (AT5G42530.1), which has a MYC2 recognition site and showed responsiveness to singling ABA in a previous study (Abe et al., 2003). The same was observed for a transmembrane protein that was not further specified (AT2G25510.1). Interestingly, it shared 49% sequence identity with the former one, indicating both play role in the crosstalk between the same two pathways. The transmembrane protein AT2G25510.1 was also found to be upregulated in a study identifying the role of Receptor-like kinase1 (RPK1) as a key player in early ABA signalling in *Arabidopsis* (Osakabe et al., 2005). Notably, none of these genes showed increased expression in the other conditions at that time point, indicating ABA signalling as an early response during *Xcc* infection (Figure 6). This is in line with a recent study that showed that calcium-dependent ABA signalling is involved in stomata closure during *Xcc* infection in *Brassica napa* (Mamun et al., 2023). Another upregulated gene also in the ABA pathways was GRP23, encoding a glycine-rich protein responsive to abscisic acid (ABA) and salicylic acid (SA). Previous studies have shown that this protein interacts with RNA polymerase II, suggesting it function as a transcriptional regulator (Ding et al., 2006). Another differentially expressed gene responsive to ABA is Arabinogalactan protein 9 (APG9), belonging to a group of membrane-bound glycosylated proteins involved in a diverse set of cell functions (Lin et al., 2022; Pereira et al., 2014). In contrast, the Pathogen and Circadian Controlled 1 (PCC1) was also highly upregulated is SA responsive and was shown to have a negative regulatory effect on ABA signalling. The upregulation of PCC1 upon Xcc infection aligns with prior studies in A. thaliana (Tan et al., 2015).

The third group of hormone signalling activated genes were linked to Jasmonate (JA) signalling, a major player in regulation of the growth-defence trait of (Li et al., 2022). The jasmonate-responsive plant defensins PDF1.2 and PDF1.3 were expressed (Penninckx et al., 1998; Sels et al., 2008), only in the pathogen treated sample at 7 dpi.

To better understand the influence of the phage Seregon in the bacteria and phage condition we focussed on upregulated genes within this samples. Allowed us to identify GRP3.1, GRP3.2 and a hypothetical protein which were upregulated at 2 dpi only if the bacterial pathogen was present in combination with the phage Seregon at MOI5, and not in the presence of the bacterium alone. Interestingly, GEP3 was proposed to negatively regulate the activity of the wall-associated kinase1 (Wak1) (Behnami and Bonetta, 2021; Gramegna et al., 2016). In literature, further function linked to GRP3 are root size determination and stress tolerance. GEP3 is upregulated in the phage treatment at 2 dpi, while being not differentially expressed in the controle and in the Xcc treatment at this time point. This could indicate that the damage associated molecular pattern (DAMP) signalling is tuned down in the presence of the phage. Never the less it worth to mention, that one study proposed a contrasting model for the GEP3-WAK interaction as modulator of defence responses (Mangeon et al., 2017). All this points to differential regulation of the SA-Pathway in the MOI5 condition or even a SA independent pathway being activated.

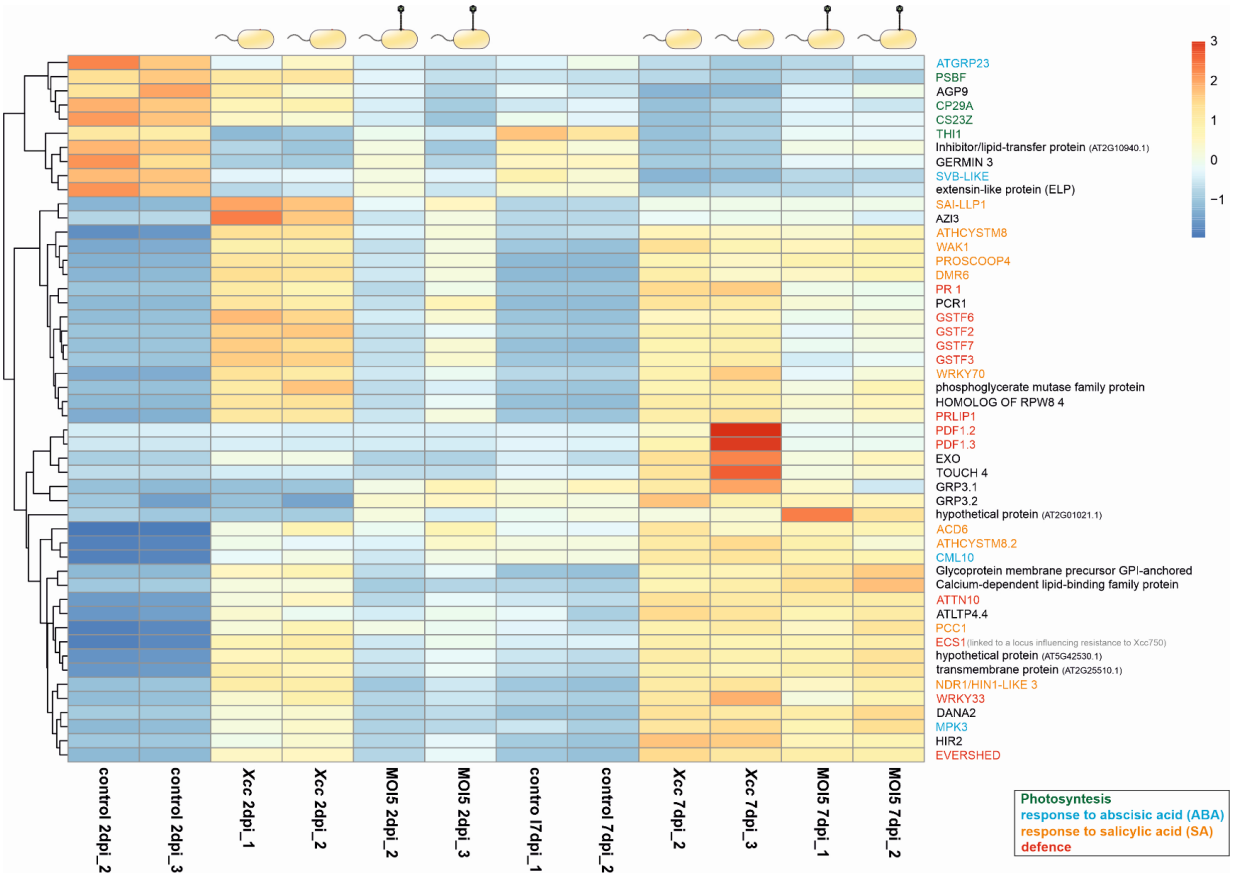


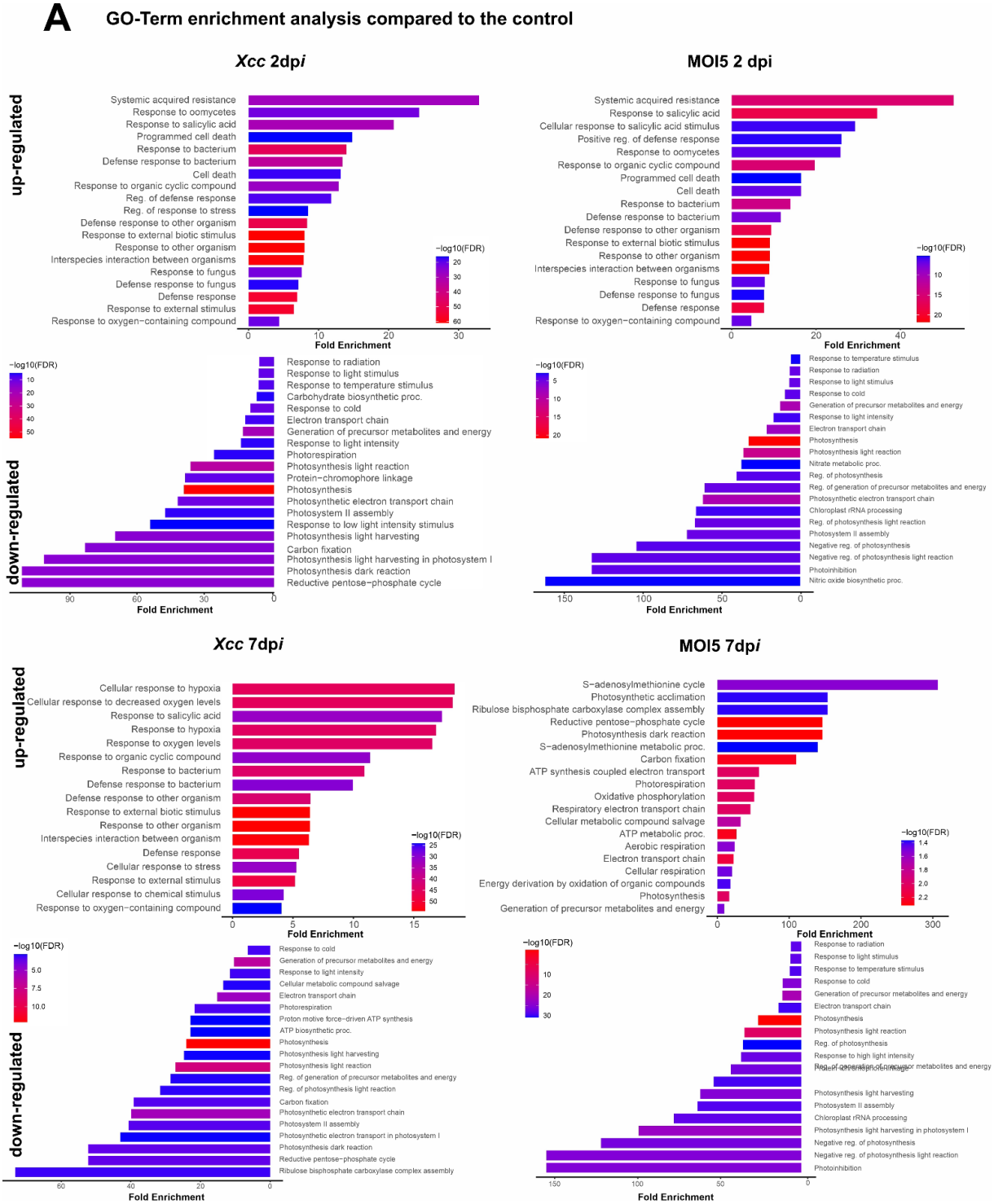
Figure 6. Global transcriptome analysis during the tripartite interaction. Heat map of the 50 most significant differential expressed genes. Gene identifiers were replaced with gene names based on TAIR entries, and further color-coding of the gene names was applied based on the biological function described for the genes. Shown are z-scores ranging from -2 (dark blue) to 3 (dark red) and data for two independent biological replicates ('control',' *Xcc*', 'MOI5' at 2 or 7 dpi).

To sum tis up in the *Xcc* inoculated plants after two days, many SA signalling genes are highly upregulated (SA-induced legume lectin-like protein 1, WAK1, WYRY70, DMR6, PROSCOOP4, NDR1). In the condition were phage and pathogen are present these genes are upregulated to a much lower extent. Still, most interestingly, three genes are upregulated that are downregulated in the pathogen-only condition (GRP3.1, GRP3.2 and a hypothetical protein). A noteworthy point is that PR1 is solely expressed in the pathogen conditions without the phage.

After the identification of several interesting genes we also aimed to gain insights into pathways that are active during the tripartite interaction. Therefore, we performed a pathway enrichment analysis using Shiny 0.8, where the significant differentially expressed genes were linked to the biological process they are involved in (Figure 7.). Compared to the control plants at 2 dpi in both the *Xcc* condition as well in the bacterium and phage condition (MOI5) primally biological processes linked to defence and salicylic acid signalling are enriched (Figure 7 A). Its noteworthy that in combination with the phage positive regulators of the defence response are enriched (GO:0031349), namely the genes SARD1, SOBIR1, PCC1, WRKY70, ACD6 and PBS3. For both conditions photo synesis relevant processes

were downregulated, highlighting the defence growth trade off and a shift to defence at this timepoint. At 7 dpi the conditions behave quite differently, while the plants inoculated with Xcc still show mostly enrichment in biological functions related to defence are upregulated, the phage treated plants containing the bacterium are enriched in photosynthesis related processes and carbon fixation.

To further dissect the changes in pathway enrichment between the bacterium only condition and the phage bacterium combination, we compared the significant genes found with the phages (MOI5) to the significant differential expressed genes in the bacteria treated plants as a reference (Figure 7 B). This was of special interest to us since we already identified changes in single genes during differential gene expression analysis. This showed that at 2 dpi the plants treated with phages participate to a higher extend in photosynthesis compared to the *Xcc* inoculated plants. Looking at the genes expressed to a lower extend in presence of the phage in combination with the bacterium, many defence related processes are active to a lower extend. The toxin metabolite production was the predominantly active in the bacteria treated plants but compared to them downregulated when the bacterium was treated with phage Seregon. At 7 dpi we see the same picture as above the phage treated plants with the bacteria have shifted to photosynthesis and growth while the Xcc only treated plants are still heavily invested in defence.


A
GO-Term enrichment analysis compared to the control
Xcc 2dpi
MOI5 2 dpi
Xcc 7dpi
MOI5 7dpi
up-regulated
down-regulated
Fold Enrichment
−log10(FDR)
Systemic acquired resistance
Response to oomycetes
Response to salicylic acid
Programmed cell death
Response to bacterium
Defense response to bacterium
Cell death
Response to organic cyclic compound
Reg. of defense response
Reg. of response to stress
Defense response to other organism
Response to external biotic stimulus
Response to other organism
Interspecies interaction between organisms
Response to fungus
Defense response to fungus
Defense response
Response to external stimulus
Response to oxygen−containing compound
Response to radiation
Response to light stimulus
Response to temperature stimulus
Carbohydrate biosynthetic proc.
Response to cold
Electron transport chain
Generation of precursor metabolites and energy
Response to light intensity
Photorespiration
Photosynthesis light reaction
Protein−chromophore linkage
Photosynthesis
Photosynthetic electron transport chain
Photosystem II assembly
Response to low light intensity stimulus
Photosynthesis light harvesting
Carbon fixation
Photosynthesis light harvesting in photosystem I
Photosynthesis dark reaction
Reductive pentose−phosphate cycle
Cellular response to hypoxia
Cellular response to decreased oxygen levels
Response to hypoxia
Response to oxygen levels
Cellular response to stress
Cellular response to chemical stimulus
Response to temperature stimulus
Response to cold
Cellular metabolic compound salvage
Proton motive force−driven ATP synthesis
ATP biosynthetic proc.
S−adenosylmethionine cycle
Photosynthetic acclimation
Ribulose bisphosphate carboxylase complex assembly
Reductive pentose−phosphate cycle
S−adenosylmethionine metabolic proc.
ATP synthesis coupled electron transport
Oxidative phosphorylation
Respiratory electron transport chain
ATP metabolic proc.
Aerobic respiration
Cellular respiration
Energy derivation by oxidation of organic compounds
Nitrate metabolic proc.
Reg. of photosynthesis
Reg. of generation of precursor metabolites and energy
Chloroplast rRNA processing
Reg. of photosynthesis light reaction
Negative reg. of photosynthesis
Negative reg. of photosynthesis light reaction
Photoinhibition
Nitric oxide biosynthetic proc.

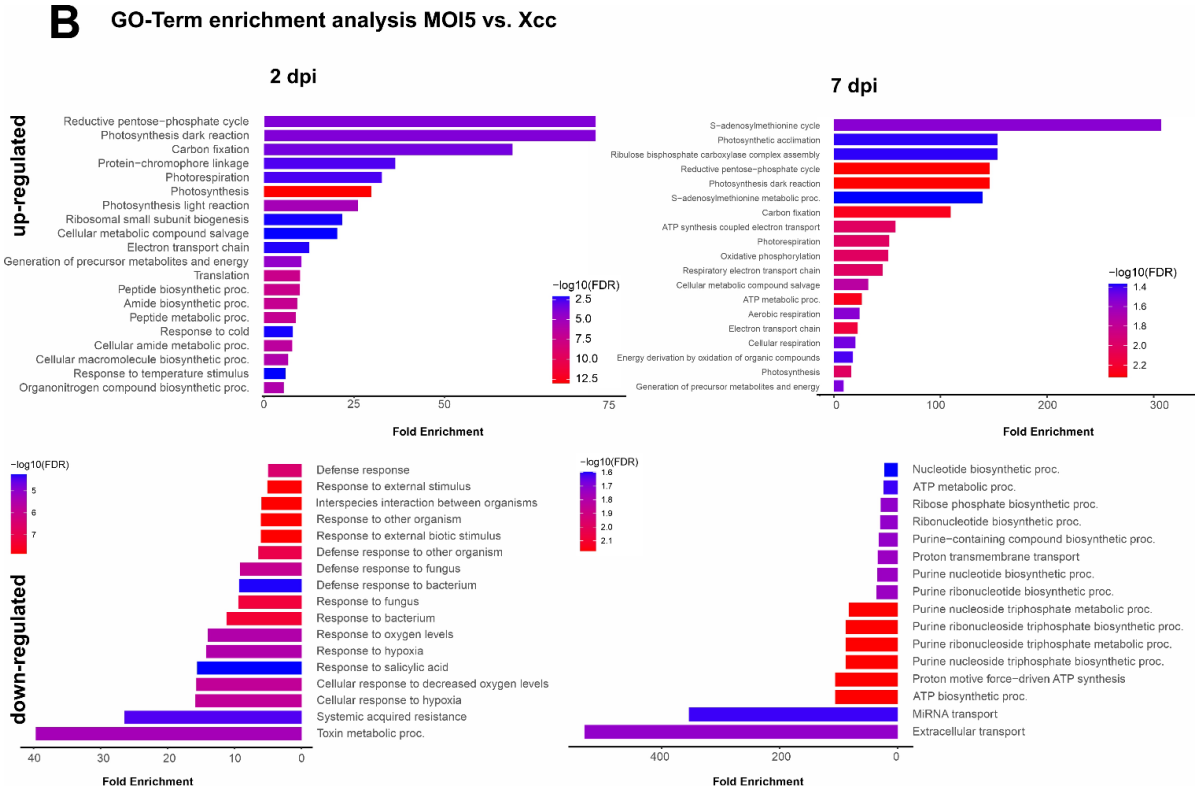


Figure 7. Gene ontology enrichment analysis. Significant differential expressed were used to identify enriched biological processes using ShinyGO (Ge et al., 2020). Displayed is the fold enrichment of the significant enriched pathways, colour coding represents the significance of the observation. **A)** Enrichment compared to the control 2dpi. **B)** Enrichment in the phage + bacterium condition compared to the bacterium only.

Excited by the regulatory changes found in the plant during the tripartite interaction we were curious about the changes in the bacterial transcripts. Not only did we find differentially expressed genes when comparing *Xcc* and *Xcc* + phage and *vice versa* (Figure 8 A). Further a PCA showed that ther is a difference expression of bacterial genes between the two conditions (Figure 8 B). At 2 dpi the half of the of significantly expressed can be found in both conditions. Interestingly a LysR-like transcription regulator is upregulated in the *Xcc* condition compared to the phage treated bacterium at MOI5. But deeper analysis of the data is needed and a plant free bacterial transcriptomic reference, would be helpful to distinguish between the response to the plant and response to the phage.

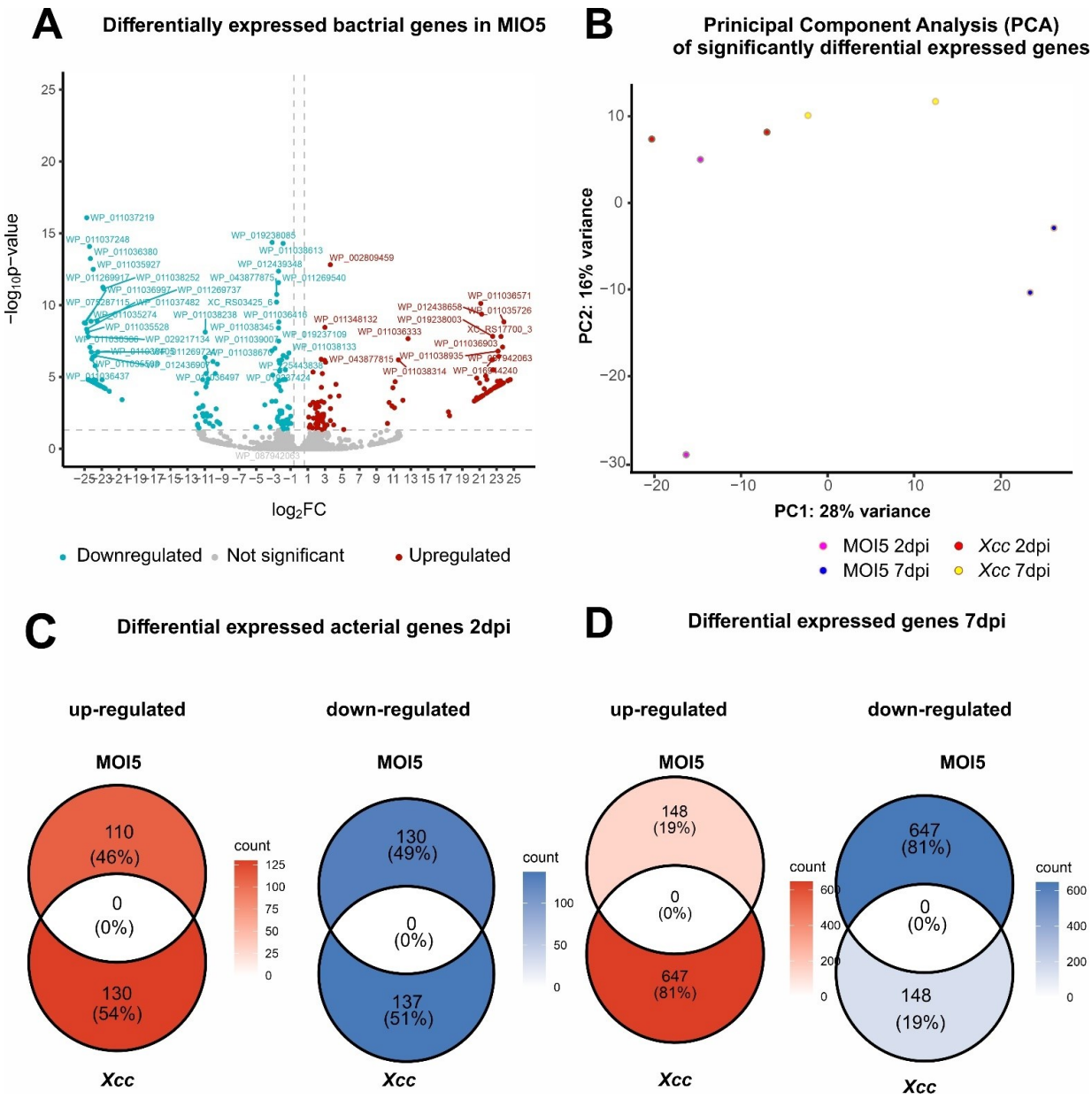


Figure 8: Differentially expressed bacterial genes were identified with DESeq2; p-val < 0.05 **A)** Vulcano-plot of differentially expressed genes. Up-regulated genes are shown in red, down-regulated genes are shown in blue **B)** Principal component analysis, samples: bacterial reads from samples MOI5 2 dpi, MOI5 7 dpi, *Xcc* 2 dpi and *Xcc* 7 dpi **C)** VennDiagram of differential expressed genes at 2 dpi in the presence of phages (MOI5), and the Xcc. Up-regulated genes are shown in red and down-regulated genes are shown in blue. **D)** Venn diagram of differentially expressed genes at 7 dpi, the mock treatment 7 dpi was used as reference. Up-regulated genes are shown in red and down-regulated genes are shown in blue.

Also, for the we obtained transcripts during the RNA sequencing (Figure 9). While the coverage of structural components like the mayor capsid protein at is the same level at 2 and 7dpi. Some hypothetical phage proteins like gene 47 generate higher transcript number at 7 dpi. Future research could focus on the initial ours of the infection to get a more nuanced expression profile for the prokaryotes.

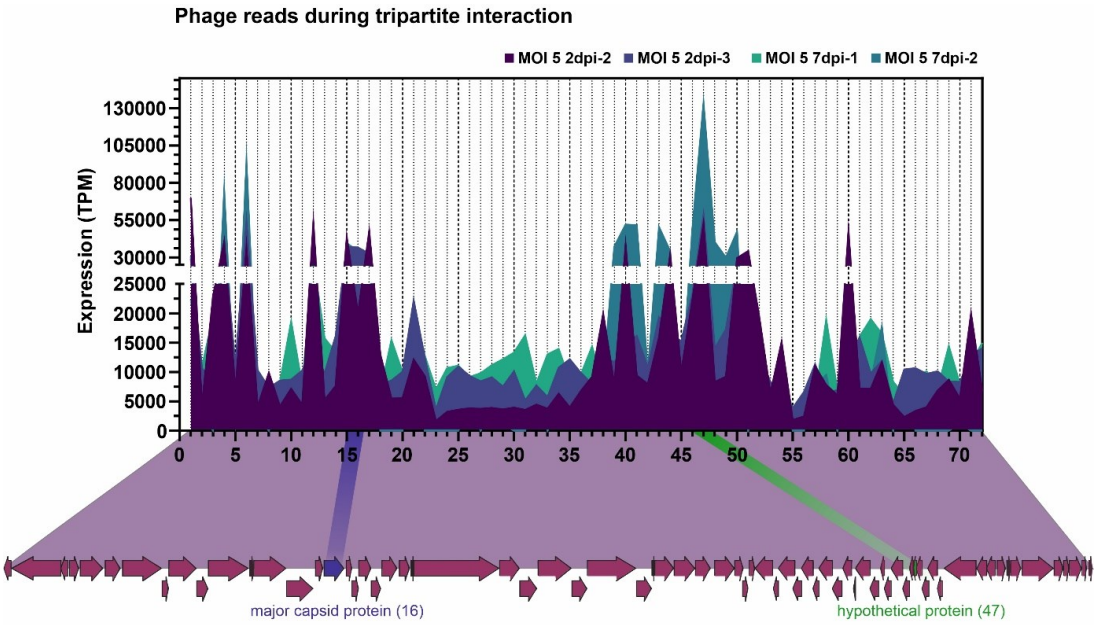


Figure 9: Phage gene expression during the tripartite interaction. Shown is the transcripts per million count of aligned reads for each gene in the genome of phage Seregon.

The observed differences in the MOI5 treatment indicate a shift from defence to growth over time with differential defence expression from early on. Future studies have to investigate the exact mechanisms for these regulatory changes during phage treatment. Also, the phage only control which could be of great interest and might give further insights how plant defence regulation is altered. This may shed light on the question of whether the bacterial reduction by the phage primarily leads to the increased fitness of the plant or if a yet not fully understood defence pathway is triggered in the presence of the phage.

4. Conclusion

Past studies showed the great potential that bacteriophages bear in controlling crucifers' black rot. From the first researchers bringing up the idea a hundred years ago (Mallmann and Hemstreet, 1924) to systematic testing of Xanthomonas phages in plant nurseries in recent years (Holtappels et al., 2022). We could show that a MOI of five of the phage Seregon was sufficient to protect *Arabidopsis thaliana* seedlings against *Xcc* infection and restore the leaf area to control levels 14 days post inoculation.

In line with previous literature, we found that at two dpi for the *Xcc* condition, the SA-Induced legume lectin-like protein one was strongly upregulated, which suggests effector-triggered SA mediated defence response (Armijo et al., 2013). In the Xcc plus phage (MOI5) condition it was also up-regulated, at 2 dpi, but to a lower extent. ATHCYSTM8, a further salicylic acid response gene belonging to the Pathogen-induced Cysteine-rich transmembrane proteins involved in disease resistance, is also upregulated from early on in the *Xcc* condition fitting to earlier reports (Pereira Mendes et al., 2021) and indicating SA signalling in the pathogen condition. In contrast, the SA induced genes are expressed weaker for the *Xcc* and phage combination. This suggests either a reduction in bacterial load or crosstalk with other pathways inhibiting SA signalling.

Early Sa signalling upon pathogen detection leads to upregulated membrane protein wall-associated receptor kinase (Wak1) located at the extracellular matrix. It detects damage-associated molecular patterns (DAMPS) like Oligogalacturonides (Ferrari, 2013). We found it up-regulated in all conditions containing the bacteria at all days tested, but also here to a weaker extent at two dpi MOI5. Interestingly, its interaction partner GEP3, which negatively regulates the activity of Wak1 (Behnami and Bonetta, 2021; Gramegna et al., 2016), is upregulated in the phage treatment at two dpi while being downregulated in all other conditions at this time point. Indicating that the DAMP signalling is tuned down in presence of the phage. GRP3 is not only involved in root size determination but also stress tolerance. Interestingly GRP3 was proposed to be activated in a salicylic acid independent pathway (Rairdan et al., 2001).

A further highly upregulated gene with is product acting at the cell wall is ECS1. It was up-regulated at 7dpi in the MOI5 and *Xcc* condition, but only found in *Xcc* at 2 dpi. Previous studies have linked ECS1 to resistance against *Xcc* not by conferring direct resistance but by interaction with a locus that confers resistance against Xcc (Aufsatz et al., 1998). It was described as SA dependent, but its exact function remains to be investigated.

The plant defence gene PR1 is expressed in the *Xcc* conditions at 2 and 7 dpi but not in all other conditions, pointing into the direction of differential regulation of the SA-Pathway in the MOI5 condition or even a SA independent pathway being activated. Past studies have shown that the T3SS-delivered effector protein AvrXccC induces PR1 expression in *Arabidopsis thaliana* Col-0 (Rong et al., 2010). Also, glutathione S-transferases (GSTs) 2 and 6 are upregulated strongly in the bacterial condition; they are involved in reactive oxygen species production (Gullner et al., 2018) and were linked to Sa and ethylene signalling in the past (Lieberherr et al., 2003). Further upregulated genes from this family under the *Xcc* treatment are GSTF7 and GSTF3.

The observed differences in the MOI5 treatment have to be investigated in future studies. The may shed light on the question if the bacterial reduction by the phage primarily leads to the resistance of the plant or if a jet not fully understand SA in depended defence pathway is triggered in the presence of the phage.

Author Contributions:

Conceptualization, All; methodology, All; validation, All; data analysis, All; investigation, S.E.; resources, B.A, U.S and J.F.; data curation, S.E and writing—original draft preparation, S.E.; writing—review and editing, All; visualisation, S.E. supervision, U.S., J.F. and B.A.; All authors have read and agreed to the published version of the manuscript.

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A Appendix

A.1 Supplementary material

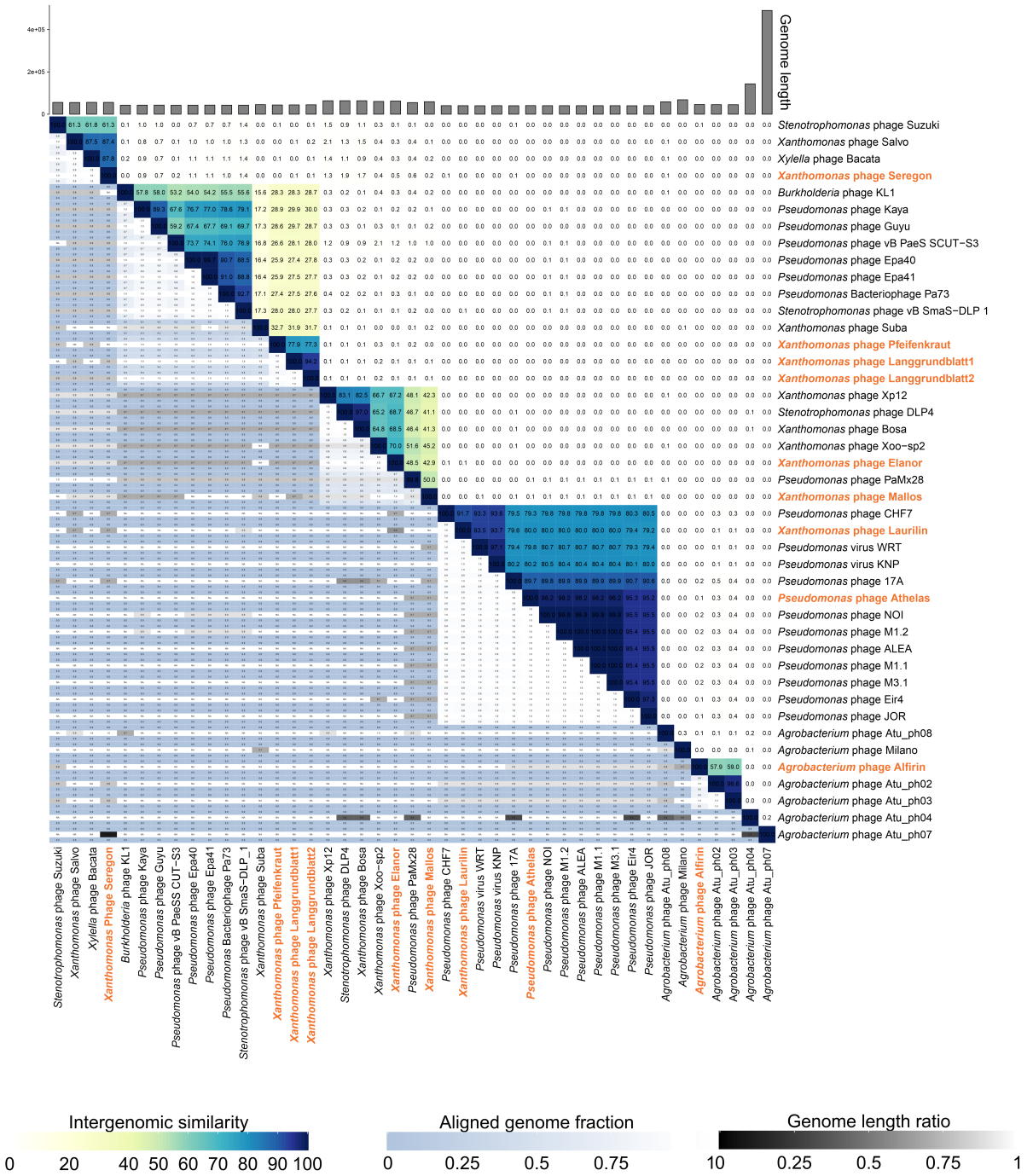

Figure A.1.1: Average nucleotide-based heatmap analysis using the phage isolates obtained during this PhD project. Genomes of closest related phages were acquired from NCBI. The analysis was performed using VIRIDICT.

A.2 Appendix to 4.1: Isolation of Novel *Xanthomonas* Phages Infecting the Plant Pathogens *X. translucens* and *X. campestris*

Supplementary Information to

Isolation of novel *Xanthomonas* phages for the plant pathogens *X. translucens* and *X. campestris*

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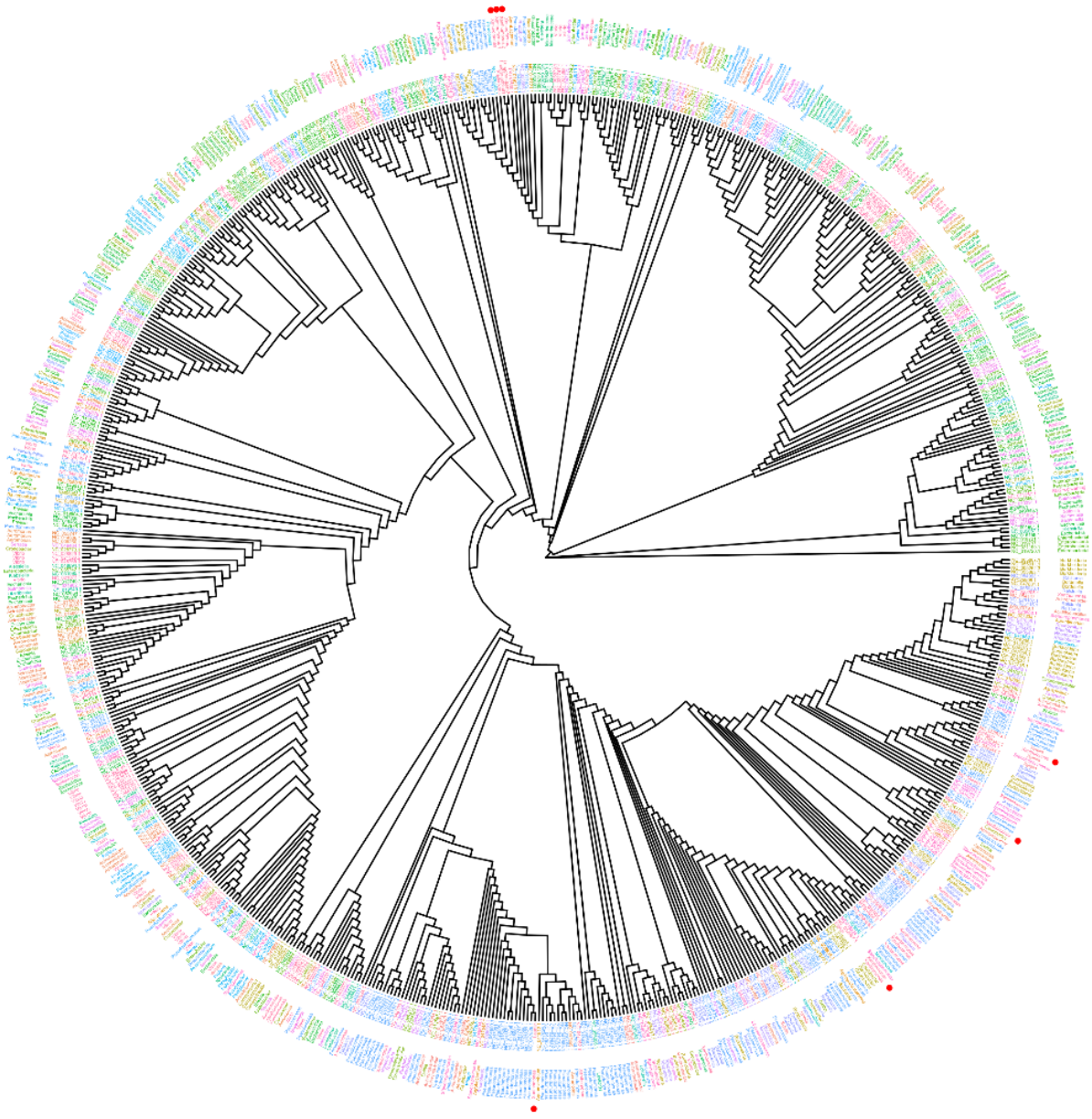

Figure S1. K-mer clustering dendrogram of Proteobacteria phages. More than 2000 Phage genomes were retrieved from VirusHost DB [29] filtered by host genus. Afterwards a clustering with 90% identity level was performed, the remaining 725 phages, as well as the closest relatives to our phages (red dots) according to NCBI Blast were used to build the dendrogram.

	Langgrundblatt1	Langgrundblatt2	Pfeifenkraut	Elanor	Laurilin	Mallos	Seregon
Xanthomonas translucens	1	1	1	1	1	1	0.000004
Xanthomonas campestris	0.00002	0.0003	0.00007			0.00002	1
Sinorhizobium meliloti							
Bacillus subtilis							
Herbaspirillum seropedicae							
Azospirillum brasilense sp245							
Pseudomonas flourecence						0.00004	
Pseudomonas koreensis							
Pseudomoas syringae pv lapsa							
Pseudomoas syringae pv tomato						0.00002	
Agrobacterum tumefaciens							

Figure S2. Host range assay. The host range of the seven phages was determined by spotting serial dilutions of the phages on lawns of different Xanthomonads, plant pathogenic bacteria and plant growth promoting bacteria (PGPB) propagated on the respective medium. Lysis is indicated as follows; plaque formation on isolation host (dark green), plaque formation on other bacteria or clearance of the lawn (light green), no plaques or lysis visible (no color). Numbers indicate the efficiency of plating (EOP).

Table S1. Phage particle size. Measurements of virion particles analyzed by Transmission electron microscopy (TEM).

Phage Name.	Capsid diamaeter	Tail length	Phage size
Langgrundblatt 1	56 nm ± 3	170 nm ± 8	226 nm
Langgrundblatt 2	57 nm ± 3	160 nm ± 18	217 nm
Pfeifenkraut	55 nm ± 3	153 nm ± 10	208 nm
Elanor	77 nm ± 5	134 nm ± 12	211 nm
Laurelin	77 nm ± 2	143 nm ± 7	220 nm
Mallos	82 nm ± 2	141 nm ± 7	223 nm
Seregon	66 nm ± 3	225 nm ± 7	291 nm

Table S2. Strains /Phages used in this study.

Organism	Source	Reference
Xanthomonas translucens (DSM 18974)	DSMZ	https://www.dsmz.de/collection/catalogue/details/culture/DSM-18974
Xanthomonas campestris pv. *campestris*	AG Narberhaus (RUB, Bochum)	[14]
Sinorhizobium meliloti 1021 (DSM 30135)	DSMZ	https://www.dsmz.de/collection/catalogue/details/culture/DSM-30135
Bacillus subtills (DSM 10)	DSMZ	https://www.dsmz.de/collection/catalogue/details/culture/DSM-10
Herbaspirillum seropedicae (DSM 6445)	DSMZ	https://www.dsmz.de/collection/catalogue/details/culture/DSM-6445
Azospirillum brasilense sp245	AG Arsova	[51]
Pseudomonas fluorescence (DSM 50090)	DSMZ	https://www.dsmz.de/collection/catalogue/details/culture/dsm-50090
Pseudomonas koreensis (DSM 16610)	DSMZ	https://www.dsmz.de/collection/catalogue/details/culture/DSM-16610
Pseudomoas syringae pv *lapsa* (DSM 50274)	DSMZ	https://www.dsmz.de/collection/catalogue/details/culture/DSM-50274
Pseudomoas syringae pv *tomato* DC3000	AG Ott (University Freiburg)	[52]
Agrobacterum tumefaciens C58	AG Narberhaus (RUB, Bochum)	[53]

A.3 Appendix to 4.2: Seed coating with phages for sustainable plant biocontrol of plant pathogens and influence of the seed coat mucilage

Supplementary Information to:

"Seed coating with phages for sustainable plant biocontrol of plant pathogens (of the genera of *Pseudomonas*, *Xanthomonas* and *Agrobacterium*) and influence of the seed coat mucilage"

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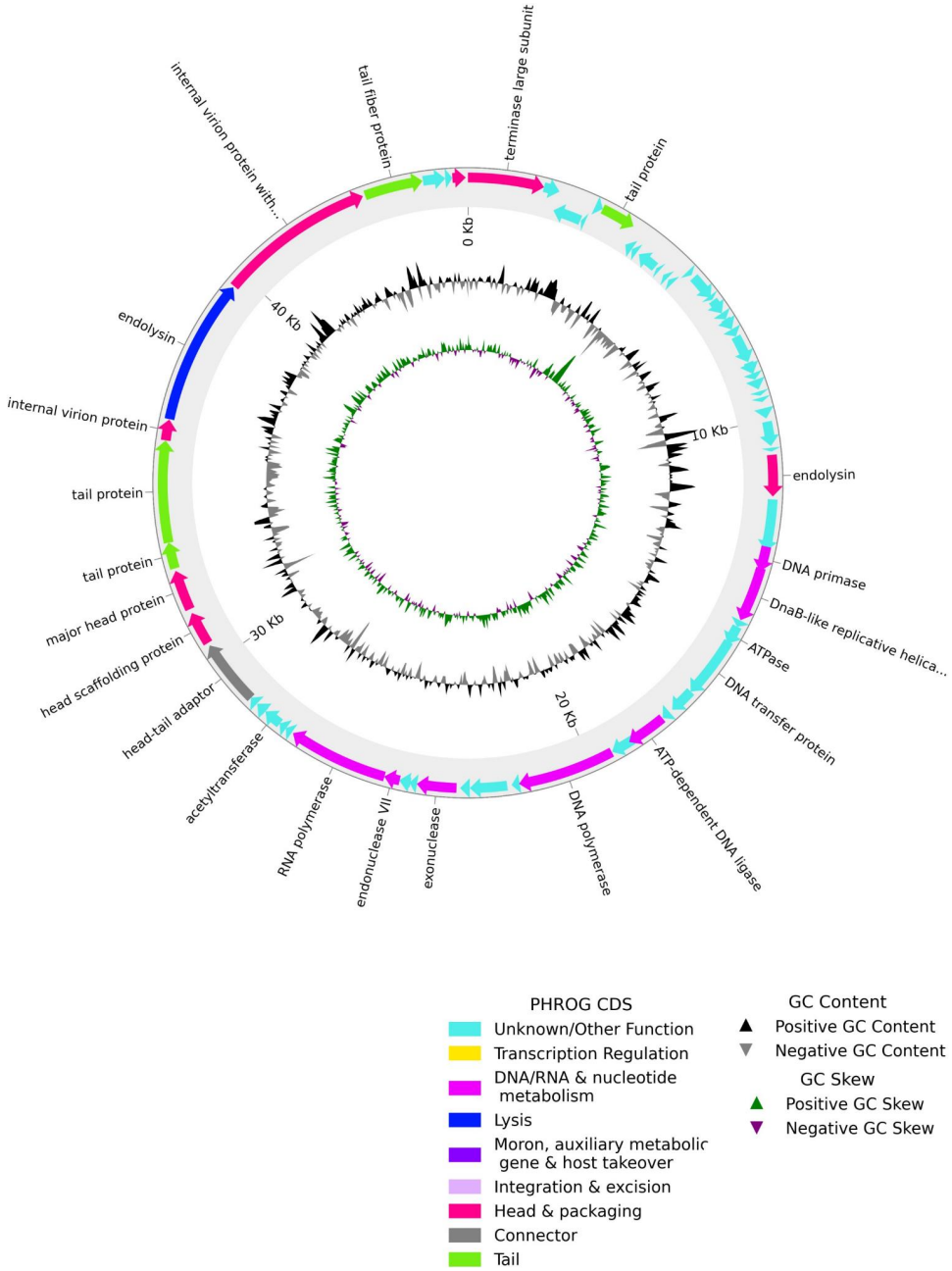
Supplementary material:

A ***Agrobacterium phage Alfirin***



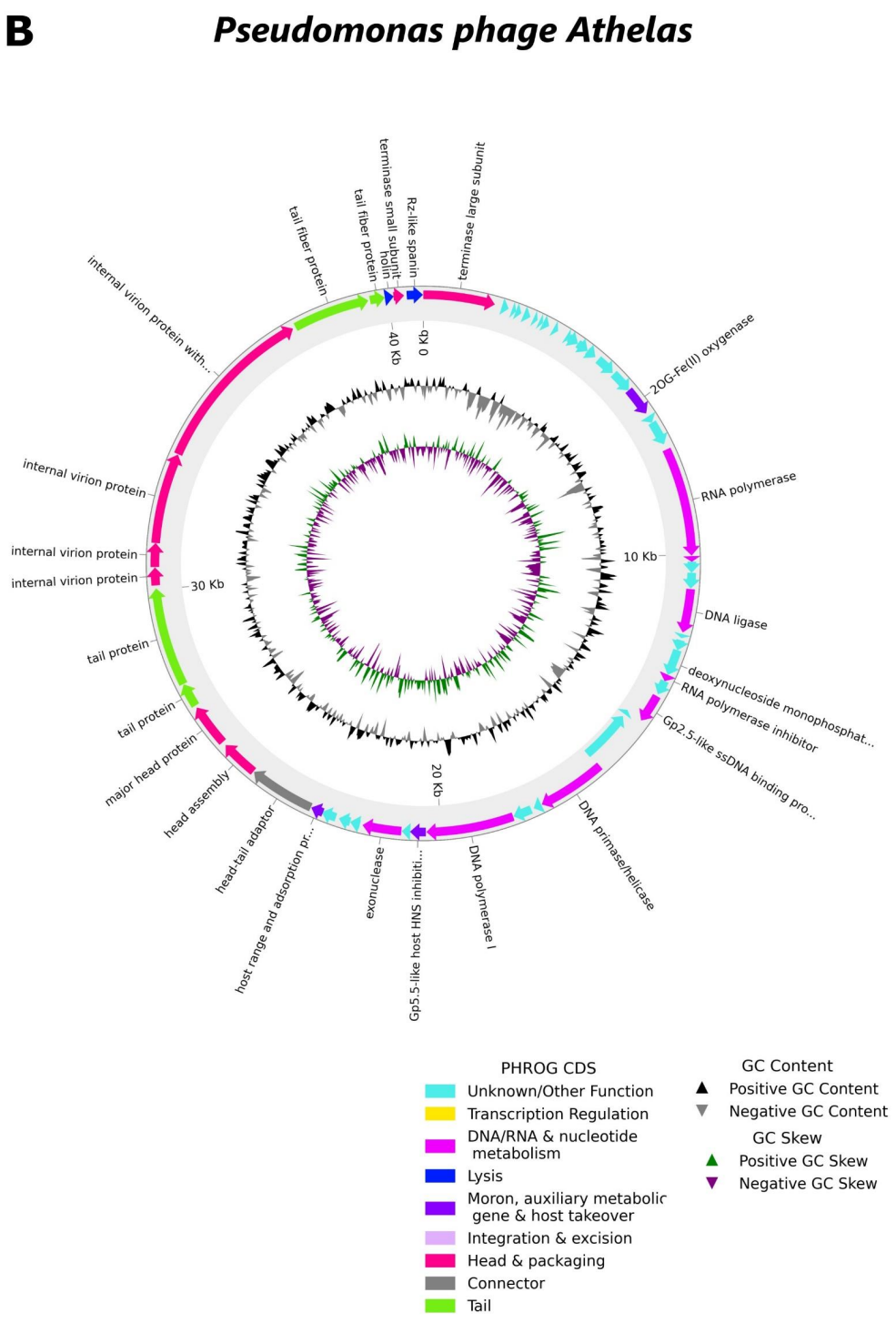


Figure S1: Phage Annotation of *Agrobacterium* Alfirin and *Pseudomonas* phage Athelas. A) genome annotation of *Agrobacterium* Alfirin B) genome annotation of *Pseudomonas* phage Athelas The phage open reading frames (ORFs) were predicted with Pharokka v 1.3.2 (Bouras et al., 2023) in terminase reorientation mode using PHANOTATE (McNair et al., 2019). 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 and Söding, 2017) and PyHMMER (Larralde and 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. The architecture of the genomes of the phages is notably characteristic, as functional units tend to cluster together, demonstrating the inherent modularity of phage genomes.

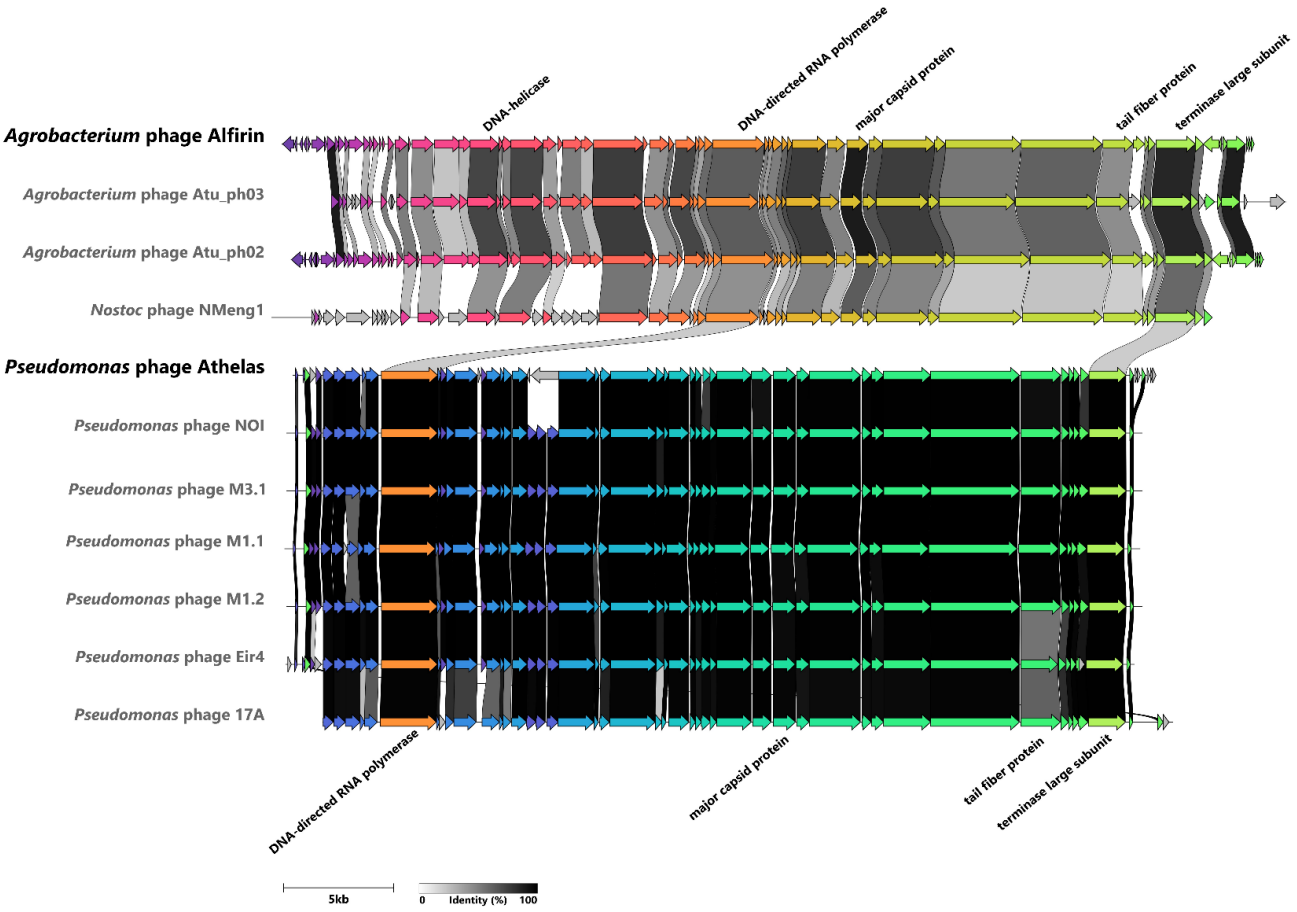


Figure S2 Genome comparison on CDS level. The coding sequences (CDS) of our isolated phages were compared to the closest relatives, retrieved from NCBI blastn, using the clinker pipeline (Gilchrist and Chooi, 2020) to cluster them in groups by similarity (each color represents one group) and percent identity of the member of one group is indicated by grey values. The circular genomes are represented linear and the direction of the arrows is in line with transcription direction of each CDS.

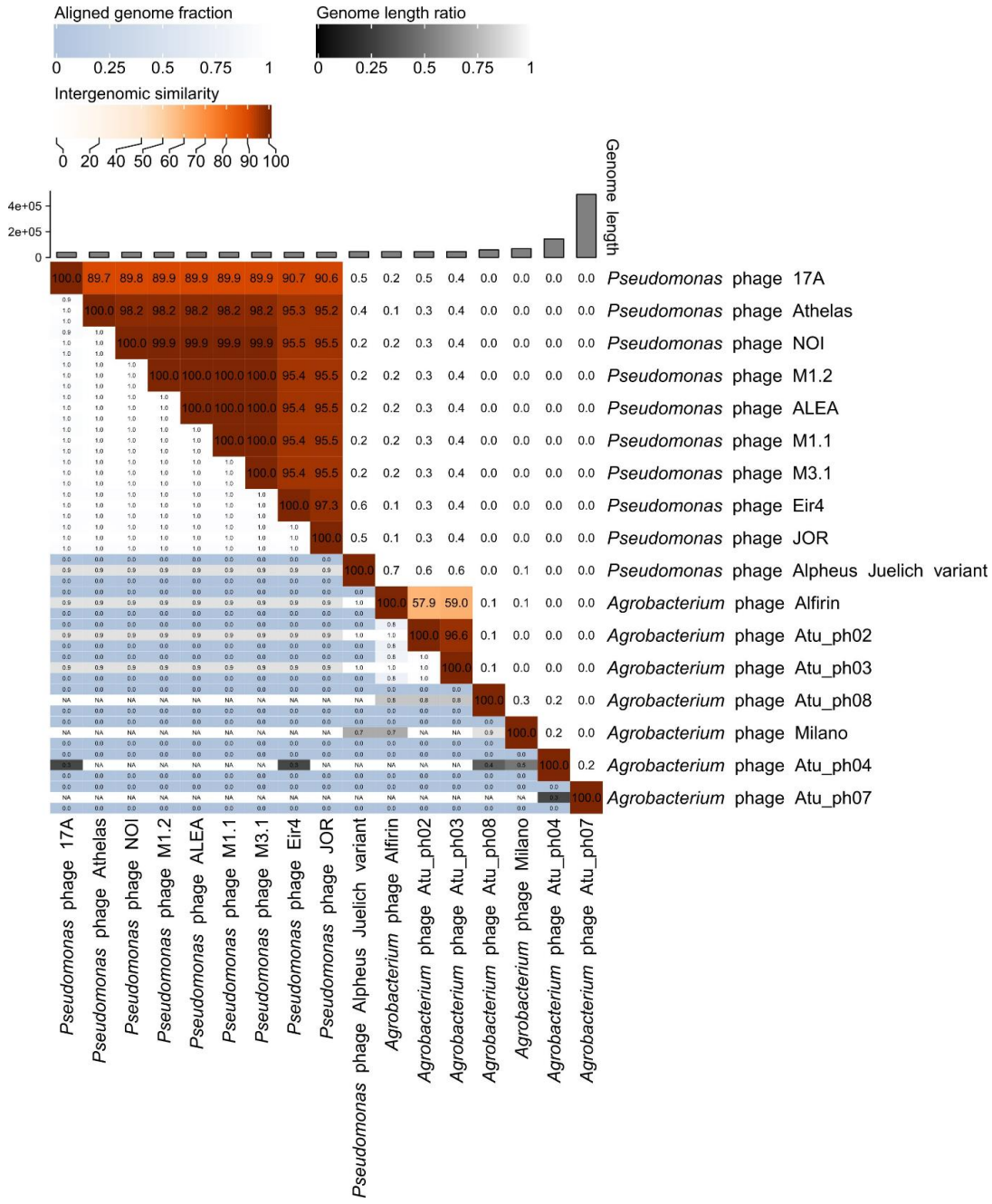


Figure S3: Viridict Heatmap. Average nucleotide-based heatmap analysis using the *Agrobacterium* Phage Alfirin and *Pseudomonas* phage Athelas. Genomes are were acquired from NCBI based on relatedness to the isolated phages in this study phages. The analysis was performed using VIRIDICT (Moraru et al., 2020).

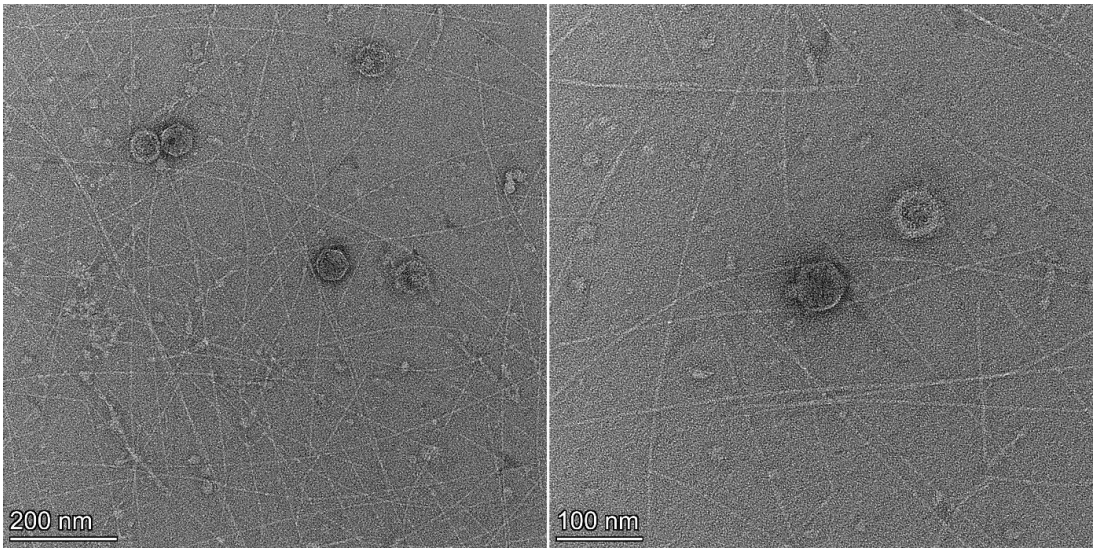


Figure S4: Electron microscopy images of phage Athelas co-incubated with *A. thaliana* mucilage. Transmission electron microscopy (TEM) images of phage Athelas virion particles, incubated with mucilage mechanically removed from 2000 Col-0 seeds. After an incubation time of 1h the phage particles were negative stained with uranyl acetate.

Table S1: Genetic features of the used *E.coli* phages.

Phage Name	Accession Number	Reference Host	Genome Size (Bp)	GC Content (%)	ORF Number [a]	Genome Termini Class [b]	Lifestyle Prediction [c]	reference
Bas64	MZ501081	*Escherichia coli K-12*	39.842	48.0	51	n.a.	virulent	(Maffei et al., 2021)
Bas65	MZ501078	*Escherichia coli K-12*	39.451	49.0	50	n.a.	virulent	(Maffei et al., 2021)
Bas66	n.a.	*Escherichia coli K-12*	n.a.	n.a.	n.a.	n.a.	virulent	(Maffei et al., 2021)
Bas67	MZ501064	*Escherichia coli K-12*	39.315	49.0	48	n.a.	virulent	(Maffei et al., 2021)
Bas68	MZ501055	*Escherichia coli K-12*	39.466	49.0	49	n.a.	virulent	(Maffei et al., 2021)
Bas69	MZ501049	*Escherichia coli K-12*	70.849	41.0	86	n.a.	virulent	(Maffei et al., 2021)
T7	NC_001604	*Escherichia coli*	39,739		59	DTR	virulent	(Studier, 1972)

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A.4 Appendix to 4.3: Molecular responses in phage biocontrol and conclusions on tripartite interactions *in planta*

Supplementary Information to:

"Molecular responses in phage biocontrol and conclusions on tripartite interactions in planta"

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Supplementary material:

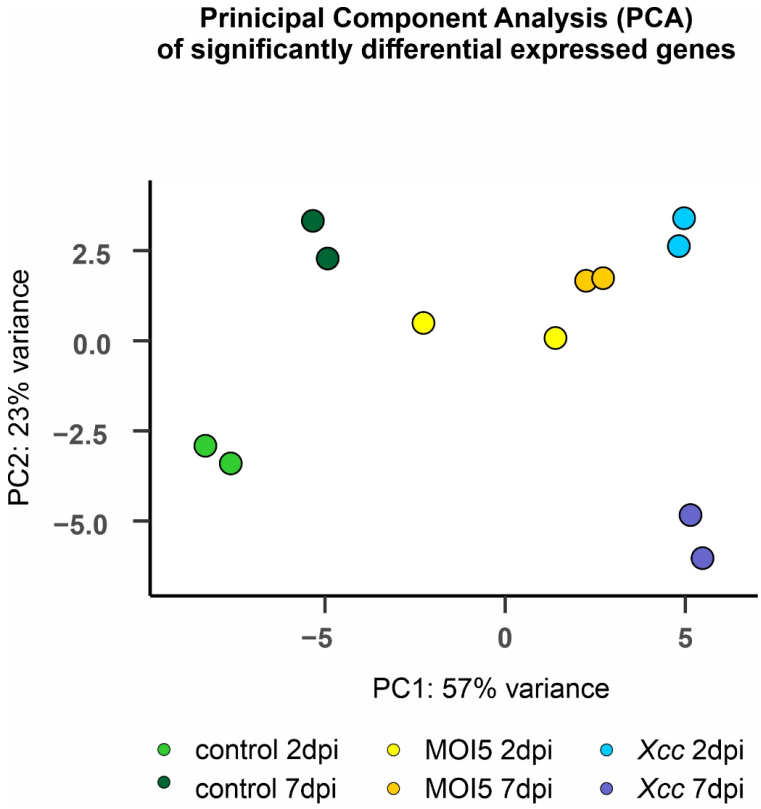

Figure S1: Principal component analysis of the detected plant transcripts within the RNA-Seg data, samples: control 2 days post inoculation (dpi), control 7 dpi, MOI5 2 dpi, MOI5 7 dpi, Xcc 2 dpi and Xcc 7 dpi.

Table S1: Primers used in this study

Name	Gene	Sequence	Source
Ef1a-F	Eukaryotic translation elongation Factor 1 alpha	AGATCAACGAGCCCAAGA	Souček et al. 2017
Ff1a-R	Eukaryotic translation elongation Factor 1 alpha	CCGTTCCAATACCACCAAT	Souček et al. 2017
UBC9-F	ubiquitin-conjugating enzyme 9	CAAGGTGCTGCTATCG	Souček et al. 2017
UBC9-R	ubiquitin-conjugating enzyme 9	ATCTCAGGGACCAAAGG	Souček et al. 2017
SARD1-F	SAR Deficient 1	TTGTTGTTAGAGATCATCGTGGA	This study
SARD1-R	SAR Deficient 1	CGAGAGGAGAGCTTCTTGTGA	This study
MYC2-F	MYC-related transcriptional activator	GGTTGGGACGCAATGATTAGAGT	This study
MYC-2-R	MYC-related transcriptional activator	CCATCTTCACCGTCGCTTGTTG	This study
PR1-F	pathogenesis-related protein 1	TCTCGTTCACATAATTCCCACG	This study
PR1-R	pathogenesis-related protein 1	ACTACAACTACGCTGCGAACA	This study
Pdf1.2-F	plant defensin 1.2	GTTTGCTTCCATCATCACCC	This study
Pdf1.2-R	plant defensin 1.2	GGGACGTAACAGATACAC TTG	This study
Seregon_VAP-F	Phage Seregon virion-associated protein	ATGTCCCTTGCCAACCTGC	This study
Seregon_VAP-R	Phage Seregon virion-associated protein	GTTGACGTTGGCGAGGTCTT	This study

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Erklärung

Hiermit versichere ich an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Es wurden bisher keine erfolglosen Promotionsversuche unternommen.

Sebastian Hermann Erdrich