

RESEARCH PAPER

Effect of phage XooG01 and flagella protein of *Xanthomonas oryzae* pv. *oryzae* on *Xa* genes expression in rice

Hardian Susilo Addy^{1,2*}, Wulan Arum Hardiyani^{2*}, Harits Ramadhan Fajrianto³

¹University of Jember, Faculty of Agriculture, Program of Plant Protection, Jember, Indonesia

²University of Jember, Graduate Program of Biotechnology, Jember, Indonesia

³University of Jember, Applied Molecular and Microbial Biotechnology Research Group, Jember, Indonesia

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Corresponding Author

Tel.: +628 214 133 1654

E-mail: hsaddy.faperta@unej.ac.id

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Abstract

Bacterial leaf blight (BLB) is caused by the phytopathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), a destructive disease that significantly threatens rice production. Controlling bacterial leaf blight on rice (*Oryza sativa*) is important through the plant resistance approach, including pathogen-derived ligands such as flagella to induce plant resistance mechanisms, in addition to using lytic bacteriophage. This study investigated the interaction between the bacteriophage XooG01 and flagellar proteins from XooH02 on the expression of resistance genes in rice. Leaves were pre-treated with a mixture of the phage and bacterial cell surface appendages before pathogen inoculation, and gene expression was measured using semi-quantitative RT-PCR. The results revealed a paradoxical transcriptional response. While the expression of genes *Xa10* and *xa13* was significantly upregulated in treated leaves after pathogen challenge, the expression of the primary immune receptor *Xa21* was, contrary to expectations, significantly decreased. The expression of *Xa4* showed a slight, non-significant increase. This unexpected suppression of *Xa21* suggests a phage-mediated interference in the host-pathogen recognition process, likely by the phage physically masking the flagellin elicitor from plant receptors. These findings reveal a complex tripartite interaction with critical implications for the design of future biocontrol strategies.

Introduction

Rice (*Oryza sativa* L.) is a cornerstone of global food security, providing the primary caloric intake for more than half of the world's population, including Indonesia, as a C3 grain crop adapted to warm climates, its productivity is fundamental to sustaining a rapidly growing global population (Mohidem et al., 2022). However, challenges such as biotic stresses, including devastating diseases like bacterial leaf blight (BLB), continually threaten its yield (Irpawa et al., 2024). Bacterial leaf blight is a significant disease in rice cultivation, caused by the pathogenic bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). The *Xoo* enters the leaf through hydathodes, causing the plant to dry

out and even die (Shen and Ronald, 2002). *Xoo* attacks rice during vegetative and generative phases, causing up to 50% damage, especially during the rainy season when the pathogen infests the fields (Niño-Liu et al., 2006; Ilsan et al., 2016). Preventative approaches are crucial in controlling BLB, since overuse of chemical substances for long-term control would negatively impact both producers and consumers (Banerjee et al., 2021). Although the use of resistant varieties is effective, it is inefficient considering the breeding efforts, due to *Xoo*'s adaptability, with more than 22 known pathotypes (Rashid et al., 2021). Consequently, an alternative technology is required for the efficacious control of BLB

that is safe and environmentally friendly. This goal can be achieved by the induction of plant resistance mechanisms based on the plant receptor and pathogen ligand interaction (Hajam et al., 2017; Liang et al., 2022).

The mechanism of ligand-receptor interaction occurs through a molecular approach known as plant pattern-recognition receptors (PRRs), which recognize microbial cell components as an induction pattern, including the flagellin component (Tonelli et al., 2020). Previous research has revealed that flagellin from *Pseudomonas syringae* pv. *tomato* significantly enhances the resistance of tomatoes (Roberts et al., 2020), and flagellin from *Ralstonia solanacearum* can elevate the production of resistance compounds in tomatoes (Nadhira et al., 2021). This occurrence is due to components of the flagella (flagellin) primarily recognized by the PRRs receptor FLS2 found in plant cells, which are connected to a co-receptor in the signal pathway for resistance activation, subsequently resistance and gene expressions of the host plant (Sanguankiatichai et al., 2022). However, the capability of the flagella of *X. oryzae* to induce resistance mechanisms in rice remains under investigation, especially regarding its specific relationship with the expression of resistance genes against *Xoo*, which is known to have about 46 genes (Yugander et al., 2018).

On the other hand, the presence of *Xoo* in the field is sometimes unknown, and in some cases, the infection does not manifest symptoms, posing a latent threat to the susceptible host. Consequently, a protective approach and bacterium eradication are necessary by utilizing non-chemical substances such as bacteriophages to support sustainable agriculture (Rejeki et al., 2021). It is known that bacteriophages can reach pathogenic bacteria that have infected plants, thereby inhibiting the pathogen from causing plant diseases (Addy et al., 2012). Moreover, bacteriophages induce lysis in infected bacteria and prevent pathogenesis. As a result, components of lysed bacterial cells, including flagella and polysaccharides, can function as ligands that trigger plant resistance mechanisms (Sausset et al., 2020). Flagella, the primary protein subunit of which is flagellin (FlhC), act as an important elicitor that plays a crucial role in the plant immune system's response to bacterial pathogen infections (Hajam et al., 2017; Meindl et al., 2000; Wang et al., 2015).

On the other hand, it is known that bacterial flagella and lipopolysaccharides (LPS) also act as receptors for bacteriophages, which may hinder the ability of bacteriophages to infect other bacterial cells (Liu et al., 2022). However, this condition has not yet been the focus of simultaneous studies regarding the interaction between bacteriophages and host bacteria in plant hosts. Previous studies have focused on interactions between the two entities, such as between bacteriophages and bacteria, like the study on *Ralstonia* phage RsoM1USA against *Ralstonia solanacearum* (Addy et al., 2019) or phage *Xoo*H01 against *X. oryzae*

pv. *oryzae* (Rejeki et al., 2021) and between bacteria and host plants, such as *R. solanacearum* on tomato (Wanget al., 2023) or *X. oryzae* pv. *oryzae* in rice (Addy et al., 2024) separately. In this study, we focus on all interactions simultaneously by examining the role of flagella and bacteriophages, on their potency as inducers of plant resistance, particularly in rice against bacterial leaf blight.

Materials and Methods

Bacteria and bacteriophage of *X. oryzae* pv. *oryzae*

X. oryzae pv. *oryzae* H02 (*Xoo*H02) and phage *Xoo*G01 were obtained from the Applied Molecular and Microbial Biotechnology Laboratory (AM2B), University of Jember. *Xoo*H02 was maintained in Yeast Dextrose Broth (YDB, which comprises 10g/L of yeast extract and 20 g/L of dextrose) and cultured at 28°C for 48 h before use. To confirm the presence of functional flagella, a motility assay was performed by inoculating 3 µL of *Xoo*H02 suspension onto a solid Yeast Dextrose Agar (YDA) (comprises 10g/L of yeast extract, 20 g/L of dextrose, and 20 g/L of Agar) and semisolid YDA media (comprises YDA with 3 g/L of Agar) media, and the motility zone (halo) was observed after 12 h (Roy et al., 2022). In addition, phage *Xoo*G01 was propagated in *Xoo*H02, as previously described by Rejeki et al. (2021). The phage infectivity was confirmed by dropping 5 µL of phage *Xoo*G01 suspension (10^8 PFU/mL) onto the *Xoo*H02 lawn on YDA, followed by incubation at 28°C for 24 h.

Extraction of bacterial extracellular appendages proteins

Cell surface appendages exposed proteins, including the flagella of *Xoo*H02, were extracted from *Xoo*H02 grown on nutrient-poor YDA media (0.25% YDA media comprises 250 mL/L of YDB medium and 20 g/L agar). Briefly, 50 µL of 48-hour-old culture was spread onto the test medium using a sterile L-glass and incubated at 28°C for 20-22 h (Addy et al., 2012). Bacterial cells (at a population of 10^8 CFU/mL) were harvested and resuspended in 8 ml of 10 mM Tris-Cl. The cell surface appendages were mechanically sheared from the cells using a 23-G syringe (Onemed, Indonesia) followed by centrifugation (Sorvall 16R-TX-400 rotor; Thermo Scientific) at 4000 rpm and 4°C for 10 m to pellet the cells. The supernatant was precipitated using 4% (w/v) cold PEG 6000 + 2.5 M NaCl and incubated at room temperature for an hour. Afterward, the suspension was centrifuged for 20 m at 4000 rpm. The resulting pellet was then collected and eluted in 10% (v/v) TE Buffer. The presence of cell surface appendage proteins, including flagella, was visualized using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Narulita et al., 2016).

Leaf infiltration, pathogen inoculation, and sampling

Four-week-old rice leaves were used for all experiments. For the treatment group, 5 µL suspension

containing both phage *XooG01* and extracted *XooH02*-free flagella proteins was infiltrated into the leaves using a needleless syringe (Lang et al., 2019). The leaf surface was first gently punctured with a sterile needle to facilitate infiltration. Control leaves were infiltrated with sterile water. Twenty-four hours after this initial treatment, all leaves (except for the control) were challenge-inoculated with a suspension of *XooH02* using the leaf clipping method (Lang et al., 2019). Leaf samples were collected at defined time points: immediately before treatment (control), one day after treatment (just before pathogen inoculation), and then at one and two days post-inoculation (dpi), for both treated and non-treated groups (Figure 1) for further analysis.

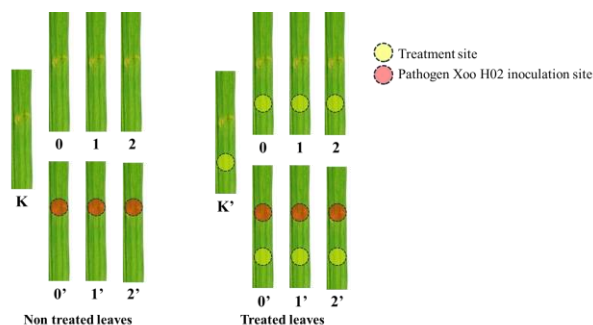


Figure 1. Scheme for leaf infiltration (treatment), pathogen inoculation, and sampling. The leaf was infiltrated with phage *XooG01* and *XooH02* cell surface appendages. One day after infiltration, leaves were inoculated with the pathogen *XooH02* and incubated for two days post-inoculation. Leaf samples were collected on the day of infiltration (K'), one day after infiltration or the day of pathogen inoculation (0'), one-day post inoculation (1'), and two days post inoculation (2'), and its control (K, 0, 1, and 2). All samples were then subjected to determine the gene expression as described in Materials and Methods.

Disease assessment and resistance genes expression analysis

Samples for gene expression analysis were taken every day up to day two. The expression data of the resistance genes were obtained through a semi-quantitative reverse transcription polymerase chain reaction (sq-RT-PCR) method with specific primers for *Xa4*, *Xa10*, *x13*, and *Xa21* genes (Nadhira et al., 2022). Reverse transcription was carried out using reverse transcriptase (Toyobo, Japan) to synthesize complementary DNA (cDNA) for further amplification of the resistance genes in rice. The amplification products were then observed on an agarose gel and recorded with GelDoc after performing RT-PCR with the RevertraAce Kit (Toyobo, Japan) and MyTaq HS Red Mix (Bioline, US) with particular cycle conditions as described by Nadhira et al. (2022). Semi-quantitative data were obtained and analyzed using Image J software (Antiabong et al., 2016). Leaf lesions were observed daily and measured 14 days post-inoculation.

Data analysis

All statistical data were analyzed using an ANOVA based on the F-value at the 5% confidence level. The

significance of the treatments was determined using the Duncan Multiple Range Test (DMRT) at the 5% confidence level.

Results and Discussion

Flagella are cellular organelles in bacteria with various protein monomers (Apel and Surette, 2008). Active bacterial flagella demonstrate the ability to move on liquid surfaces (swimming) as well as on semisolid or solid surfaces (swarming) (Jarrell and McBride, 2008). In pathogenic *Xoo*, a solitary flagellum enables motility of the bacterium (Tian et al., 2015). The motility of *XooH02* was assessed to confirm the presence of functional flagella. The results demonstrated that *XooH02* exhibited significant swimming motility on semisolid media, forming a diffuse, expanded colony from the point of inoculation (Figure 2A).

In contrast, on solid YDA media, the colony remained localized at the inoculation site with minimal expansion (Figure 2B). Lippolis et al. (2014) also showed that the bacterium swims on low-concentration agar media because of flagella rotation, resulting in bacterial movement. Palma et al. (2022) and Xu and Wozniak (2015) also evaluated the motility of bacteria and conclude that swimming motility appears as "motility halo" extensively from the inoculation area on semisolid or 0.3% soft agar media. In addition, the essential role of the flagellum in *Xoo* motility was demonstrated by Tian et al. (2015), who showed that a mutant, *Xoo* strain PXO99A, defective in genes *rpoN2* and *fleQ*, responsible for flagellar activity and motility, led to a significant reduction in the swimming motility in a semisolid agar plate.

The infectivity of phage *XooG01* against its host, *XooH02*, was demonstrated in a spot-test assay. The spot of *XooG01* suspension onto a lawn *XooH02* resulted in a clear plaque, at the inoculated site at a concentration of 10^8 PFU/mL (Figure 2C). These plaques indicate successful bacterial lysis, confirming that phage *XooG01* is undergoing its lytic life cycle within the host cells, *XooH02*. As described by Schofield et al. (2012) and Vu and Oh (2020), bacteriophages targeting either human or plant bacterial pathogens share a similar life cycle, particularly from attachment to the cell target, replication of the genetic material, and ultimately causing the lysis and the release of new phage particles.

To confirm the presence of flagellin, the extract of the cell surface protein from *XooH02* was analyzed using SDS-PAGE. The analysis revealed a prominent protein band with an approximate molecular weight of 43 kDa (Figure 2D). This band size is consistent with the predicted molecular weight for flagellin protein (FliC) in *X. oryzae* pv. *oryzae* and *oryzicola* (FliCXoo and FliCXoc, respectively) as reported by Wang et al. (2015).

This study observed and measured the length of leaf lesions (Figure 3A) to determine the plant's response to *Xoo* infection. Notably, the pre-treatment with the suspension of *XooH02* cell surface protein and

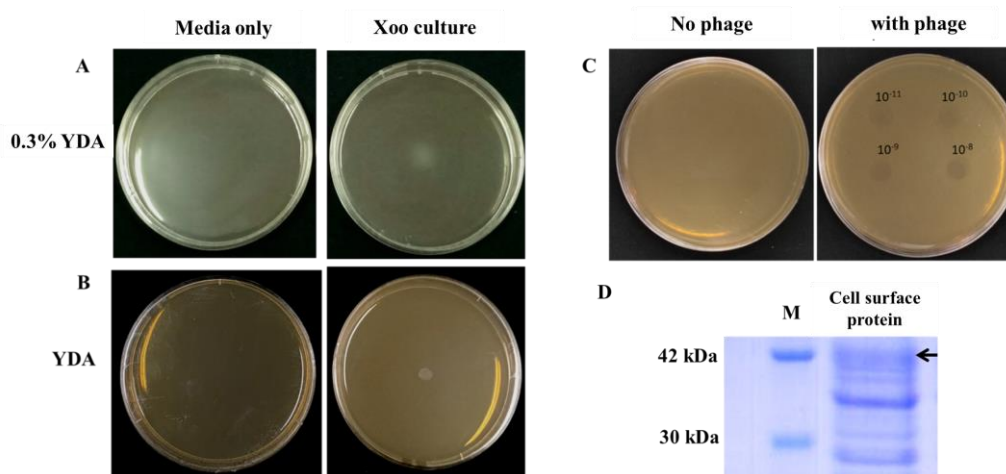


Figure 2. The activity of flagellar *XooH02* and bacteriophage *XooG01*. **A)** The colony showed the diffusion in media semisolid (0.3% YDA); **B)** The colony remains on the drop site; **C)** Presence of a clear zone on the drop site of bacteriophage *XooG01*; **D)** Flagellar protein (FliC) was visualized through SDS-PAGE analysis. A predicted FliC is shown in the size of band 43 kDa.

phage *XooG01* did not confer a significant change in necrotic symptom on the leaf tip (Figure 3A), as the average lesion length (Figure 3B) was statistically similar from that of the non-treated control. This result indicates a resistance phenotype in rice after treatment with extracellular appendage protein and phage before inoculation with the pathogen. The degree of plant resistance phenotype is strongly influenced by the expression of resistance genes in rice against *X. oryzae*, which is known to have at least 46 genes (Yugander et al., 2018). Variations in the expression level of these genes, such as dominant genes such as *Xa4*, *Xa5*, *Xa10*, or *Xa21*, or recessive genes such as *xa3*, *xa26*, or *xa13*, have been confirmed to affect the severity of disease symptoms on rice leaves infected with *Xoo* (Jiang et al., 2020). Thus, we hypothesized that the treatment induced significant transcriptional reprogramming that is not visible at the phenotypic level. Therefore, we subsequently analyzed the expression profiles of these specific genes to dissect the three-way interaction among the host, pathogen, and bacteriophage.

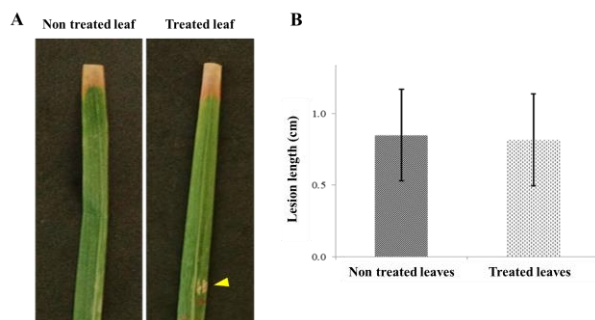


Figure 3. Symptom **A)** and the lesion length **B)** of bacterial leaf blight on treated and non-treated leaves with cell surface protein of *XooH02* and phage *XooG01* 14 days post pathogen inoculation. The head arrow (in yellow) represents the cell surface protein of *XooH02* and phage *XooG01* infiltration site.

Furthermore, this study targets particular genes in Near Isogenic Lines (NILs), such as *Xa4*, *Xa10*, *xa13*, and *Xa21* (Nadhira et al., 2022), to investigate the interaction between *XooH02*'s cell surface protein and

phage *XooG01* and the host plant in inducing resistance genes. The results revealed a complex and paradoxical pattern of gene expression (Figures 4 and 5).

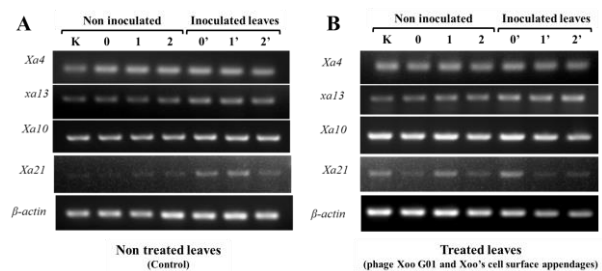


Figure 4. Band presence in sqRT-PCR; **A.** Genes expression on non-treated leaves with *XooH02* inoculation; **B.** Genes expression on flagellin protein and bacteriophage *XooG01* treated leaves following by inoculation *XooH02*. Numbers represent sampling described in the material and methods (Figure. 1).

The *Xa4* belongs to the *Receptor-Like Kinase (RLKs)* gene (pattern recognition receptor) group that encodes the wall-associated kinases (WAKs) protein, which helps strengthen the plant cell wall and prevent *Xoo* infection. (Jiang et al., 2020). The expression of the *Xa4* gene in non-treated leaves with cell surface proteins and phage *XooG01* was slightly higher than in treated leaves (Figure 5). In addition, the non-treated leaves showed a significant expression at 1 to 2 days post inoculation with *XooH02* (Figure 5A). However, the treated leaves showed no significant expression even after *XooH02* inoculation (Figure 5B). A similar phenomenon was also observed in a previous study where the quality of *Xa4* expression decreased after pathogen inoculation, presumably due to the high incidence of disease and weakening of plant cell walls (Nadhira et al., 2022). In contrast, the increase in gene expression in treated leaves in *Xoo*-inoculated leaves at two days post-inoculation indicates the enhancement of the activity of the *Xa4*, which repairs its cell wall following the attack by *Xoo*.

In contrast, *Xa10* and *xa13* expression were significantly upregulated in treated leaves only after

challenge with *Xoo*H02 (Figure 5B). This upregulation is likely triggered by pathogen effectors (e.g., TALEs) delivered into the plant cell during infection, activating downstream defense or susceptibility pathways independently of the initial PAMP recognition. A similar phenomenon was observed in rice inoculated with the virulent *Xoo* strain PXO99A and not in uninoculated rice, revealing that the effector *avrXa10* from PXO99A is responsible for the high expression of *Xa10* in IRBB10A rice line (Yang et al., 2022). The *Xa10* acts as an executor gene, with its activation being a form of hypersensitive response (HR) or cell death when infected by *Xoo*. Through interaction between the plant transmembrane domain and bacterial TALEs, *Xa10* can trigger a plant defense response. *AvrXa10* directly binds to the *Xa10* promoter, inducing a hypersensitive response (HR) or systemic cell death to reduce pathogen development and infection (Tian et al., 2014; Yang et al., 2022).

Besides, the *xa13* is a susceptible gene of R genes with recessive resistance activity (Ji et al., 2018). Li et al. (2019) reported that *X. oryzae* secretes the effector that recognizes the binding element site in the promoter region of *xa13* during infection, in the sequence of producing sugar required for *Xoo* growth in plants. In addition, Antony et al. (2010) demonstrated that *X. oryzae* secretes the effector *AvrXa7* or *PthXo3* via a type 3 secretion system that interacts and binds specifically to an effector binding element within the *Os-11N3* (*xa13*) promoter and increases *xa13* expression. The *xa13* encodes a sucrose efflux transporter, while the transcription-like effectors (TALEs) bind to effector binding elements (EBEs) found in the promoter region of the *OsSWEET11* gene, resulting in the sugars produced by the plant cells, which are utilized by pathogens as a nutritional requirement for their growth. Consequently, the plant becomes more susceptible to *Xoo* (Chen et al., 2012; Zafar et al., 2020).

On the other hand, the gene expression data of the *Xa21* showed the opposite result on treated and non-treated leaves, either before or after pathogen inoculation (Figure 5). In the non-treated control leaves, *Xa21* expression was significantly induced following pathogen inoculation, which is the canonical response for a primary defense gene (Figure 5A). However, this response was inverted entirely in the pre-treated leaves. Despite a known elicitor, pathogen challenge led to a drastic and progressive suppression of *Xa21* expression (Figure 5B).

Previous studies have suggested that, in BLB-resistant rice plants, *Xa21* supports the plant for a quick and efficient response against *Xoo* infection through cellular activities. In addition to resource and energy allocation, *Xa21* activates biotic stress pathways upon infection by *Xoo* (Peng et al. 2015). Peng et al. (2015) also reported that *Xa21* can inhibit *Xoo* growth in the indica rice variety 9311, which carries the *Xa21* gene, compared to those without *Xa21*. However, the increase in expression of genes during the initial application of *Xoo*H02 cell surface proteins and phage *Xoo*G01

indicates an interaction between flagellin protein and plant receptors that activate early defense via pattern-triggered immunity (PTI). However, the data indicated an opposite phenomenon: *Xa21* expression was reduced after *Xoo* inoculation. It indicates a fundamental failure or blockade of the PAMP-Triggered Immunity (PTI) pathway in rice. The *Xa21* encodes for the leucine-rich repeat receptor-like kinase (LRR-RLKs) protein in the *XA21* region. During the interaction, pathogens enter plants and release *AvrXa21* (*RaxX*) effector, which is recognized by the LRR region of *XA21* in rice; the defense signal complex is formed, triggering the PTI process (Jiang et al., 2020; Yang et al., 2022).

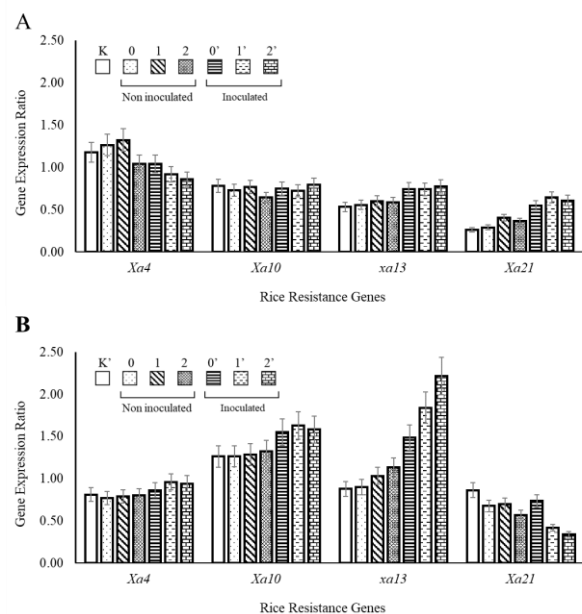


Figure 5. Resistance Gene expression on each treatment. A. Genes expression on leaves without infiltration of phage *Xoo*G01 and cell surface proteins; B. Genes expression on treated leaves with phage *Xoo*G01 and cell surface proteins. K refers to the control treatment, 0, 1, and 2 or 0', 1', and 2' refer to the day post-treatment.

It is known that interaction between the cell surface appendages, including flagella protein as a ligand and its receptor on the host cell surface, also known as plant pattern-recognition receptors (PRRs), can trigger plant defense and resistance systems (Dodds and Rathjen, 2010). Interaction between flagellin (Flc) classified as pathogen-associated molecular patterns (PAMPs) and the receptor FLS2/co-receptor BRI1-associated kinase (BAK1) occurs at the plasma membrane and connects to the activation of the signaling pathway for systemic acquired resistance induction via the activity of Mytocin-associated Protein Kinase (MAPK) and its derivatives, which subsequently trigger a complex signal transduction pathway to activate (PAMP)-Triggered Immunity (PTI) (Hajam et al., 2017; Roberts et al., 2020; Malik et al., 2020; Sanguankiattichai et al., 2022). However, this study did not show a correlation with this information. We suggested that the decrease and no significant elevation of expression levels of the *Xa4* and *Xa21* genes in

treated leaves may be due to recognition interference of activation of host-pathogen recognition through ligand-receptor interaction in plants due to the presence of phage XooG01 in the mixture treatment. The presence of phage mixed with flagella-containing extracellular appendages protein may interfere with recognition because phage may also occupy or use flagella receptors. Consequently, the plant receptor is difficult to interact with and prevents ligand (flagellin) recognition from adhering to plant PRRs (Dunne et al., 2021).

Conclusion

This study concludes that the combination of bacteriophage XooG01 and its host cell surface protein influenced the expression of resistance genes in rice plants. The co-application of phage XooG01 and its host's flagellar proteins did not enhance resistance but led to a paradoxical transcriptional response in rice. While downstream effector-triggered genes (*Xa10* and *xa13*) were induced post-infection, the primary pattern-recognition receptor gene *Xa21* expression was suppressed. Suggesting that suppression is likely caused by a phage-mediated interference mechanism, where the phage physically masks flagellin epitopes, preventing their recognition by plant receptors, and thereby blocking the initiation of PAMP-triggered immunity. These findings have critical implications for developing biocontrol strategies, demonstrating that combining phages and elicitors can have antagonistic, rather than synergistic, effects. It underscores the necessity of understanding the specific receptors used by a bacteriophage before deploying them in a biocontrol cocktail. Future research should focus on validating this masking hypothesis, for instance, by using phages that do not use flagella as a receptor or by employing advanced imaging to visualize phage-flagellin interactions. A deeper understanding of these molecular interactions is essential for designing more effective and predictable biocontrol solutions for plant diseases.

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Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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