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ORIGINAL ARTICLE

Flg22-facilitated PGPR colonization in root tips and control of root rot

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Abstract

Plant root border cells (RBCs) prevent the colonization of plant growth-promoting rhizobacteria (PGPR) at the root tip, rendering the PGPR unable to effectively control pathogens infecting the root tip. In this study, we engineered four strains of *Pseudomonas* sp. UW4, a typical PGPR strain, each carrying an enhanced green fluorescent protein (EGFP)-expressing plasmid. The UW4E strain harboured only the plasmid, whereas the UW4E-flg22 strain expressed a secreted EGFP-Flg22 fusion protein, the UW4E-Flg(flg22) strain expressed a non-secreted Flg22, and the UW4E-flg22-D strain expressed a secreted Flg22-DNase fusion protein. UW4E-flg22 and UW4E-flg22-D, which secreted Flg22, induced an immune response in wheat RBCs and colonized wheat root tips, whereas the other strains, which did not secrete Flg22, failed to elicit this response and did not colonize wheat root tips. The immune response revealed that wheat RBCs synthesized mucilage, extracellular DNA, and reactive oxygen species. Furthermore, the Flg22-secreting strains showed a 33.8%–93.8% higher colonization of wheat root tips and reduced the root rot incidence caused by *Rhizoctonia solani* and *Fusarium pseudograminearum* by 24.6%–35.7% compared to the non-Flg22-secreting strains in pot trials. There was a negative correlation between the incidence of wheat root rot and colonization of wheat root tips by these strains. In contrast, wheat root length and dry weight were positively correlated with the colonization of wheat root tips by these strains. These results demonstrate that engineered secretion of Flg22 by PGPR is an effective strategy for controlling root rot and improving plant growth.

KEYWORDS
colonization in root tips, Flg22, immune response, plant root border cells, root rot

1 | INTRODUCTION

Root rot occurs commonly in crops, vegetables, flowers, medicinal herbs, fruit trees, and forests. Root rot pathogens can infect plants throughout their growth period and cause significant annual damage. It is primarily caused by soilborne pathogenic oomycetes and fungi, including *Pythium*, *Phytophthora*, *Rhizoctonia*, and *Fusarium*. Pathogenic mycelia invade the root tip and subsequently infect the entire root and stem base, resulting in brownish-black discolouration and rot of the root tips, rotting and black spots at the bases of the roots and stems, yellowing and wilting of the leaves, and plant damping-off (Miller et al., 2008; Rodríguez-Gálvez & Mendgen, 1995).

Wheat root rot is primarily caused by the soilborne pathogens *Pythium*, *Phytophthora*, *Rhizoctonia*, *Fusarium*, and *Gaeumannomyces*. In recent years, *Fusarium culmorum*, *Fusarium pseudograminearum*, and *Fusarium graminearum* emerged as the main culprits of Fusarium crown rot (FCR) of wheat in China. These three fungal pathogens are responsible for Fusarium root rot and Fusarium head blight in wheat (Kazan & Gardiner, 2018; Obanor & Chakraborty, 2014; Scherm et al., 2013). Among these, *F. culmorum* and *F. graminearum* first infect the roots and then cause other symptoms (Beccari et al., 2011; Voss-Fels et al., 2018; Wang et al., 2015). The exact site of *F. pseudograminearum* infection in wheat has yet to be determined, and the exact site of infection may depend on the distribution of the pathogen in the soil.

Biological control is widely recognized as an efficient and favoured method for effectively managing root rot. However, there are no reports of biocontrol strains capable of colonizing root tips and competing with root rot pathogens for nutrients or ecological niches. A lack of beneficial or pathogenic microbial colonization at the plant root tip has long been observed. Microbial colonization at the root tip is significantly lower than in other parts of the root (Curl & Truelove, 1986; Dong et al., 2020; Lagopodi et al., 2002; Nagler et al., 2018; Zentmyer, 1961). The root tip, which is 1–2 mm from the root apex and comprises the root cap meristem and the root cap, is surrounded by root border cells (RBCs) produced by the root cap meristem. In the absence of RBCs, microorganisms colonize and infect root tips. Thus, RBCs prevent microbial colonization and infection of the root tips (Gunawardena & Hawes, 2002; Humphris et al., 2005).

Multiple mechanisms that use RBCs to prevent pathogen colonization of root tips have been discovered. RBCs act as physical and chemical barriers to pathogens (Cannesan et al., 2011; Hawes & Pueppke, 1987). The root extracellular trap (RET), formed by mucilage secretion by RBCs, can clear pathogens. Extracellular DNA (exDNA) and associated histone H4 are essential for RET-mediated killing of pathogens. Reactive oxygen species (ROS) and nitric oxide (NO) synthesized by RBCs are important components that inhibit pathogens (Park et al., 2019; Tran et al., 2016). RBCs exhibit chemotactic attraction to certain pathogens, trap and kill them, and stimulate fungal spore germination, followed by clearance to prevent mycelial invasion (Hawes et al., 2016; Ropitaux et al., 2020; Zhao et al., 2000). Pathogens or their elicitors, such as the bacterial flagellin Flg22, increase the number of RBCs and induce the secretion of RBC metabolites. For example, the concentration of phytosulfokines in the root tip is significantly higher than in susceptible root elongation zones (Cannesan et al., 2011), and there is an increase in the production of ROS and mucilage (Plancot et al., 2013; Tran et al., 2016). Proteins secreted by RBCs, including proteinases, peroxidases, histone H4, and other defence-related proteins, may be involved in the resistance to pathogen invasion at the root tips (Wen et al., 2007).

However, when rainfall or irrigation leads to excessive soil moisture, RBCs disperse from root tips, rendering them vulnerable to pathogen invasion. Numerous pathogens secrete DNases, which degrade the exDNA generated by RBCs, facilitating root tip colonization and resulting in disease development (Park et al., 2019; Tran et al., 2016; Wen et al., 2009). Consequently, an effective biocontrol strategy involves the use of plant growth-promoting rhizobacteria (PGPR) to compete for ecological niches (root tips) and nutrients (Kamilova et al., 2005).

The mechanisms used by RBCs to resist pathogen colonization in root tips may not apply to PGPR. *Agrobacterium tumefaciens*, the causal agent of crown galls, shows chemotaxis toward pea RBCs and binds to them; however, its toxigenic mutants lose this ability (Hawes & Pueppke, 1989). Some nonpathogenic bacteria and fungi show neither chemotaxis toward RBCs nor induction of RET secretion (Gunawardena & Hawes, 2002; Tran et al., 2016). Nonpathogenic bacteria utilize the mucilage produced by RBCs as their sole carbon source but exhibit weak growth (Knee et al., 2001). Alternatively, some nonpathogenic bacteria do not utilize mucilage but the mucilage induces spore formation (Gochnauer et al., 1990).

Flg22 is a 22-amino acid peptide originally identified in the flagellin protein of *Pseudomonas aeruginosa*. It is highly conserved among plant-pathogenic bacteria and acts as an elicitor, triggering immune responses in aerial plant tissues such as ROS bursts, ethylene production, and alkalinization responses (Felix et al., 1999; Wyrsch et al., 2015). Flg22 is the most extensively studied pathogen-associated molecular pattern (PAMP) (Jin et al., 2022) and microbe-associated molecular pattern (MAMP) (Aslam et al., 2009). Moreover, Flg22 triggers immune responses in RBCs, similar to those observed in aerial tissues, and alters the distribution of extensin epitopes in the cell walls, leading to callose deposition in RBCs (Plancot et al., 2013). Additionally, it induces RBCs to produce RETs and exDNA (Tran et al., 2016). However, Flg22, located within the flagella, cannot initiate plant immune responses. Plants secrete glycosidases, such as BGAL1, to hydrolyse the glycosyl groups of flagellin proteins. BGAL1 is induced under hypoxia, salt stress, and pathogen attack (Schmid et al., 2005). Subsequently, proteinases secreted by plants cleave and release Flg22 from flagellin, and the resulting Flg22 triggers a plant immune response (Sanguankiattichai et al., 2022).

The plant pathogen *Ralstonia solanacearum* induces pea RBCs to produce RETs, and its extracellular DNase degrades exDNA of RETs and promotes root tip infection. Notably, the N-terminal *R. solanacearum* flagellin sequence, which corresponds to Flg22, differs from that of Flg22 (Tran et al., 2016). The elicitor of *R. solanacearum*

may be a type III secreted effector, RipAC (Yu et al., 2022). Accordingly, we hypothesized that engineered expression of Flg22 from PGPR would induce immune responses in RBCs and that extracellular DNase would facilitate the evasion of RETs, thereby enabling the colonization of the root tips and competition for ecological niches and nutrients with root rot pathogens, enhancing the effectiveness of root rot control.

Pseudomonas sp. UW4 is a typical PGPR strain isolated from the rhizosphere of reeds that produces 1-aminocyclopropane-1-carboxylic acid deaminase, which enhances plant resistance to biotic and abiotic stresses and promotes growth (Cheng et al., 2007; Glick et al., 1995; Nascimento et al., 2013; Shah et al., 1998). The sequence corresponding to Flg22 in the flagellin protein (GenBank accession number: AFY21079.1) differed from Flg22 by only three amino acids (Figure S1). We constructed engineered UW4 strains expressing Flg22 to control wheat root rot, overcome the defence of RBCs against PGPR, and enable PGPR colonization of root tips.

2 | RESULTS

2.1 | Flg22 promoted colonization by *Pseudomonas* sp. UW4 in wheat root tips

The fluorescence-expressing plasmid pBBR1MCS2-pAmp-EGFP was introduced into UW4 by triparental mating to generate the fluorescent strain UW4E to facilitate the observation of colonization by *Pseudomonas* sp. UW4 in the rhizosphere (Figures S5 and S6).

The UW4 and UW4E culture supernatants hydrolysed the plasmid DNA (Figure S7a). The extracellular DNase activities were 10.18 ± 0.13 U/mL and 10.17 ± 0.07 U/mL for the two strains, respectively, with no significant difference (Figure S7b). UW4, UW4E, and Flg22_UW4 did not trigger mucilage, exDNA, or ROS production in wheat root tips or RBCs when co-incubated with wheat root tips and RBCs. In contrast, Flg22 triggered immune responses in wheat root tips and RBCs (Figure 1a).

UW4E was inoculated onto wheat roots and co-cultured in growth pouches for 7 days. Confocal laser scanning microscopy revealed that UW4E could not colonize the root tips but only established colonization in the root elongation zone. However, co-inoculation of UW4E with Flg22 (1 μM) onto wheat roots resulted in successful colonization of the root tips and the root elongation zone (Figure 1b). Quantitative determination of colonization size was conducted for UW4, UW4E, and UW4E supplemented with Flg22 in 1 cm wheat root tips. No difference was observed between UW4 and UW4E, whereas UW4E supplemented with Flg22 showed a 67.3%–69.1% increase compared to UW4 and UW4E. There were no differences in wheat root length or dry weight between wheat roots inoculated with UW4 and UW4E. However, when wheat roots were inoculated with UW4E supplemented with Flg22, root length and root dry weight increased by 12.5%–16.9% and 12.4%–17.0%, respectively, compared with those of UW4 and UW4E (Figure 1c).

2.2 | Construction of engineered strains of *Pseudomonas* sp. UW4 for Flg22 expression

To further explore the use of Flg22 in promoting root-tip colonization by *Pseudomonas* sp. UW4, three engineered UW4 strains were generated to express Flg22. These strains were UW4E-flg22, which secreted the fusion protein EGFP-Flg22 by harbouring the plasmid pBBR1MCS2-pAmp-flg22-EGFP; UW4E-Flg(flg22), where the *flg22* sequence in the UW4 genome was substituted with *flg22* from *P. aeruginosa*; and UW4E-flg22-D, which secreted a fusion protein of Flg22-DNase by *flg22* from *P. aeruginosa* knocked in upstream of the DNase gene on the UW4E chromosome. Colony PCR validation results for the three strains are shown in Figure S6. Green fluorescent proteins expressed in the three strains were observed by confocal laser scanning microscopy (Figure S8). No differences were observed in the growth of UW4, UW4E, or the three strains in Luria Bertani (LB) medium (Figure S9).

The culture supernatants of the three strains were incubated with plasmid DNA and analysed by agarose gel electrophoresis, as UW4 and UW4E can degrade DNA (Figure S7a). The extracellular DNase activity of the five strains was similar (Figure S7b). Western blot analysis demonstrated the secretion of the histidine-tagged Flg22-EGFP fusion protein by UW4E-flg22 cells (Figure S10a). Flg22-DNase secreted by UW4E-flg22-D and its DNase activity was confirmed by native PAGE (Figure S10b), after which the gel was incubated with plasmid DNA (Figure S10c). However, no additional secreted proteins were observed in UW4E-Flg(flg22) compared to UW4E (results not shown).

2.3 | Interaction of *Pseudomonas* sp. UW4 engineered strains expressing Flg22 with wheat RBCs

Pseudomonas sp. UW4E-flg22 was based on the plasmid expression and secretion of the free peptide Flg22. UW4E-Flg(flg22) was based on the genomic expression of Flg22 located in flagellin. UW4E-flg22-D was based on the genomic expression and secretion of the Flg22 fusion protein with DNase. UW4E-flg22, UW4E-Flg(flg22), and UW4E-flg22-D cells were incubated with wheat root tips and RBCs to investigate whether these strains induced immune responses in wheat RBCs. UW4E-flg22 and UW4E-flg22-D induced RBC-produced mucilage, exDNA, and ROS, whereas UW4E-Flg(flg22) did not (Figure 2a).

2.4 | Colonization of wheat root tips by engineered *Pseudomonas* sp. UW4 strains expressing Flg22 and promotion of wheat seedling growth

To test engineered *Pseudomonas* sp. UW4 strains expressing Flg22 to colonize wheat root tips and promote wheat seedling growth, after the inoculation of wheat roots with *Pseudomonas* sp. UW4 strains and

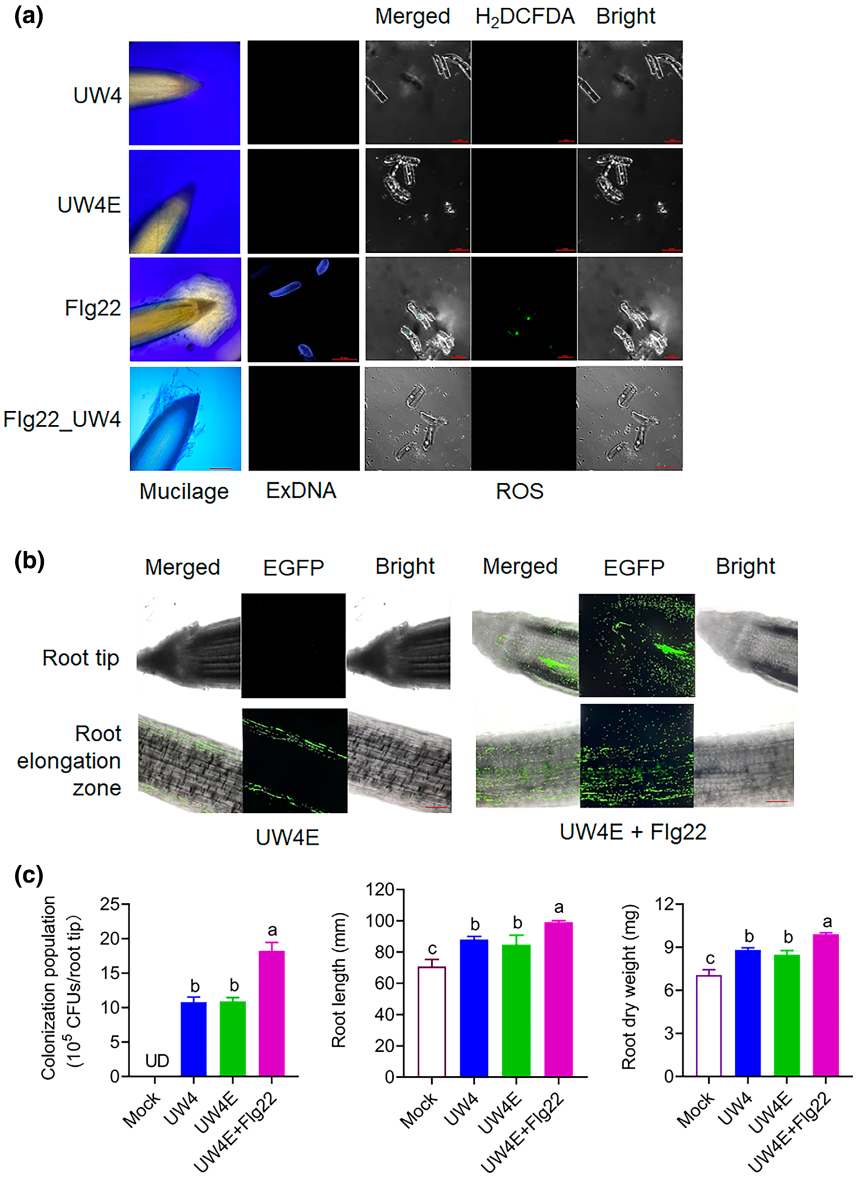

FIGURE 1 Flg22 triggered the immune response in wheat root border cells (RBCs) and promoted the colonization of wheat root tips by *Pseudomonas* sp. UW4. (a) Interaction of Flg22, Flg22_UW4 and *Pseudomonas* sp. UW4 with wheat RBCs. Wheat root tips or RBCs obtained from 3-day-old seedlings were co-incubated with bacterial broth or peptides at room temperature. Mucilage was observed by optical microscope at 10× magnification and taken pictures from the beginning of the co-incubation. exDNA and reactive oxygen species (ROS) were observed by confocal laser scanning microscopy after co-incubation for 20 and 15 min. Scale bars are 100 μm. (b) Confocal laser scanning microscopy observing the influence of Flg22 on the wheat root tip colonization by *Pseudomonas* sp. UW4. Wheat roots were inoculated with bacterial suspension or bacterial suspension supplemented with 1 μM Flg22, and then incubated for 7 days in growth pouches at 28°C. Colonization of wheat root tip and elongation zone by *Pseudomonas* sp. UW4 was observed by confocal laser scanning microscopy. Scale bars are 100 μm. (c) Quantitative analysis of the influence of Flg22 on the wheat root tip colonization and the promotion of wheat seedling growth by *Pseudomonas* sp. UW4E. Colonization of 1 cm root tips by UW4 was counted by the agar dilution plate count method using Luria Bertani agar containing 100 μg/mL ampicillin. Data are presented as mean ± *SD* (from three independent replicate experiments, with six seedlings per replicate). Bars with different letters were significantly different with $p < 0.05$ based on one-way analysis of variance.

incubation in growth pouches for 7 days, confocal laser scanning microscopy revealed that UW4E-flg22 and UW4E-flg22-D colonized the root tips and elongation zone, whereas UW4E and UW4E-Flg(flg22) colonized the root elongation zone but not the root tips (Figure 2b). Quantitative analysis of the colonization of 1 cm wheat root tips indicated 23.6%–54.2% higher colonization by UW4E-flg22 and UW4E-flg22-D than by UW4, UW4E, and UW4E-Flg(flg22) (Figure 2c). The promotion of wheat root length and dry weight by these five strains displayed trends similar to those of their root tip colonization (Figure 2c) and showed a significant positive correlation ($p < 0.05$).

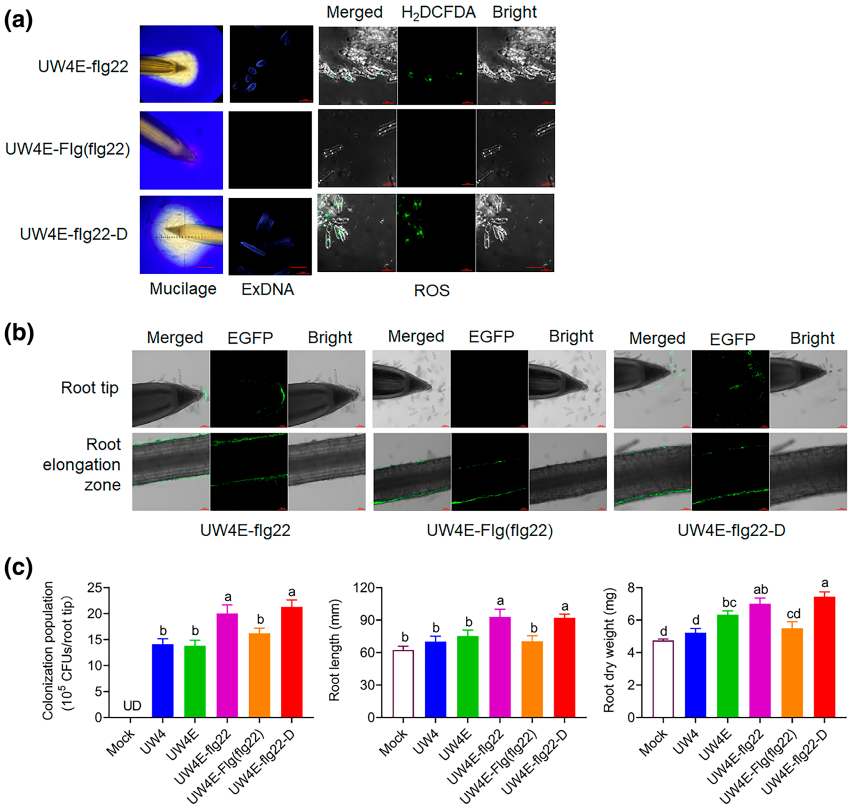

FIGURE 2 Interaction of *Pseudomonas* sp. UW4 engineered strains expressing Flg22 with wheat root border cells (RBCs) and roots. (a) Mucilage, exDNA, and reactive oxygen species (ROS) derived from wheat RBCs induced by *Pseudomonas* sp. UW4 engineered strains expressing Flg22. Wheat root tips or RBCs obtained from 3-day-old seedlings were co-incubated with bacterial broth at room temperature. Mucilage was observed by optical microscope under 10× magnification and pictures were taken from the beginning of the co-incubation. exDNA and ROS were observed by confocal laser scanning microscopy after co-incubation for 20 and 15 min. Scale bars are 100 μm. (b) Wheat root tip colonization by *Pseudomonas* sp. UW4 engineered strains expressing Flg22 observed using confocal laser scanning microscopy. Wheat roots were inoculated with bacterial suspension, and then incubated for 7 days in growth pouches at 28°C. Colonization of wheat root tip and elongation zone by *Pseudomonas* sp. UW4 strains was observed by confocal laser scanning microscopy. Scale bars are 100 μm. (c) Quantitative analysis of the wheat root tip colonization and the promotion of wheat growth by *Pseudomonas* sp. UW4 strains in growth pouches. Colonization of 1 cm root tips by UW4 strains was counted by the agar dilution plate count method using Luria Bertani agar containing 100 μg/mL ampicillin. Data are presented as mean ± *SD* (from three independent replicate experiments, with six seedlings per replicate). Bars with different letters were significantly different with $p < 0.05$ based on one-way analysis of variance.

2.5 | Interaction of fungal root rot pathogens with wheat roots

To detect the two fungal root rot pathogens that secret DNase and induce immune responses in wheat RBCs, the culture supernatants of *R. solani* and *F. pseudograminearum* degraded DNA (Figure S11) with DNase enzyme activities of 4.38 ± 0.17 U/mg protein and 3.48 ± 0.05 U/mg protein, respectively. Incubation of spores with wheat RBCs induced mucilage, exDNA, and ROS production (Figure 3a). Inoculation of wheat roots with spores and incubation in growth pouches for 7 days resulted in root rot incidences of 29% and 21%, respectively, and 26.2%–29.0% and 15.3%–16.7% reductions in root length and root dry weight, respectively, compared with the mock control. The symptoms of wheat root rot include yellowing and decay of root tips. Adding 1.2 U of DNase I to the spore suspension increased root rot incidence by 65.9% and 161.9% and reduced root length and dry weight by 16.0%–19.0% and 11.4%–25.7%, respectively, compared with no enzyme addition (Figure 3b,c). Inoculation of wheat roots with Flg22 (1 μM) and spores of *Rhizoctonia solani* or *F. pseudograminearum* did not result in changes in the root rot incidence, root length, or root dry weight compared to inoculation with the pathogenic spores alone (Figure S16).

2.6 | Fungal root rot control by engineered *Pseudomonas* sp. UW4 strains expressing Flg22 in the pot trial

To evaluate the engineered *Pseudomonas* sp. UW4 strains expressing Flg22 to colonize wheat root tips and control fungal root rot in a non-axenic pot trial, wheat seeds were inoculated with *Pseudomonas* sp. UW4 strains and sown in pots containing *R. solani* or *F. pseudograminearum* spores. After 15 days of cultivation, confocal laser

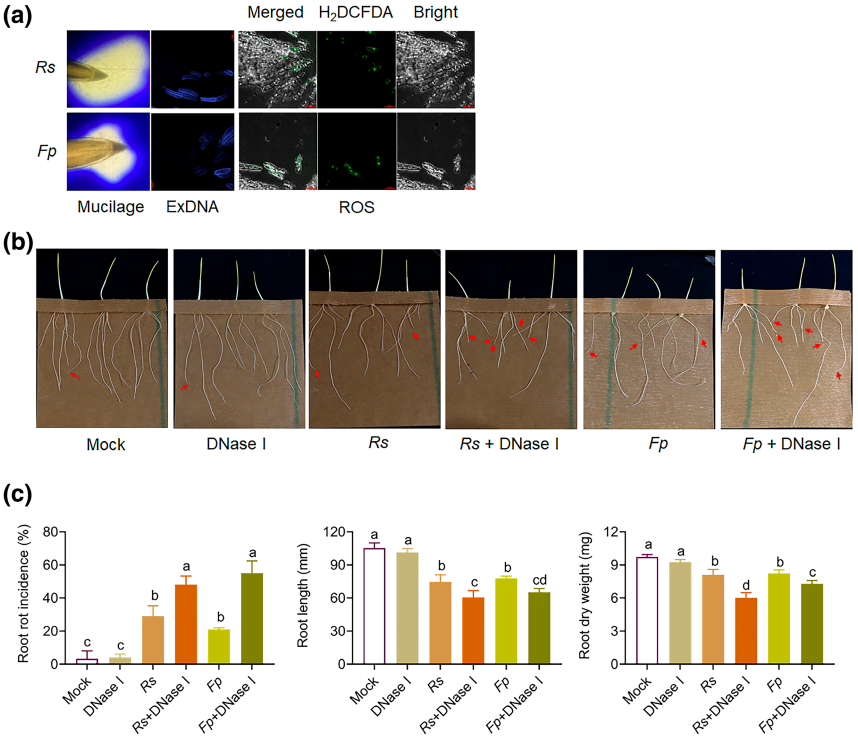

FIGURE 3 Interaction of fungal spores and DNase I with wheat root border cells (RBCs) and roots. (a) Mucilage, exDNA, and reactive oxygen species (ROS) derived from wheat RBCs induced by fungal spores. Wheat root tips or RBCs obtained from 3-day-old seedlings were co-incubated with fungal spore suspension (10^5/mL) at room temperature. Mucilage was observed by optical microscope under 10× magnification and taken pictures from the beginning of the co-incubation. exDNA and ROS were observed by confocal laser scanning microscopy after co-incubation for 20 and 15 min. Scale bars are 100 μm. (b) The occurrence of wheat root rot and the reduction in wheat seedling growth caused by fungal spores and DNase in growth pouches. Wheat roots were inoculated with fungal spore suspension (10^5/mL) or fungal spore suspension supplemented with 1.2 U of DNase I, and then incubated for 7 days in growth pouches at 28°C. Red arrows indicate roots affected by root rot. (c) Quantitative analysis of the influence of inoculation of fungal spores and DNase I on wheat root rot and root growth. *Rs*, *Rhizoctonia solani*; *Fp*, *Fusarium pseudograminearum*. Data are expressed as mean ± *SD* (from three independent replicate experiments, with six seedlings per replicate). Bars with different letters were significantly different with $p < 0.05$ based on one-way analysis of variance.

scanning microscopy revealed that UW4E-flg22 and UW4E-flg22-D colonized the root tips and elongation zone. In contrast, the UW4E and UW4E-Flg(flg22) strains did not colonize the root tips but only the root elongation zone (Figures 4a and 5a). Quantitative determination of colonization of 1 cm root tips showed that UW4E-flg22 and UW4E-flg22-D colonization at the root tips was 33.8%–93.8% higher than that of UW4, UW4E, and UW4E-Flg(flg22) in substrates inoculated with *R. solani*, and the incidence of root rot was reduced by 24.6%–35.7% (Figure 4b; Figure S12). UW4E-flg22 and UW4E-flg22-D colonization of the root tips was 41.5%–70.4% higher in soil inoculated with *F. pseudograminearum* than UW4, UW4E, and UW4E-Flg(flg22), and the root rot incidence was reduced by 24.7%–35.3% (Figure 5b; Figure S13). The five strains showed a significant negative correlation ($p < 0.05$) with their colonization at the root tips and rot incidence, and a significant positive correlation ($p < 0.05$) between root tip colonization and root length and root dry weight of wheat (Figures 4b and 5b). Similar results were obtained with co-inoculation of *Pseudomonas* sp. UW4 strains with spores of *R. solani* and *F. pseudograminearum* were cultivated in the growth pouches (Figures S14 and S15).

3 | DISCUSSION

Methods for detecting microbial colonization of root tips include microscopic observation (Lagopodi et al., 2002; Olivain & Alabouvette, 1999; Smith et al., 1992; Wen et al., 2009; Zentmyer, 1961), plate counting (Kamilova et al., 2005) or quantitative PCR (Dong et al., 2020) to determine the microbial population in roots 0.5–1.0 cm away from the root tip. Confocal laser scanning microscopy showed that GFP-labelled *Bacillus subtilis* NCD-2 did not colonize the root tips of cotton, while high colonization in roots located 0.5 cm away from the root tips was demonstrated using traditional plate counting and quantitative PCR analysis (Dong et al., 2020). This indicated that the microscopic observation accurately revealed the occurrence of microbial colonization in the root tips. In this study, our combined microscopic observations and the plate counting method confirmed that *Pseudomonas* sp. UW4E-flg22 and UW4E-Flg(flg22)-D, which express and secret Flg22, colonize wheat root tips, whereas strains UW4, UW4E, and UW4E-Flg(flg22), which do not express or secrete Flg22, are unable to colonize wheat root tips.

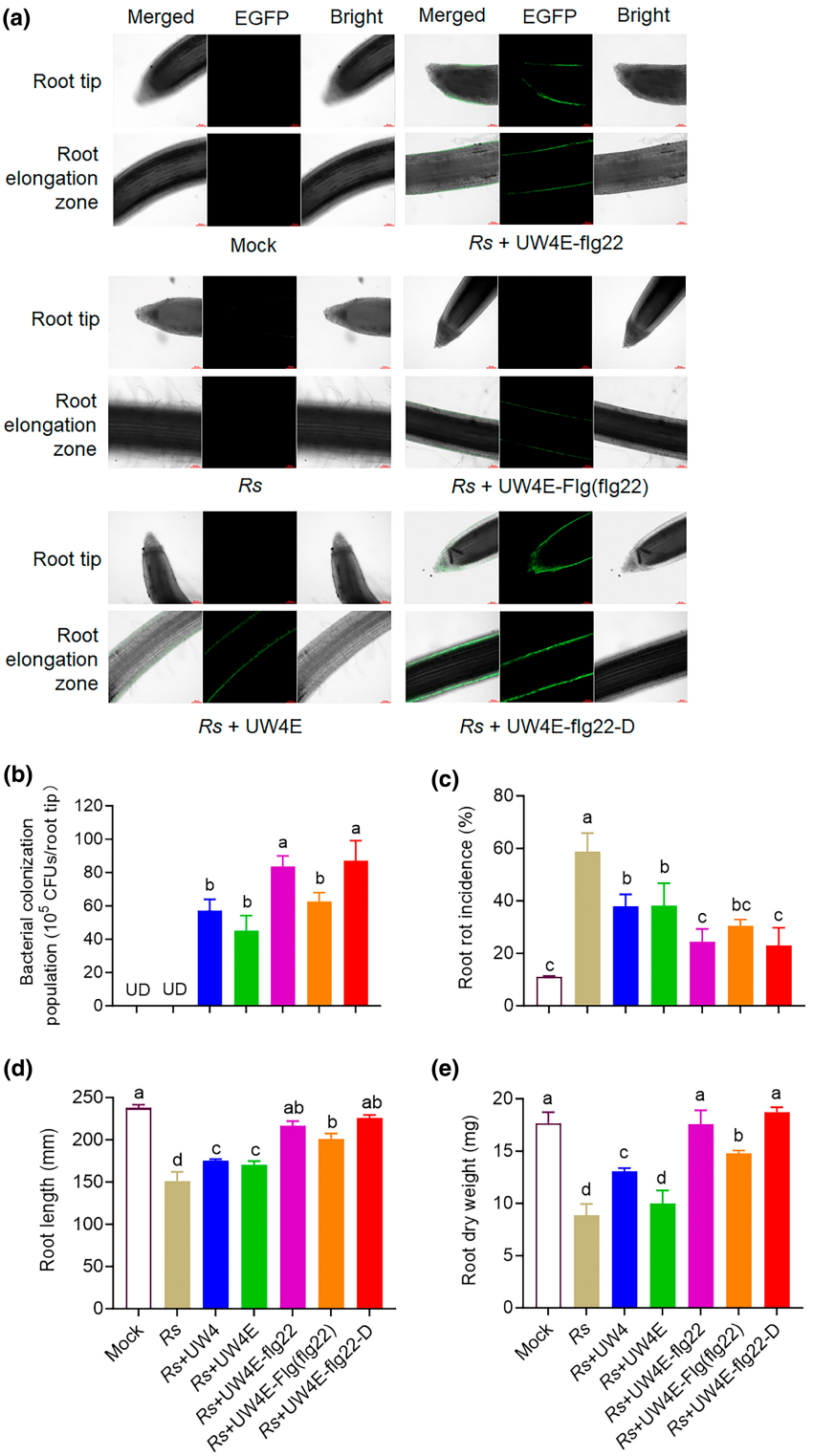

FIGURE 4 Influence of *Pseudomonas* sp. UW4 strains co-inoculated with *Rhizoctonia solani* spores on wheat root tip colonization by *Pseudomonas* sp. UW4 strains and wheat root growth in pot trial. (a) Confocal laser scanning microscopy observation of wheat root colonization by *Pseudomonas* sp. UW4 strains co-inoculated with *R. solani* spores onto wheat roots in pot trial. Colonization of wheat root tip and elongation zone by *Pseudomonas* sp. UW4 strains was observed by confocal laser scanning microscopy. Scale bars are 100 μm. (b) Colonization population of *Pseudomonas* sp. UW4 strains in wheat tips. After incubation for 15 days, colonization of 1 cm root tips by UW4 strains was counted by the agar dilution plate count method using Luria Bertani agar containing 100 μg/mL ampicillin. (c) Wheat root rot incidence. Following a 15-day incubation, the number of roots affected by root rot and the number of healthy roots were counted, and the percentage of roots affected by root rot relative to the total number of roots was calculated as the root rot incidence. (d) Wheat root length after incubation for 15 days. (e) Wheat root dry weight after incubation for 15 days. *Rs*, *R. solani*; UD, undeterminable. Data are expressed as mean ± *SD* (from three independent replicate experiments, with six seedlings per replicate). Bars with different letters were significantly different with $p < 0.05$ based on one-way analysis of variance.

Various attempts have been made to improve PGPR that are capable of colonizing root tips in competition with root rot pathogens for niches and nutrients. Cyclic enrichment has been used to screen and identify strains capable of efficiently colonizing the root tip, degrading contaminants (Kuiper et al., 2001), and effectively controlling root rot (Kamilova et al., 2005). The colonization ability of rhizobacterial strains at root tips (1–2 cm) can be increased 8–40-fold by introducing the colonization gene *sss* (site-specific recombinase) (Dekkers et al., 2000). Deficiency in the chemotaxis-associated genes *gac* or *kinB sadB wspR* of *Pseudomonas fluorescens* F113rif (F113) results in increased colonization of root tips (last centimetre of the main root) and improved effectiveness in controlling root rot (Barahona et al., 2011; Martínez-Granero et al., 2006). However, the aforementioned studies assessed the colonization of these strains at

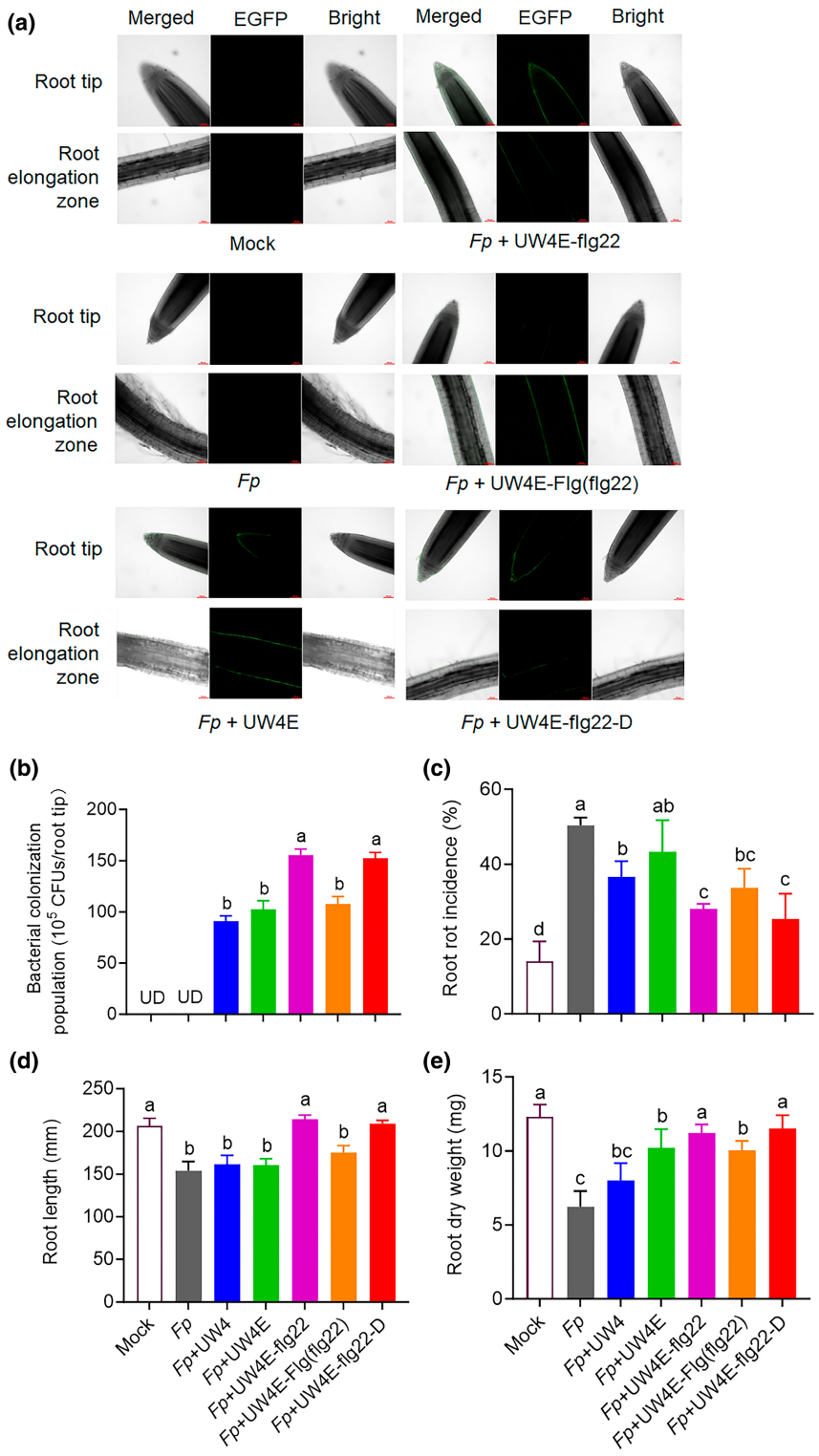

FIGURE 5 Influence of *Pseudomonas* sp. UW4 strains co-inoculated with *Fusarium pseudograminearum* spores on wheat root tip colonization by *Pseudomonas* sp. UW4 strains and wheat root growth in pot trial. (a) Confocal laser scanning microscopy observation of wheat root tip colonization by *Pseudomonas* sp. UW4 strains co-inoculated with *F. pseudograminearum* spores onto wheat roots in pot trial. Colonization of wheat root tip and elongation zone by *Pseudomonas* sp. UW4 strains was observed by confocal laser scanning microscopy. Scale bars are 100 μm. (b) Colonization population of *Pseudomonas* sp. UW4 strains in wheat tips. After incubation for 15 days, colonization of 1 cm root tips by UW4 strains was counted by the agar dilution plate count method using Luria Bertani agar containing 100 μg/mL ampicillin. (c) Wheat root rot incidence. Following a 15-day incubation, the number of roots affected by root rot and the number of healthy roots were counted, and the percentage of roots affected by root rot relative to the total number of roots was calculated as the root rot incidence. (d) Wheat root length after incubation for 15 days. (e) Wheat root dry weight after incubation for 15 days. *Fp*, *F. pseudograminearum*; UD, undeterminable. Data are expressed as mean ± *SD* (from three independent replicate experiments, with six seedlings per replicate). Bars with different letters were significantly different with $p < 0.05$ based on one-way analysis of variance.

root tips by conducting plate counts on a 1–2 cm section of the root tips and could not confirm that these strains could overcome the barrier of RBCs to colonize the root tips.

The root rot bacterium *R. solanacearum* (Tran et al., 2016) and the fungi *Nectria haematococca* (Wen et al., 2009) and *Cochliobolus heterostrophus* (Park et al., 2019) induce host RBCs to produce RETs and secrete extracellular DNases, or are treated with DNase I to degrade the exDNA in RETs, thereby infecting the root tips of their hosts. However, the mechanisms through which they induce immune responses in RBCs remain unclear. In this study, we found that *Pseudomonas* sp. UW4 secreted extracellular DNase, whereas Flg22_UW4 did not induce an immune response in wheat RBCs. Supplementation with Flg22 promoted UW4 colonization of wheat root tips and enhanced the plant-growth-promoting effects of UW4. Strains UW4E-flg22 and UW4E-flg22-D, which express and secrete Flg22, induced mucilage, exDNA, and ROS synthesis in

wheat RBCs and colonized root tips, as observed by confocal laser scanning microscopy. However, the non-secretory expression of the Flg22 strain UW4E-Flg(flg22) did not induce the synthesis of mucilage, exDNA, or ROS in wheat RBCs and did not colonize the root tips. Correspondingly, the colonization of wheat root tips by *Pseudomonas* sp. UW4 strains expressing and secreting Flg22 and their plant growth-promoting effects on wheat seedlings were dramatically higher than those of the strains with non-secretory Flg22 expression.

Flg22 is a well-characterized bacterial MAMP sensed by the plant cell surface FLAGELLIN SENSING 2 (FLS2) and elicits a MAMP-triggered immune response (Chinchilla et al., 2006; Gómez-Gómez & Boller, 2000). FLS2 is a plant pattern-recognition receptor (PRR). Each amino acid in Flg22 induces a different intensity of interaction with *Arabidopsis thaliana* FLS2. Changing the seventh amino acid in Flg22, S to L, as in Flg22_UW4, results in a binding free energy loss of 0.31 kcal/mol with FLS2 (Wei et al., 2020). Additionally, the immune response induced by Flg22 depends on the plant species and Flg22 dosage. Flg22 recognition specificities are widespread in higher plants (Saijo et al., 2018), but not in monocotyledonous *Brachypodium distachyon* cells (Murakami et al., 2022). FLS2 recognition of Flg22 is highly conserved in plants (Hudson et al., 2024). However, whether plants recognize Flg22 and the strength of the response are probably related to the function and abundance of FLS2 (Bauer et al., 2001; Vetter et al., 2012; Zhang et al., 2017). The dosage of Flg22 also affects the immune response of plants to Flg22. For example, 200 nM Flg22 does not induce H_2O_2 production in rice cells (Murakami et al., 2022), whereas 500 nM Flg22 does induce H_2O_2 production in rice cells (Takai et al., 2008). Flg22-1 from the plant growth-promoting bacterium *Pseudomonas kilonensis* F113, which differs from Flg22_UW4 only in the first amino acid, induces a hypersensitive response in *Nicotiana benthamiana* leaves (Luo et al., 2023), whereas Flg22_UW4 does not induce an immune response in wheat RBCs, suggesting that both amino acid differences and plant species may be involved.

The spores of *R. solani*, a typical root rot pathogen, induced the synthesis of mucilage, exDNA, and ROS in wheat RBCs, secreted extracellular DNase, and infected wheat root tips in this study. Supplementation with DNase I increased *R. solani* root rot incidence. *F. pseudograminearum* is a crown rot pathogen in wheat. However, its spores also induce immune responses in wheat RBCs, secrete extracellular DNase, infect wheat root tips and supplementation with DNase I increases its root rot incidence, similar to that of *R. solani*. Thus, *F. pseudograminearum* is a root rot pathogen. Furthermore, the addition of Flg22 did not increase the pathogenicity of the two pathogens. Previous studies also found that the pathogenic fungi *N. haematococca* (Gunawardena et al., 2005; Wen et al., 2007, 2009) and *F. culmorum* (Jaroszuk-Ściseł et al., 2009) induce mucilage generation in pea and rye RBCs. None of these fungi contain Flg22 and the mechanisms by which they induce immune responses in RBCs remain unknown. Several saccharide and protein elicitors have been identified as fungal-secreted elicitors recognized by PRRs to induce immune responses. Examples include lipochito-oligosaccharides (LCOs) from arbuscular mycorrhizal fungi (AMF) (Maillet et al., 2011), fungal chitin/chitosan (Cao et al., 2014; Erwig et al., 2017), RSAG8_07159 and FGSG_11487 from *R. solani* (Anderson et al., 2017), and Fg02685 from *F. graminearum* (Xu et al., 2022). However, whether these fungal elicitors induce immune responses in RBCs has not yet been reported. In this study, *R. solani* and *F. pseudograminearum* induced immune responses in RBCs similar to Flg22. Supplementation with Flg22 did not increase the pathogenicity of these two pathogens, suggesting that the elicitors secreted by them share similarities with Flg22.

When *Pseudomonas* sp. UW4 strains were co-inoculated with *R. solani* and *F. pseudograminearum* in wheat roots, Flg22-secreting UW4 strains colonized wheat root tips and exhibited high efficacy in controlling root rot and promoting wheat growth in axenic growth pouches and non-axenic pot cultivation. Conversely, the UW4 strains that did not secrete Flg22 failed to colonize wheat root tips and exhibited considerably lower efficacy in controlling root rot and promoting wheat growth than the UW4 strains that secreted Flg22. There was a significant negative correlation between the incidence of wheat root rot and the colonization of the UW4 strains on 1 cm root tips. In contrast, the growth-promoting effects on wheat seedlings were significantly positively correlated with the colonization of UW4 strains on 1 cm root tips.

Extracellular DNase activity, induction of immune response in wheat RBCs, colonization of wheat root tips, and control of wheat root rot did not differ between the Flg22-secreting strains of *Pseudomonas* sp. UW4E-flg22 and UW4E-flg22-D. The difference between the strains was that UW4E-flg22 had a plasmid-based expression of Flg22 while UW4E-flg22-D was based on genomic expression and secretion of the Flg22 fusion protein with DNase. Considering the environmental safety and stability of genetically modified organisms (GMOs), UW4E-flg22-D is an appropriate choice, and elimination of the fluorescence-expressing plasmid is recommended to obtain UW4-flg22-D.

The mechanism by which *Pseudomonas* sp. UW4E-flg22 and UW4E-flg22-D successfully colonized the root tips is probably because their secreted Flg22 induced an immune response in RBCs to produce RET, while the extracellular DNase produced by the strains degraded the exDNA in RET, thereby overcoming the barrier of RET. Furthermore, these strains exhibited no virulence in the induction of other plant defence responses. Conversely, high Flg22 expression in bacterial pathogens may increase disease risk. *R. solanacearum* induces pea RBCs to produce RET and release exDNA. However, approximately 25% of the bacterial population is still killed by RET because the degradation of exDNA by the extracellular DNase secreted by *R. solanacearum* is not sufficient to release it from RET, and only supplemental DNase can increase its infection of the root tips (Tran et al., 2016). Plant defence responses are induced by the expression of other virulence genes that interfere with pathogen infection. Therefore, pathogens use multiple methods to overcome plant flagellin pattern recognition and to evade immune responses. For example, Flg22 of *R. solanacearum* is mutated and cannot induce an immune response in RBCs but is induced by another elicitor

(Yu et al., 2022). *Pseudomonas syringae* downregulates flagellin expression (Bao et al., 2020). Thus, high Flg22 expression in bacterial pathogens may not increase the risk of disease.

In the present study, we engineered PGPR strains of *Pseudomonas* sp. UW4E-flg22 and UW4E-flg22-D, which secreted the expressed bacterial PAMP Flg22 and improved the ability of PGPR to control root rot. These strains possess natural extracellular DNase activity, secrete Flg22 to induce immune responses in wheat RBCs to produce RETs, and use secreted extracellular DNases to degrade the exDNA in RETs to cross the RBC barrier to colonize root tips and biocontrol root rot. These strains exhibited 33.8%–93.8% higher colonization on wheat root tips (1 cm) compared to the UW4 strains that did not secrete Flg22 and reduced the root rot incidence caused by *R. solani* and *F. pseudograminearum* by 24.6%–35.7% compared to the UW4 strains that did not secrete Flg22 in the pot trial. There was a negative correlation between the incidence of wheat root rot and the colonization of the root tips by these strains. In contrast, wheat root length and dry weight positively correlated with the colonization of these strains at the root tips. These results demonstrate that the engineered secretion of Flg22 by PGPR is an effective strategy to compete for the root tip niche and nutrition, effectively controlling root rot and promoting plant growth.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plasmids, peptides, strains, and plants

The plasmids, strains, and plants used in this study are listed in Table S1. Flg22 from *P. aeruginosa* and Flg22_UW4 from *Pseudomonas* sp. UW4 were synthesized by BGI Tech (Beijing, China). *Pseudomonas* sp. UW4 was donated by Professor Bernard R. Glick, University of Waterloo (Waterloo, ON, Canada). *R. solani* and *F. pseudograminearum* were generous gifts from Professor Hongxia Yuan, Henan Agricultural University (Zhengzhou, China).

4.2 | Microbial cultivation

Escherichia coli and *Pseudomonas* sp. UW4 were cultivated on LB agar plates at 37°C and 30°C and in shake flasks of LB medium at 37°C and 30°C at 220 rpm, respectively. The pathogenic fungi were grown on plates with potato dextrose agar (PDA) at 25°C and in shake flasks of potato dextrose (PD) medium at 25°C at 150 rpm.

4.3 | Vector construction

To construct a vector that expressed and secreted the Flg22-EGFP fusion protein, the DNA fragment flg22-EGFP was synthesized by BGI Tech (Beijing, China). The fragment had the XhoI and BamHI sites on the two ends, the ampicillin resistance gene promoter sequence, and the sequences encoding Flg22 of *P. syringae* (also acting as a signal peptide sequence), EGFP and a 6×His tag (Figure S2). The pAmp-EGFP fragment in pBBR1MCS2-pAmp-EGFP was replaced with the synthesized flg22-EGFP fragment by enzyme digestion and enzyme ligation, resulting in the plasmid pBBR1MCS2-pAmp-flg22-EGFP, which expressed and secreted Flg22-EGFP.

To construct a homologous recombination plasmid for replacing the non-*flg22* on the UW4 chromosome with *flg22*, the DNA fragment Flg(flg22) was synthesized by BGI Tech. The DNA fragment carried a 761 bp left arm homologous to the upstream sequence of the *non-flg22* gene on the UW4 chromosome, a *flg22* sequence, and a 699 bp right arm homologous to the sequence downstream of *non-flg22* on the UW4 chromosome (Figure S3). The fragment was ligated with suicide plasmid pEX18Gm by using the Seamless Cloning Kit (Beyotime) to obtain the resulting plasmid pEX18Gm-flg22.

To generate an additional homologous recombination plasmid for knocking *flg22* into the sequence upstream of the DNase gene on the UW4 chromosome, another DNA fragment, flg22-DNase, was synthesized by BGI Tech. The DNA fragment contained a 684 bp left arm homologous to the sequence upstream of the DNase gene signal peptide on the UW4 chromosome, *flg22*, a linker sequence, and a 951 bp right arm homologous to the sequence downstream of the DNase gene signal peptide on the UW4 chromosome (Figure S4). The fragment was ligated with suicide plasmid pEX18Gm by employing the Seamless Cloning Kit (Beyotime) to obtain the plasmid pEX18Gm-flg22-DNase.

4.4 | Triparental mating

pBBR1MCS2-pAmp-EGFP and pBBR1MCS2-pAmp-flg22-EGFP were introduced into *Pseudomonas* sp. UW4 by using the triparental mating method (Winstanley et al., 1989). The donor strains were *E. coli* DH5α strains harbouring pBBR1MCS2-pAmp-EGFP and pBBR1MCS2-pAmp-flg22-EGFP, and the helper strains were both *E. coli* JM101 (pRK2013). The generated transformants were named *Pseudomonas* sp. UW4E and UW4E-flg22. The transformants were verified by colony PCR and confocal laser scanning microscopy observation. The primer pair used in the colony PCR was P1/P2 flanking the EGFP gene to verify UW4E. The primer pair used in the colony PCR was P3/P4 flanking the *flg22* gene to verify UW4E-flg22 (Table S2).

4.5 | Markerless genetic replacement

Markerless genetic replacement was conducted using a standard method for two-step allelic exchange as described by Schweizer and Hoang (1995) to generate the engineered strains *Pseudomonas* sp. UW4E-Flg(flg22) and UW4E-flg22-D. Briefly, *E. coli* S17-1 strains carrying pEX18Gm-flg22 and pEX18Gm-flg22-DNase were co-incubated with *Pseudomonas* sp. UW4E on LB plates. Single-crossover colonies in which plasmid integration into the chromosome of UW4E had occurred were obtained by growth on LB agar

supplemented with gentamicin (25 μg/mL) and ampicillin (100 μg/mL). After the single-crossover colonies were spread on LB plates containing 20% sucrose and ampicillin (100 μg/mL) to induce a second crossover event, the growing colonies were confirmed by colony PCR as the engineered strains. The primer pair used for colony PCR verification of UW4E-Flg(flg22) was P5/P6 flanking the Flg(flg22) replacement fragment. The primer pair used for colony PCR verification of UW4E-flg22-D was P7/P8 flanking the flg22-DNase replacement fragment (Table S2).

4.6 | DNase activity assays

The crude enzyme solution was prepared by collecting the supernatant of *Pseudomonas* sp. UW4 shake flask culture for 12 h or the 10-fold concentrated ultrafiltered supernatant of fungal shake flask culture grown for 5 days. Gel analysis of the DNase activity was conducted by incubating an aliquot (7 μL) of the crude enzyme solution or DNase I (1 U/μL) (Solarbio) with 2 μL pBBR1MCS-2 (50 ng/μL) at 37°C for 1 h. The resulting solution was subjected to agarose gel electrophoresis and Super GelRed staining.

DNase activity was quantitatively analysed using a modified version of the method previously described by Yu et al. (2021). A 0.5 mL crude enzyme solution was mixed with 1 mL of 0.1 M HAc-NaAc buffer (pH 5.0) and 1.5 mL of calf thymus DNA (0.15 mg/mL) and incubated at 35°C for 10 min. The absorbance $(A)_{260}$ value was monitored in real time, and one unit (U) of enzyme activity was defined as the amount of enzyme needed to increase the A_{260} by 0.001 per minute at 35°C and pH 5.0.

To identify the Flg22-DNase protein secreted by the UW4E-flg22-D strain, the supernatants of UW4E-flg22-D and UW4E shake flask cultures grown for 12 h were concentrated 100-fold using ultrafiltration membranes with molecular weight cut-offs of 3 kDa, 10 kDa, and 30 kDa. After the concentrated supernatants were separated by electrophoresis on native-PAGE gels and stained with Coomassie Brilliant Blue, the resulting gels were then transferred onto agarose gels supplemented with 0.1 μg/mL pBBR1MCS-2 and incubated at room temperature for 2 h. Following incubation, the native-PAGE gels were removed, and the agarose gels were stained with Super GelRed.

4.7 | Western blotting

Overnight culture supernatants of *Pseudomonas* sp. UW4 strains were collected and concentrated 100-fold with a 0.45 μm microporous membrane. After the concentrated supernatants were separated by SDS–PAGE and electrophoretically transferred to nitrocellulose membranes, the secreted protein expressed by the UW4E-flg22 strain was stained using mouse anti-His tag monoclonal antibody (Invitrogen, 1:2000 dilution) as the primary antibody and peroxidase-conjugated goat anti-rabbit IgG (H + L) (Invitrogen, 1:5000 dilution) as the secondary antibody.

4.8 | Wheat seed disinfection and germination

Wheat seeds were surface disinfected using 70% ethanol and 1% sodium hypochlorite as described elsewhere (Herrera et al., 2016). The disinfected seeds were then evenly placed on a 1% agar plate precovered with germination paper and incubated at 28°C to facilitate germination.

4.9 | Observation of mucilage, exDNA and ROS from RBCs

The aerial root tips of the wheat seedlings cultured on the agar plate for 3 days were shaken gently in solution for 5–8 min to collect RBCs. The solution was 100 μL of bacterial broth at $OD_{600} = 1.0$, fungal pathogen spore suspension (10^5/mL), or 1 μM Flg22. Mucilage around RBCs in a mixture of RBC suspension and blue ink at a ratio of 20:7 was observed using an E100 microscope (Nikon) (Cai et al., 2011).

The RBC suspension was mixed with an equal volume of 1 μM H_2DCFDA and incubated at room temperature for 15 min. The ROS generated by the RBCs were visualized using a confocal laser scanning microscope (A1HD25; Nikon). Additionally, the exDNA secreted by the RBCs was observed using a confocal laser scanning microscope after the RBC suspension was mixed with an equal volume of 10 μg/mL DAPI and incubated at room temperature for 20 min.

4.10 | Growth pouch incubation

After germinating on the plate for 3 days, the wheat seedlings were transplanted into a growth pouch (14 × 2.5 cm, Qwbio) containing 15 mL of sterile water and cultured further at 28°C. To determine bacterial colonization in wheat roots, the wheat roots were immersed in a 5 mL bacterial suspension ($OD_{600} = 1.0$) or bacterial suspension supplemented with 1 μM Flg22 for 30 min prior to transferring the seedlings to growth pouches. Subsequently, the seedlings were incubated for 7 days. Sterile water was used as a mock control. Root tip colonization by the UW4 strains was observed using a confocal laser scanning microscope (A1HD25; Nikon) and was counted using the agar dilution plate count method. The agar dilution plate count was performed by grinding three to five root tips 1 cm in length and then diluting in a gradient. The resulting bacterial suspension was spread on LB agar containing 100 μg/mL ampicillin (UW4 has inherent resistance to penicillin) (Gao et al., 2020).

To determine the infection of fungal pathogens on wheat roots, the roots of the seedlings germinating on the plate for 3 days were immersed in 5 mL of DNase I solution (1.2 U), 10^5/mL of fungal spore suspension, a mixture of fungal spore suspension (10^5/mL) and 1.2 U of DNase I, or fungal spore suspension (10^5/mL) mixed with bacterial suspension ($OD_{600} = 1.0$) for 5 min, and then the seedlings were transferred into growth pouches containing 10 mL of sterile water.

Sterile water was used as a mock control. The pouches were cultured in a growth chamber at 28°C for 7 days. The root length, root dry weight, root disease incidence, and bacterial colonization in the root tips were measured.

4.11 | Pot trial

To determine the effectiveness of bacteria against fungal root rot, wheat seeds were disinfected and immersed in sterile water or a bacterial suspension ($OD_{600}=1$) of UW4, UW4E, UW4E-flg22, UW4E-Flg(flg22), or UW4E-flg22-D until the seeds were swollen and then sown in pots with 5 kg substrate (1:1 peat and vermiculite) containing 100 mL of spore suspension at a concentration of 10^5/mL. Sterile water was used as a mock control. The pots were incubated in a growth chamber with a light cycle of 16 h light/8 h dark at 25°C for 15 days. The root length, root dry weight, root disease incidence, and root tip colonization by the UW4 strains were determined.

4.12 | Statistical analysis

Three independent biological replicates were conducted for all experiments, with each experiment performed in triplicate. The significance of the data was assessed using one-way analysis of variance, followed by Bonferroni multiple comparison tests using Prism v. 9.0 software (GraphPad). A test value of $p<0.05$ was considered significant.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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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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