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RESEARCH ARTICLE

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Improvement of fitness and biocontrol properties of *Pseudomonas putida* via an extracellular heme peroxidase

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Abstract

The extracellular 373-kDa *PehA* heme peroxidase of *Pseudomonas putida* KT2440 has two enzymatic domains which depend on heme cofactor for their peroxidase activity. A null *pehA* mutant was generated to examine the impact of *PehA* in rhizosphere colonization competence and the induction of plant systemic resistance (ISR). This mutant was not markedly hampered in colonization efficiency. However, increase in *pehA* dosage enhanced colonization fitness about 30 fold in the root and 900 fold in the root apex. In vitro assays with purified His-tagged enzymatic domains of *PehA* indicated that heme-dependent peroxidase activity was required for the enhancement of root tip colonization. Evaluation of live/dead cells confirmed that overexpression of *pehA* had a positive effect on bacterial cell viability. Following root colonization of rice plants by KT2440 strain, the incidence of rice blast caused by *Magnaporthe oryzae* was reduced by 65% and the severity of this disease was also diminished in comparison to non-treated plants. An increase in the *pehA* dosage was also beneficial for the control of rice blast as compared with gene inactivation. The results suggest that *PehA* helps *P. putida* to cope with the plant-imposed oxidative stress leading to enhanced colonization ability and concomitant ISR-elicitation.

INTRODUCTION

Part of the *photosynthetically* fixed carbon in plants is released as plant root exudates. Apart from nutrients, root exudates also contain numerous chemical signals that mediate plant–soil bacteria interaction processes (Bais et al., 2006; Uren, 2007). The composition of the exudates may vary depending on the microorganisms encountered (Kamilova et al., 2006).

The presence of some non-pathogenic bacteria in the plant roots and/or in the surrounding soil area under the influence of the root exudates (rhizosphere) may trigger an indirect mechanism of suppressing a broad spectrum of pathogenic agents. This mechanism, called induced systemic resistance (ISR) activates specific plant defence pathways (Bakker et al., 2003; Pieterse et al., 2014). Alternatively, direct biocontrol mechanisms are exerted directly by the beneficial bacteria against pathogenic agents (Lugtenberg & Kamilova, 2009; Thomashow & Bakker, 2015).

Rhizosphere competence has been shown to be required for bacteria to exert their beneficial effects.

In particular, an efficient rhizosphere colonization capacity was revealed to be an essential factor for soil-borne non-pathogenic bacteria to exert antagonism against pathogens (Chin-A-Woeng et al., 2000; Matilla et al., 2010). Numerous bacterial colonization determinants have been identified which, when inactivated, diminish fitness of *P. putida* in the rhizosphere (Espinosa-Urgel & Ramos-González, review in preparation). Of particular importance are cell envelope components such as large adhesins and lipopolysaccharides (Martínez-Gil et al., 2010; Yousef-Coronado et al., 2008) and exopolysaccharides (EPS) (Martínez-Gil et al., 2013).

Bacterial ISR-traits have been identified, including flagella, lipopolysaccharides and secreted metabolites such as siderophores, cyclic lipopeptides, volatiles, antibiotics, phenolic compounds and quorum sensing molecules (De Vleesschauwer & Höfte, 2009). Although the mechanisms of ISR are better understood in Dicotyledoneae than in Monocotyledoneae, including the graminaceous crop plants, numerous microorganisms known to induce ISR in monocots and diverse bacterial determinants have been identified as ISR elicitors in monocots. As with dicots, the potential resistance induced in monocots depends on the host-bacteria combination and on the pathogen (Balmer et al., 2013).

Rice is a monocot model plant and rice blast is considered to be a model disease for the study of genetics, epidemiology, biology and the molecular pathology of host–parasite interactions. The hemibiotrophic pathogen *Magnaporthe oryzae* is the causal agent of rice blast which is by far the most important disease that affects rice (Fernandez & Orth, 2018; TeBeest et al., 2007). Interestingly, several plant proteins related to reactive oxygen species (ROS) signalling and scavenging have been identified as being involved in the pathogen perception by the plant (Meng et al., 2019). Treatment with specific strains of *Pseudomonas aeruginosa*, *P. fluorescens* and *Serratia plymuthica* spatially separated from the pathogen have been shown to induce resistance against *M. oryzae* in rice (De Vleesschauwer et al., 2006, 2008, 2009). The siderophore pseudobactin and antibiotics such as pyocyanin produced by *Pseudomonas* spp. are important molecules required for resistance to *M. oryzae* (De Vleesschauwer et al., 2006, 2008).

Plant peroxidases play a role in lignin production (Marjamaa et al., 2009), the formation of which may increase plant resistance to pathogenic agents. The induction of plant peroxidases following the induction of systemic defence responses in rice has been reported (Taheri & Höfte, 2007; Vidhyasekaran et al., 2001). Moreover, salicylic acid produced by *P. aeruginosa* triggers peroxidases accumulation in rice leading to an increased resistance to the necrotrophic fungus *Rhizoctonia solani* (Saikia et al., 2006). In addition, EPS produced by *Pantoea* are known to induce defence responses in wheat cells by triggering the accumulation of hydrogen peroxide and increased peroxidase activity (Ortmann & Moerschbacher, 2006).

The root tip is a metabolically active zone where levels of ROS are higher than in other root areas (Dunand et al., 2007). It was previously shown that *P. putida* mutants lacking Fe-superoxide dismutase are less competitive in root tip colonization (Kim et al., 2004); an indication that ROS-scavenging enzymes play an important role in colonization of this niche. A similar role of these enzymes has been reported in diazotrophic bacteria (Alquéres et al., 2013). An implication of bacterial peroxidases in inducing resistance in plants was only recently unveiled. Previous work in our laboratory revealed that a transposon mutant derivative of *P. putida* KT2440R in the locus PP2561 which encodes the extracellular heme peroxidase *PehA* was hampered in the elicitation of systemic resistance in Arabidopsis against the bacterial phytopathogen *P. syringae* pv. *tomato* DC3000. The *P. putida* KT2440R truncated *pehA* mutant was also less efficient in competitive colonization of the rhizosphere (Matilla et al., 2010). The 3619 amino acid *PehA* protein contains two animal heme peroxidase (ANHEMP)-like domains that are likely the result of sequence duplication. It is known that after reconstitution with heme both domains of *PehA* have peroxidase activity (Santamaría-Hernando et al., 2012).

In this study, we investigated the role of this bacterial extracellular heme peroxidase in colonization competence and ISR-triggering in monocots. For this, we generated a null *pehA* mutant to avoid any putative accumulation of a truncated *PehA* protein in the bacterial cell. In addition, we overexpressed *pehA* and found enormous improvement in regard to bacterial cell survival, colonization fitness, especially of the root tips and protection of rice plants against the foliar phytopathogenic fungus *M. oryzae*. Furthermore, using heme-reconstituted his-tagged purified enzymatic domains of *PehA*, we confirmed that the peroxidase activity of this protein is essential for enhancing root tip colonization. Taken together, these results indicate an important role for *PehA* in the colonization and ISR-triggered protection of monocots against pathogenic infection.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas putida* strains were routinely grown at 28 or 30°C as indicated in either Luria-Bertani (LB) medium (Bertani, 1951) with no

TABLE 1 Bacteria and plasmids used

Strains and plasmids	Relevant characteristics	Reference or source
Pseudomonas putida		
KT2440 (or KT)	Derivative of *P. putida* mt-2, cured of pWWO	Regenhardt et al. (2002)
KT2440 *pehA*	Km^r, null PP2561 mutant	This study
KT2440R (or KTR)	Rif^r derivative of KT2440	Espinosa-Urgel and Ramos (2004)
KT2440Tn*7*-ΩSm1	Sm^r, site-specific insertion of miniTn*7*	Matilla et al. (2007)
KT2440RTn*7*-ΩSm1	Rf^r, Sm^r, site-specific insertion of miniTn*7*	Matilla et al. (2007)
KT2440R *pehA*	Rif^r, Km^r, null PP2561 mutant	This study
KTRPP2561	Rif^r, Km^r; miniTn*5* interrupting PP2561	Matilla et al. (2007)
Escherichia coli		
CC118λ*pir*	Rif^r, λ*pir*	Herrero et al. (1990)
DH5α	*supE44 lacU169* (*Ø80lacZΔ M15*) *hsdR17* (r_k-m_k-) *recA1 endA1 gyrA96 thi-1 relA1*	Woodcock et al. (1989)
HB101	F^- *Δ(gpt-proA)62 leuB6 supE44 ara-14 galK2 lacY 1Δ (mcrC-mrr) rpsL20* (Sm^r) *xyl-5 mtl-1 recA 13 thi-1*	Boyer and Roulland-Dussoix (1969)
Enterobacter cloacae	Resistant to λ phage, growth in citrate	S. Molin
Plasmids		
p34S-Km3	Km^r, Ap^r, *km3* antibiotic resistance cassette	Dennis and Zylstra (1998)
pKNG101	Sm^r, *oriR6K mobRK2 sacBR*	Kaniga et al. (1991)
pLAFR3	Tc^r, derivative from the cosmid pLAFR1, P_{lac} fused to the fragment encoding the α peptide of β-galactosidase	Vanbleu et al. (2004)
pMBL-T	Ap^r, PCR cloning vector, P_{lac} fused to the fragment encoding α peptide of β-galactosidase	Canvax
pRK600	Cm^r, *oriColE1 mobRK2 traRK2*	Finan et al. (1986)
pUC18Not	Ap^r, identical to pUC18 but with two NotI sites flanking pUC18 polylinker	Herrero et al. (1990)
pBBR1MCS-5	Gm^r, oriRK2 mobRK2	Kovach et al. (1995)
pBBR1MCS-2	Km^r, oriRK2 mobRK2	Kovach et al. (1995)
pCSSH1	Tc^r, cosmid of *P. putida* genebank, derivative of pLAFR3, containing the cluster *pehABCD*	This study
pCSSH3	Tc^r, Km^r, derivative of pCSSH1 harbouring *pehA* null	This study
pMIR160	Ap^r, pMBL-T with a 1-kb PCR fragment upstream of *rup2561,* obtained using primers 1PehAf and 2PehAr	This study
pMIR161	Ap^r, pMBL-T with a 0.94-kb PCR fragment downstream of *rup2561*, obtained using primers 3PehAfw and 4PehAr	This study
pMIR162	Ap^r, 1-kb SalI/XbaI fragment of pMIR160 cloned at the same sites in pUC18Not	This study
pMIR163	Ap^r, 1-kb SalI/XbaI fragment of pMIR160 and 0.92-kb XbaI/SacI fragment of pMIR161 cloned at the same sites in pUC18Not	This study
pMIR164	Ap^r, Km^r, *km3* cassette of p34S-Km3 inserted into XbaI site of pMIR163	This study
pMIR166	Sm^r, Km^r, 2.9-kb NotI fragment of pMIR164 inserted into pKNG101. Plasmid construction to generate the chromosomal and cosmid encoded *pehA* null mutation	This study
pMIR185	Gm^r, *pehA* expressed from P_{lac} of pBBR1MCS-5	This study

Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Rif, rifampin; Sm, streptomycin; Tc, tetracycline; MCS, multiple cloning site.

glucose or in King's B (KB) medium (King et al., 1954). *Escherichia coli* strains were grown at 37°C in LB. When appropriate, antibiotics were added to the medium at the following concentrations in (μg/ml): ampicillin, 100; chloramphenicol, 30; kanamycin 25 or 50 (*E. coli* and *Enterobacter cloacae* or *P. putida* strains); rifampin, 10; streptomycin, 50 or 100 (*E. coli* and *E. cloacae* or *P. putida* strains); tetracycline, 7.5.

Molecular biology techniques

Chromosomal DNA was prepared with the Promega Wizard Genomic DNA Purification Kit (cat. no. A1120). Plasmid DNA was isolated with the Qiagen spin miniprep kit (cat. no. 27106). Cosmid isolation was carried out by the alkaline lysis method (Sambrook & Russell, 2001). DNA restriction, dephosphorylation, ligation and electrophoresis were performed using standard protocols (Sambrook & Russell, 2001) and manufacturer instructions (Roche and New England Biolabs). DNA fragments were recovered from agarose gels using the Roche High Pure PCR Cleanup Micro Kit. Competent cells were prepared using calcium chloride and transformations were carried out by standard procedures (Sambrook & Russell, 2001). Southern Blot and colony hybridization techniques were performed by standard methods and DNA probes were labelled with digoxigenin-11-dUTP (Roche, cat. no. 11573179910). Electrotransformation of *P. putida* cells was performed as previously reported (Enderle & Farwell, 1998). Expand high fidelity Taq polymerase (Roche) was used for the amplification of DNA fragments.

Isolation and characterization of cosmid pCSSH1

Cosmid pCSSH1 was isolated from a *P. putida* KT2440 genebank that was constructed in pLAFR3 (Ramos-González, 1993) by colony hybridization against *pehA* using the digoxigenin-labelled probe obtained with the oligonucleotides 2561-Tn5Fw 5′GACAGCCAGCTCAA GCCCTAC3′ and 2561-Tn5Rw 5′GTAGAAGCGGTCAC CGTTCTGC3′. The presence of the complete gene cluster encoding a Type 1 Secretion System (T1SS) and *pehA* was confirmed following amplification of two fragments from pCSSH1 with two independent oligonucleotides pairs. The first pair was PP2558F 5′ATAGCCTG AAGGAATACGTCGC3′ and PP2558R 5′CTGAACCAG CGCAACCAC3′, and second pair was PP2561F 5′CG GTAACGACAACCTGAACGG3′ and PP2561R 5′TCAA CCGGCCAGGATGAAGTC3′. Sequencing from the extremes of the cosmid pLAFR3 with oligonucleotides Uni 5′GTTTTCCCAGTCACGACG3′ and Rev 5′GCG GATAACAATTTCACACAG3′ allowed elucidation of the extremes of the insertion in pCSSH1.

Construction of *pehA* null derivatives in the chromosome of *P. putida* and in the cosmid pCSSH3

A null allele of *pehA* was generated in the suicide vector pKNG101 for the inactivation by homologous recombination of the chromosomal or cosmid encoded wild-type gene. To obtain the region upstream to *pehA* a 1029 bp segment was amplified by PCR from cosmid pCSSH1 using primers 1PehAf (5′GATCAGGTCGACAATCGTGGTG3′) and 2PehAr (5′CTGGCAGTCTAGAGACCTCAGGCAAC AGGGTCCTTCCCG3′) and cloned into pMBL-T to generate plasmid pMIR160. To obtain the region downstream to *pehA* a 942 bp segment was similarly amplified via PCR with primers 3PehAfw (5′CTGAGGTCTCTAG ACTGCCAGATCCTGGCCGGTTGAGG3′) and 4PehAr (5′CAGGTGCGGTGAGCTCTTC3′). This latter PCR product was cloned into pMBL-T and the resulting plasmid was named pMIR161. The absence of missense mutations was confirmed by sequencing. Plasmids pMIR160 and pMIR161 were digested at the sites incorporated by the primers (underlined). The insert SalI-XbaI of pMIR161 was cloned into the same sites of pUC18Not to generate the plasmid pMIR162 and the insert XbaI-SacI of pMIR161 was cloned at the corresponding sites of pMIR162 in such a way that in this construction, named pMIR163, upstream and downstream flanking regions of *pehA* were brought together linked by an XbaI site. A 0.97 kb-XbaI Km resistance cassette from p34S-Km3 (Table 1) was introduced into this site to generate the plasmid pMIR164. Finally, the NotI fragment including the above-described insert containing the deletion of the complete *pehA* gene with a Km insertion was cloned into the same site of pKNG101 and this plasmid (pMIR166) was used for replacement of the wild-type *pehA* for the null allele.

For the replacement of *pehA* in the chromosome of *P. putida* the suicide plasmid pMIR166 was transferred to the host by triparental conjugation using *E. coli* CC118λ*pir* as donor and *E. coli* HB101 (pRK600) as helper strain. Mutants were selected based on resistance to Km and sensitivity to Sm and confirmed by PCR and Southern Blot using standard procedures (Sambrook & Russell, 2001).

Allelic replacement of *pehA* in cosmid pCSSH1 was also accomplished by triparental mating to generate the mutated cosmid pCSSH3, which harbours the null *pehA* allele. However, for this process, *E. cloacae* was used as an intermediate host for pCSSH1 given its insensitiveness to the λ phage and thus the inability to develop λ-lysogenic derivatives that stably maintained pMIR166 as an independent replicon. Exconjugants of *E. cloacae* harbouring pCSSH3 were selected on M9 minimal medium supplied with citrate 15 mM based on double KmTc resistance and Sm

sensitivity. The incorporation of the *pehA* null mutation in pCSSH3 was confirmed by PCR and restriction pattern.

Construction of pMIR185

For the construction of pMIR185, a 12.2 kb BspEI/BsrGI fragment of pCSSH1 with *pehA* was cloned into XmaI/XbaI sites of the pBBR1MCS-5 vector. This fragment contains 24 bp upstream the start codon of *pehA* that includes its own Shine-Dalgarno. In this construction, *pehA* is deprived of its promoter and its expression is controlled by the P*lac* promoter present in the cloning vector. Given the absence of *lacI* in *Pseudomonas*, *pehA* expression from pMIR185 does not require isopropyl β-D-1-thiogalactopyranoside (IPTG).

Cell viability

Determination of viable cell number was performed using the LIVE/DEAD BacLight kit (Invitrogen) following the directions of the manufacturer. This procedure makes use of two nucleic acid stains, SYTO9 green-fluorescent stain, which efficiently enters into bacteria cells, and propidium iodide (PI) red-fluorescent stain that only enters into membrane-compromised bacterial cells, which frequently correspond to dead cells. Bacteria from LB-overnight cultures were washed in NaCl 0.85% (w/v), incubated in this solution for 1 h at room temperature and again washed in NaCl 0.85%. One milliliter of this bacterial suspension was incubated with 3 μl of a mixture of SYTO9 and PI (1:1, v/v) for 15 min in darkness at room temperature. All bacteria in the samples were stained with green fluorescent, whereas only unviable cells were also stained with the red fluorescent. Bacteria were analysed under a Zeiss Axioscope fluorescence microscope coupled to a Nikon DSS-Mc CCD camera.

Surface sterilization and germination of seeds

Maize and alfalfa seeds were surface sterilized and germinated on Musharige and Skoog MS-phytagel (0.2%) medium supplemented with glucose (0.5%) to detect microbial contamination as described previously (Matilla et al., 2007). Maize seeds were incubated for 2 days at 30°C and seedlings were subsequently used for exudates collection or root colonization assays. Alfalfa seeds were first maintained for 1 day at 4°C then incubated for 1 day at 30°C and seedlings subsequently were used for root colonization assays.

Plant root colonization assays

For competitive colonization assays, overnight bacterial cultures grown in LB were diluted down to an OD_{660} = 1 in M9 and both bacterial strains under investigation were mixed in a 1:1 proportion (~5×10^6 CFU per ml for each strain). Forty-eight-hour seedlings were incubated in this bacterial suspension under static conditions at 30°C for 30 min. Seedlings were then rinsed and planted in 50 ml Sterilin tubes filled with 40 g of sterilized silica sand and 10 ml of rich-PNS (maize) or with perlite substrate containing 20 ml of rich-PNS (alfalfa). The inoculated plants were maintained in a controlled chamber at 24°C/18°C (day/night) and 55%–65% humidity with a daily light period of 16 h. Six days after planting the shoots were discarded. For the recovery of bacterial cells from the rhizosphere, roots with adhered sand were placed in a 50 ml Sterilin tube containing 4 g of glass beads (diameter, 3 mm) and 10 ml of M9 salts (Sambrook & Russell, 2001). For recovery from root tips (0.7 cm) 2 ml Eppendorf tubes filled with seven glass beads and 1 ml of M9 salts were used. In both cases, the tubes were vortexed for 2 min and colony-forming units (CFU) enumerated by drop plating on LB-agar medium supplemented with the appropriate antibiotics.

For colonization assays in the presence of His-tagged recombinant proteins, the same procedure as described above was used except that seedlings were incubated in a bacterial suspension of null *pehA* mutant (~5×10^6 CFU per ml) at 30 °C for 30 min. An additional incubation step was performed for 10 min at room temperature using His-tagged PehA-Nter and PehA-Cter (1 μM), without heme and heme-reconstituted proteins which were obtained as reported in the supporting file. Control seedlings were incubated in a buffer containing 10 mM Tris–HCl, 50 mM NaCl, 10% DMSO, 10% glycerol and pH 7.5. The same buffer was used for reconstitution of proteins and further dialysis. After 4 days of incubation in the plant chamber bacterial cells were recovered from roots or root tips using the methods described above and CFU were determined by drop plating on LB-agar medium plates supplemented with kanamycin.

ISR assay for *Oryza sativa* spp. *indica* C039

ISR assays were performed essentially as described by De Vleesschauwer et al., 2008. Rice seeds were surface sterilized with 70% ethanol for 5 min, followed

by a treatment with 1% sodium hypochlorite solution for 3 min, and then repeatedly rinsed with sterile distilled water to completely remove the hypochlorite. *Pseudomonas putida* bacterial strains were cultured overnight in King's B liquid medium at 28°C and then diluted to the desired concentration in 0.85% NaCl. Surface-sterilized seeds were subsequently soaked in a bacterial suspension of ~5×10^7 CFU per ml for 10 min and incubated on wet sterile filter paper in sealed Petri dishes, to maintain humidity, at 28°C in darkness for 3 days and then exposed to light for 2 days. Potting soil (Structural type 1; Snebbout, Kaprijke, Belgium), which had been autoclaved twice on alternative days, was also mixed with the bacterial suspension, to reach a final concentration of 5×10^7 CFU per g, and was distributed in perforated plastic trays (23 × 16 × 6 cm). Roots of the 5-day seedlings were individually dipped in a bacterial suspension (5×10^7 CFU per ml) prior to sowing (12 per tray). Plants were incubated under non-sterile conditions at 28 ± 4°C with a daily light period of 12 h. The soil substrate was again inoculated with bacteria cells (5×10^7 CFU per g) 10 days after sowing. In control treatments, saline solution without bacteria was used. Fertilization solution containing Fe-EDDHA (7.6 g/L) and $(NH_4)_2SO_4$ (1.8 g/L) was added to each tray weekly. Induction of resistance in positive control plants was accomplished by spraying leaves 2 days before challenge inoculation with the pathogenic agent with 500 μM benzo(1, 2, 3) thiadiazole-7 carbonic acid S-methyl ester (BTH, BION 50 WG) in an aqueous solution containing 0.02% Tween 20. Rice leaves involved in other treatments were sprayed with an equal volume of aqueous solution containing 0.02% Tween 20.

Pathogen inoculation and disease rating

Magnaporthe oryzae isolate Guy11 was grown in a complete medium (CM) (Talbot et al., 1993). After 1 week of incubation at 28°C, mycelium was flattened onto the medium and exposed to blue light for 7 days to induce sporulation. Four-week-old leaves of five-leaf stage rice plants were sprayed with a solution of conidial suspension adjusted to a final concentration of 10^4 spores per ml in 0.5% gelatin (type B from bovine skin; Sigma-Aldrich G-6650) and maintained at 28°C under conditions of high humidity for 24 h. Six days after this challenge, the severity of disease was assessed on the second youngest mature leaf of each plant by using a 0–6 scale based on the type and size of lesions on the leaves (Roumen et al., 1997). The number of susceptible-type lesions presenting a grey centre (3–6), which was indicative of fungus sporulation was also quantified as previously described (De Vleesschauwer et al., 2008).

RESULTS

A null *pehA* mutant is not hampered in rhizosphere colonization or oxidative stress

A transposon-generated mutant of *P. putida* KT2440R named KTRPP2561 was previously used to investigate the role of the extracellular heme peroxidase *PehA* (Matilla et al., 2010). This mutant produced a truncated *PehA* protein of 733 aa, representing a fragment of the N-terminal heme peroxidase domain (Figure S1). For this current study, we generated a null *pehA* mutant by allelic replacement through homologous recombination in order to avoid any intracellular accumulation of a truncated *PehA* protein that might cause interference in our assays. This new *pehA* mutant showed the same growth kinetics as the wild-type strain (Figure S2). In contrast to that observed with the truncated *pehA* mutant, the null mutant exhibited similar colonization efficiency to the wild-type KT2440 in a competitive colonization assay performed in the maize rhizosphere; however, the fitness of the null mutant in the root tip was slightly reduced compared to the wild type (Figure S3A). The null *pehA* mutant's capacity for colonization when inoculated alone was indistinguishable from that of the wild type (not shown).

We also evaluated the effect of deleting *pehA* upon the survival of *P. putida* in the presence of hydrogen peroxide and found that this mutant behaved like the wild type (Figure S3B). In addition, growth curve inhibition assays and growth inhibition halo tests using various oxidizing agents such as methyl viologen, cumene hydroperoxide, K_2TeO_3, $CdCl_2$ and tert-butyl hydroperoxide indicated that the null *pehA* mutant did not have an increased sensitivity to these oxidants (data not shown).

The root exudates profile of gnotobiotic *Zea mays* plants incubated with KT2440R or its *pehA* null mutant were compared by using HPLC analysis. Although they were found to be highly comparable, some mostly quantitative differences could be observed (Figure S4). The enzymatic activity of *PehA* upon root exudates compounds or, alternatively, differences in the root exudation in response to different bacterial strains might explain these differences.

The results described above suggest that the removal of *pehA* by itself does not appear to be responsible for the pleiotropic phenotype observed in the transposon insertion generated mutant KTRPP2561 (Matilla et al., 2010). A plausible explanation for the discrepancy observed is that the truncated *PehA* protein produced in the transposon mutant had a deleterious effect on *P. putida*.

Increased dose of *pehA* enhances fitness in the rhizosphere

We evaluated the impact of increasing the *pehA* dosage on the colonization ability of KT2440. For this, we used cosmid pCSSH1 which is a derivative of the 20.5 kb pLAFR3, whose copy number per cell is known to be 3–7 copies depending on the host (Kües & Stahl, 1989). Cosmid pCSSH1 contains a 22.1 kb insertion (Figure 1) that includes almost the complete genomic island number 24 described by Wu et al., 2011. Thus, besides *pehA*, this cosmid contains a flanking cluster of three genes encoding a type 1 secretion system (T1SS). A strain harbouring the empty cosmid pLAFR3 was used as a control in all competitive colonization assays. Cosmids pCSSH1 or pLAFR3 were introduced into *P. putida* strains by triparental conjugation using pRK600 as a helper plasmid as reported previously (Ramos-Gonzalez & Molin, 1998). Pregerminated seedlings of maize or alfalfa were incubated with a bacterial suspension containing a 1:1 proportion of strains carrying each cosmid (pCSSH1 or pLAFR3) and the number of CFU in the inoculate was monitored. Plants were incubated for 6 days as described in Experimental procedures. Bacteria were then recovered from the complete rhizosphere or the root tip and the proportion of each strain was determined per plant. Results showed that the KTRPP2561 transposon mutant harbouring pCSSH1 was consistently more competitive than the strain carrying the empty cosmid pLAFR3 (Figure 2A). Thus, the extra 22.1 kb of the DNA insertion in pCSSH1 not only was not a burden but actually improved the previously observed defective colonization capacity for KTRPP2561 (Matilla et al., 2007). When pCSSH1 was introduced into the *pehA* null mutant the competitiveness level of this strain was increased (Figure 2B) above that reached by the truncated mutant harbouring this cosmid. The greatest enhancement in competitiveness was observed when pCSSH1 was introduced into the wild-type strain. In this case, the proportion of this strain increased from 50% up to 80% after 6 days in the maize rhizosphere, and almost displaced the strain harbouring pLAFR3 in the root tip, increasing its ratio up to 97% (Figure 2C). Therefore, the colonization ability of *P. putida* KT2440 and its corresponding *pehA* mutants was clearly increased when carrying pCSSH1. In order to ascertain that no other gene contained on pCSSH1 was responsible for the observed enhancement in colonization fitness, a *pehA* null derivative of pCSSH1, termed pCSSH3, was generated by allelic replacement, using the same plasmid pMIR166 that was used to generate the null *pehA* mutant in the chromosome of KT2440. Competitive colonization experiments were then performed with strains harbouring this null *pehA* mutant cosmid pCSSH3 or the empty vector pLAFR3 and the proportion of CFU carrying each cosmid was similar (Figure S5). Cosmid pCSSH1 enhanced the bacterial fitness in competition with pCSSH3 also in dicotyledonous plants (Figure S6). Taken together, these results suggest that an increased dose of *pehA* improves the fitness of *P. putida* in the rhizosphere. This suggestion was confirmed by expressing *pehA* ectopically from the plasmid pMIR185. This plasmid is a derivative of the stable vector pBBR1MCS-5, which presents a higher number of copies than pLAFR3. In contrast to cosmid pCSSH1, pMIR185 only contains *pehA*. In the presence of this plasmid, the colonization index of *P. putida* was increased up to 28-fold in the rhizosphere and an enormous improvement (up to 880-fold) was observed in the root tip environment (Figure 3A). Interestingly, an increase in *pehA* dose enhanced oxidative stress resistance of the wild-type strain (Figure 3B).

Increased *pehA* dose enhances cell viability in the stationary phase

In order to document whether the increased fitness conferred by pCSSH1 in the rhizosphere was also manifest in laboratory cultures, we performed co-culture assays with *P. putida* strains carrying either cosmid pCSSH1 or pCSSH3. Experiments were carried out in LB-medium supplemented with a positive selection for the cosmids (tetracycline) and the number of CFU of each strain was determined at different stages of bacterial growth. The results indicated

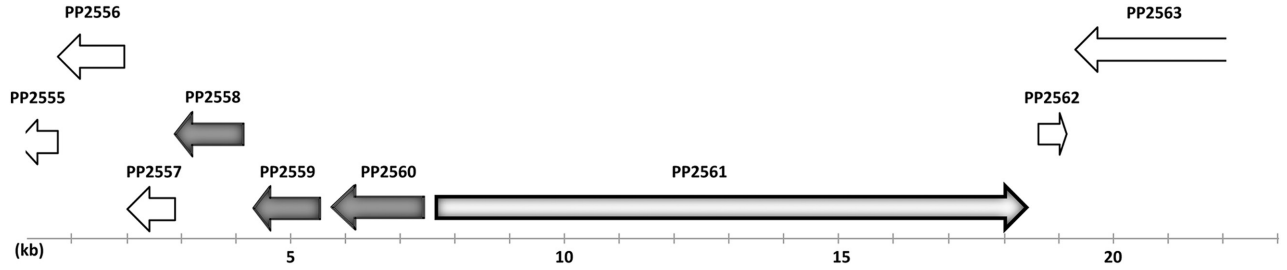

FIGURE 1 Genetic organization of the fragment inserted into the pLAFR3-derivative cosmid pCSSH1. The inserted DNA is a 22.1 kb DNA fragment (2902955–2925123) from *Pseudomonas putida* KT2440. The NCBI annotation of loci includes PP2555, a 'putative SAM-dependent methyltransferase'; PP2556, a 'chromate transporter'; PP2557, a 'PAS/PAC sensor-containing diguanylate cyclase'; PP2558, a 'outer membrane efflux protein'; PP2559, a 'a type 1 secretion membrane fusion protein'; PP2560, a 'T1SS ATPase'; PP2561, a 'heme peroxidase'; PP2562, a 'hypothetical protein'; and PP2563, an 'antibiotic biosynthesis protein'.

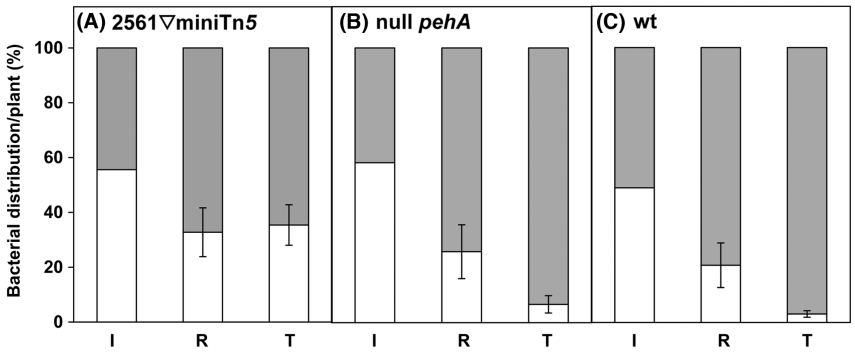

FIGURE 2 Rhizosphere colonization capacity conferred by pCSSH1 to *Pseudomonas putida* strains. Proportions of CFUs carrying cosmids pCSSH1 (grey) or pLAFR3 (white) in the initial inoculum (I) and recovered after 6 days in the corn rhizosphere (R) and the root tips (T) are plotted. A. KTRPP2561 (pCSSH1) vs KT2440RTn*7*-ΩSm1 (pLAFR3). KTRPP2561 appears as 2561▽minitn*5* in this panel. B. KT2440 *pehA* (pCSSH1) vs KT2440Tn*7*-ΩSm1 (pLAFR3). C. KT2440Tn*7*-ΩSm1 (pCSSH1) vs KT2440 (pLAFR3). The parental cosmid pLAFR3 was used as control. Data represent the average results from six plants and standard deviation is shown. One-way analysis of variance coupled to a Bonferroni post hoc test was used to compare means ($p < 0.05$). Whereas the sizes of bacterial inoculum were similar in the beginning of the experiment, differences between strains were significant at the end.

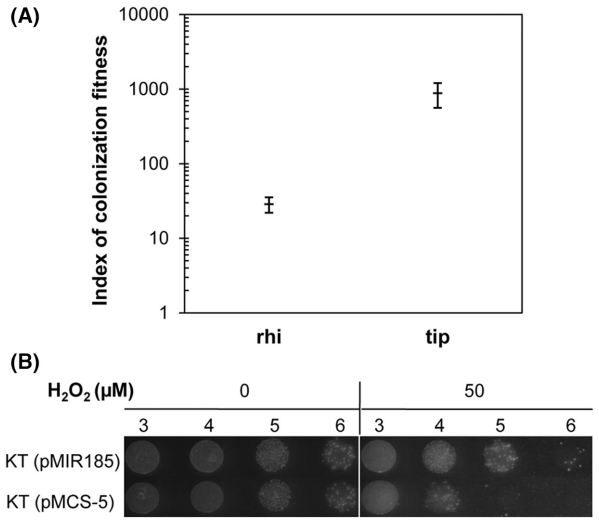

FIGURE 3 Overexpressing *pehA* provides an advantage to *Pseudomonas putida*. (A) Colonization capacity of *P. putida* expressing *pehA* ectopically in competition with wild type. The index of colonization fitness (Ramos-González et al., 2013) is measured as 1/[(percentage of recovered KT2440 (pMIR185) vs KT2440 (pBBR1MCS-2) per plant at 6 days post inoculation)/(percentage KT2440 (pMIR185) vs KT2440 (pBBR1MCS-2) in the initial inoculum)]. The index of colonization fitness for KT2440 (pBBR1MCS-5) was 0.98 ± 0.05; thus, this strain and KT2440 (pBBR1MCS-2) are equally competitive. Gm and km resistance markers allowed strain-specific selection. Seed adhesion rate was similar for both strains (0.5% attached bacteria after 30 min). Data represent the average results from six plants and standard deviation is shown. (B) Hydrogen peroxide resistance. Bacterial strains were cultivated overnight in liquid LB medium and subsequently adjusted to an OD_{600nm} of 1 in M9 buffer (Sambrook & Russell, 2001). Serially diluted bacterial suspensions (10 μl) ranging from 10^3–10^6 as indicated were spotted on agar plates containing plant nutrient solution (PNS) supplemented with Fe-EDTA and MS micronutrients (Matilla et al., 2007); as C source citrate 15 mM was used. Gm 50 μg/ml was added to select for pBBR1MCS-5 and pMIR185 and hydrogen peroxide 50 μM was added as stressor. Plates were incubated at 28°C for 36 h.

that the bacterial population with pCSSH1 became dominant upon entrance into the stationary phase of growth and it reached 70% after 24 h. As a consequence of this, a major proportion of the recovered population carried pCSSH1 at the beginning of this experiment (Figure 4A). A possible explanation for this result is that increased dosage of *pehA* enhances cell viability. To test this hypothesis quantification of viable *P. putida* cells carrying cosmids pCSSH1, the null *pehA* cosmid pCSSH3 or the empty pLAFR3 was carried out using the LIVE/DEAD BacLight assay as indicated in Experimental procedures. Bacterial cells from LB cultures were analysed at the beginning of the stationary phase under fluorescence microscopy; no statistically significant differences between strains were observed. However, in the late stationary phase, the number of dead cells for the strain carrying pCSSH1 was about 25% lower when compared to the same strain harbouring pCSSH3 or pLAFR3 (Figure 4B). These results are compatible with a transition period between exponential and stationary phases wherein viable but non-culturable cells can emerge by oxidative stress accumulation, such as that reported for *E. coli* (Desnues et al., 2003). Taking these results together, we conclude that a higher dose of *pehA* positively contributes to bacterial fitness in natural and laboratory environments, although the effect was more remarkable in the rhizosphere and especially in the root apex.

Pseudomonas putida colonization capacity is increased in the presence of heme-containing PehA-Nter and PehA-Cter

We aimed to determine whether exogenous application of *PehA* might also improve the colonization

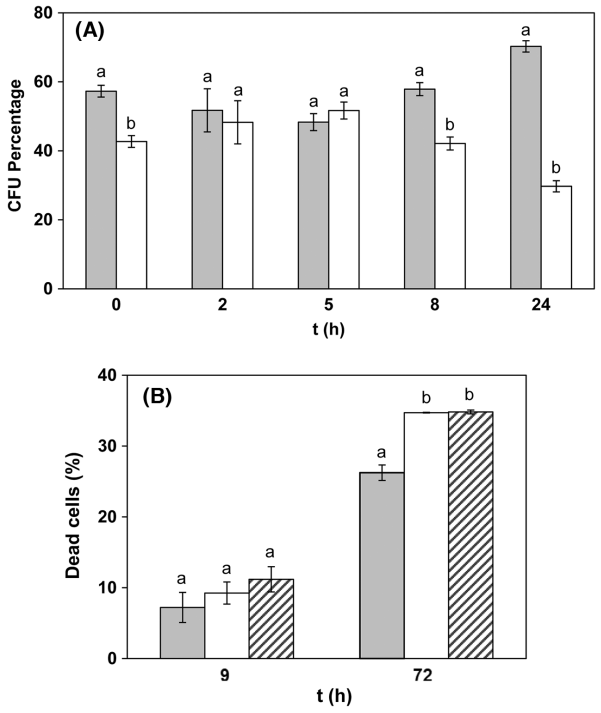

FIGURE 4 Effect of *pehA* dosage on *Pseudomonas putida* efficiency and viability. (A) Efficiency conferred by *pehA* from pCSSH1 to *P. putida* strains in culture. *Pseudomonas putida* strains harbouring derivative cosmids of pLAFR3 were individually grown for 16 h in LB medium with the appropriate antibiotics. Each strain was then diluted to an initial OD_{660} = 0.05 and mixed with a second strain in fresh LB medium supplemented with tetracycline (7.5 μg/ml). At the indicated times samples of the mixed cultures were serially diluted and plated in LB-agar supplemented with SmTc for selective CFU counting of KT2440Tn7-ΩSm1 (pCSSH1) (grey) and KmTc for the selective CFU counting of KT2440 (pCSSH3) (white). (B) Percentages of dead cells in strains carrying pCSSH1 (grey), pCSSH3 (white) or pLAFR3 (stripped white) are shown after 9 and 72 h of culture in LB-medium. Determination was made by counting the number of red cells, considered as dead, in relation to the number of green cells (total). For each data point, at least 300 bacterial cells were measured for viability using fluorescence microscopy. Statistical analysis was carried out using IBM SPSS statistics software (version 19 for windows). One-way analysis of variance coupled to a Bonferroni post hoc test was used to compare means ($p < 0.05$). Different letters indicate significant differences between treatments and standard deviation is shown.

ability of *P. putida*. Given the large size of *PehA* (373 kDa) we generated the recombinant His-tagged peptides PehA-Nter and PehA-Cter that correspond to the N-and C-terminal fragments of *PehA* (Figure S1). These peptides were purified by affinity chromatography and their spectral analysis indicated an absence of bound heme. We previously confirmed that these peptides do not present peroxidase activity unless they are reconstituted with heme (Santamaría-Hernando et al., 2012; Figure S7). Heme-free and heme-reconstituted proteins obtained as described in the supporting material were both assayed to evaluate the role of the exogenously supplied peroxidase activity on the colonization ability of the null *pehA* mutant. This strain was used to avoid any interference between the purified proteins and the full-length endogenously encoded *PehA* protein. In analyses performed in the whole rhizosphere no colonization improvement was observed as a result of the addition of peroxidase active domains (not shown). However, incubation with heme-reconstituted PehA-Nter resulted in an increase in the ability of the mutant strain to colonize the root tip; almost 20 fold compared to that acquired with the heme-free PehA-Nter. In fact the root tip colonization capacity of the null *pehA* mutant bacteria after incubation of the seedlings with heme-free PehA-Nter protein was the same as when a solution of control buffer was used (Figure 5A). In a similar treatment heme-reconstituted PehA-Cter doubled the colonization capacity of the null *pehA* mutant (Figure 5B). Given that an enhancement in the colonization capacity was only observed with heme-reconstituted proteins, we propose that the peroxidase activity of *PehA* is essential for the augmentation of the null *pehA* mutant in the root tip of maize plants.

Pseudomonas putida KT2440 triggers ISR to *M. oryzae* in rice

Previous work in our laboratory indicated that *P. putida* KT2440 was able to elicit ISR against the bacterial phytopathogen *P. syringae* pv. *tomato* DC3000 while colonizing the rhizosphere of Arabidopsis (Matilla et al., 2010). To assess if *P. putida* KT2440 is also able to trigger ISR in plants of agronomic interest, we examined whether colonization of rice rhizosphere with this bacterium generated a protective effect against leaf blast disease, caused by *M. oryzae*. In addition, we also investigated the role of *pehA* in triggering ISR. Thus, for this set of assays, we used a null *pehA* mutant and a wild-type strain carrying the cosmid pCSSH1 (conferring 3–7 additional copies of *pehA*) along with wild-type KT2440. To ensure good colonization of the rhizosphere a combined seed-root-soil inoculation method was used, as described in Experimental procedures. Non-inoculated plants and a subset of plants treated with BTH, a synthetic salicylic acid analogue that confers disease resistance in rice (Shimono et al., 2007), were also included in the experiments as negative and positive controls respectively.

Data obtained by counting the numbers of susceptible-type lesions 6 days after challenge inoculation (see Experimental procedures) are presented as relative infection values compared to that of non-inoculated control plants (Figure 6). Pooled data from six independent experiments revealed that the treatment with *P. putida* KT2440 reduced leaf blast severity by 65% compared to non-treated plants. Interestingly, the treatment with BTH reduced rice blast severity by

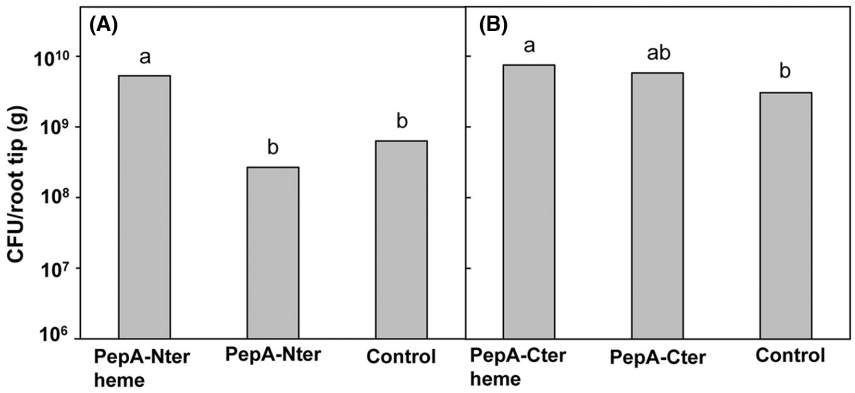

FIGURE 5 Effect of PehA-derived peptides on colonization capacity of the *P. putida* null *pehA* mutant. Two-day-old seedlings were incubated with a bacterial suspension for 30 min and subsequently incubated with a protein solution containing 1 μM of his-tagged PehA-Nter (A), his-tagged PehA-Cter (B), or buffer solution as a control. Bacterial cells were recovered from the root tips (0.7 cm; 10 plants per data point) at 4 days post planting and plated on LB-medium supplemented with the appropriate antibiotics. Experiments shown in panels (A) and (B) were performed separately. Statistical analysis was carried out using IBM SPSS statistics software (version 19 for windows). After checking normality using the Kolmogorov–Smirnov test, one-way analysis of variance with the Bonferroni post hoc test was used to compare means ($p < 0.05$). Different letters indicate significant differences between treatments.

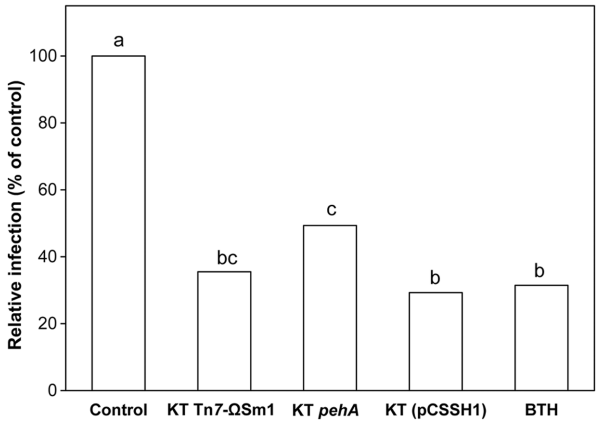

FIGURE 6 Quantification of systemic resistance induced by *Pseudomonas putida* KT2440 derivative strains against *Magnaporthe oryzae*. Four-week-old rice plants were challenged by spraying a spore suspension of virulent *M. oryzae* isolate Guy11 at 1×10^4 spores/ml. Control plants were treated with saline solution. For chemical induction of blast resistance, plants were sprayed with BTH 3 days before challenge. Disease index was determined 6 days after challenge by counting the number of susceptible-type lesions per leaf (scale >3) and expressed relative to challenged controls. Averages are shown and represent six independent experiments (with 12 plants per treatment per experiment). Different letters indicate significant differences between treatments according to Kruskal–Wallis followed by Wilcoxon comparison tests ($\rho = 0.05$).

almost 70% showing a level of protection similar to that observed in plants whose rhizosphere was colonized by the wild type or the strain carrying pCSSH1. Since no statistically significant differences in disease reduction were observed in plants inoculated with the null *pehA* mutant strain or the strain carrying cosmid pCSSH1 when compared to the wild type, we concluded that they were as effective as the parental strain in reducing the number of susceptible-type lesions produced by *M. oryzae* in rice. Nevertheless, the protection level was 20% higher with the strain overexpressing *pehA* in comparison to the null mutant (Figure 6), and the subset of plants colonized with the strain harbouring the cosmid pCSSH1 was found to be as much protected as that treated with BTH. These results indicate that *PehA* dose positively influences ISR.

Further evaluation of disease by scoring the highest lesion type present in each leaf (by using the 0–6 scale described in the experimental procedures) revealed significant differences between treated plants and the non-inoculated plants wherein a greater number of plants with severe lesions were observed if the plants were not treated (Figure 7). Although no significant difference was observed between bacterial treated plants, the trend of infection in those plants colonized by the strain harbouring cosmid pCSSH1 was similar to that observed in plants treated with BTH. Of particular interest in crop protection were the similarities found between these two groups: a smaller number of plants (15% less) with the most severe type of lesions found (type 5), and in particular a greater number of plants (more than triple) with non-susceptible-type lesions (type 0 to 2).

DISCUSSION

The mutant strain KTRPP2561 contains a transposon insertional inactivation of the gene *pehA*, previously named *pepA* (Matilla et al., 2007, 2010). We changed the name of the gene because a different gene of *P. putida* KT2440 appeared assigned with '*pepA*' in the Pseudomonas database (Winsor et al., 2016). KTRPP2561 is predicted to produce a truncated

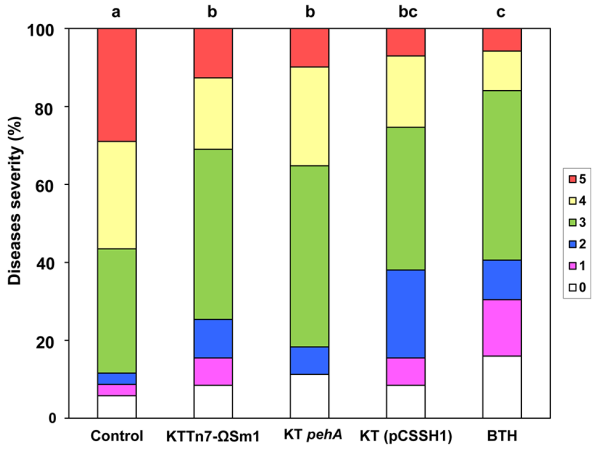

FIGURE 7 Influence of root treatments with *Pseudomonas putida* KT2440 strains containing different *pehA* doses on rice blast severity. Disease severity was assessed by scoring individual leaves from plants described in the legend of Figure 6. The severity scale (0–6) was measured by assessing the most severe lesion present on each leaf, as described in the experimental procedures. Type 6 lesions were not observed in any of the treatments. The percentage of leaves belonging to each severity group is shown. Different letters indicate significant differences between treatments according to Kruskal–Wallis followed by Wilcoxon comparison tests ($\rho = 0.05$).

protein of 733 aa that lacks the C-terminal part of *PehA*. The truncated *PehA* protein would therefore not contain a Repeat in Toxin (RTX) motif, a tandemly repeated calcium-binding sequence thought to be important for the secretion of this protein by a T1SS (Delepelaire, 2004), and presumably would be maintained intracellularly. For this current study, we generated a null *pehA* mutant to rule out any putative influence of the predicted truncated *PehA* protein on the mutant phenotype. This possibility could not be excluded with the previously reported mutant KTRPP2561.

The genome of *P. putida* KT2440 contains several genes encoding H_2O_2 scavenging enzymes (Nelson et al., 2002). Thus, the fact that a null *pehA* mutant was unaffected in its sensitivity to hydrogen peroxide was not unexpected given that a putative deleterious effect of *pehA* deletion upon resistance to the stressor might have been compensated by the induction of alternative resistance genes. For example the inactivation of the alkylhydroperoxide reductase (Ahp) system in *E. coli* did not affect the level of resistance to hydrogen peroxide because it correlated with a 10-fold induction of the catalase-peroxidase KatG (Seaver & Imlay, 2001).

Upon testing in the rhizosphere of maize no major phenotype was observed for the null *pehA* mutant, neither in colonization ability nor in fitness. However, slight mainly quantitative differences were detected in the profile of secondary metabolites present in the maize root exudates of plants incubated with the mutant strain when compared with that of plant roots incubated with the wild-type bacteria. Similarly, minor differences were previously detected in the root exudates of Arabidopsis plants colonized with KT2440 compared with those of plants colonized by the KTRPP2561 transposon mutant (Matilla et al., 2010). The influence of associated microorganisms on root exudates composition has been reported previously (Kamilova et al., 2006). Quantitative differences in the components of root exudates were previously observed when sweet basil plants were infected with wild-type or quorum-sensing mutant strains of *P. aeruginosa* (Walker et al., 2004). The changes observed in the exudation of monocotyledonous and dicotyledonous plants in the presence of KT2440 *pehA* mutants suggest that PehA may alter plant secretion and thus be involved in communication with plants. Alternatively, the peroxidase activity exhibited by PehA firstly reported by Santamaría-Hernando et al., 2012 may modify specific compounds in the exudates. In either case, we suggest that these variations might have a role in modulating the interaction of bacteria with the plant. The extracellular location of PehA, which has been immunologically detected on the bacterial surface of a *pehA* over-expressing strain (Figure S8) and is likely exported by the T1SS encoded by the flanking genes *pehBCD*, supports this suggestion. Anchoring to the bacterial cell of large adhesins secreted by T1SS, that is LapA from *P. fluorescens* (Newell et al., 2011), LapF from *P. putida* (Martínez-Gil et al., 2010) and SiiE from *Salmonella enterica* (Wagner et al., 2011) has been shown previously.

Genomic islands provide bacteria with different advantages by facilitating their adaptation to various niches and thus increasing their fitness and competitiveness (Dobrindt et al., 2004; Lawrence & Roth, 1996). As a relevant example, a genomic island of *Synechococcus* sp. CC9311 provided an increased tolerance to copper and oxidative stress to this marine organism (Stuart et al., 2013). The 22.1 kb insertion of cosmid pCSSH1 contains *pehA* and the *pehBCD* genes. These genes are part of genomic island number 24 of *P. putida* KT2440 (Wu et al., 2011). In spite of its size being twice that of the empty pLAFR3 from which is derived, introduction of pCSSH1 into *P. putida* provided strains with a significant competitive advantage in the rhizosphere. The conferred benefit was higher for the wild type and for the null *pehA* mutant strain than for the transposon insertional *pehA* mutant suggesting that factors other than *pehA* inactivation were causing the fitness decrease observed for this latter mutant (Matilla et al., 2007, 2010). One obvious possibility is that mutant KTRPP2561, besides lacking an active *PehA*, carries a load of truncated *PehA* protein and the subsequent protein aggregation reduces the fitness of bacterial cells (Bednarska et al., 2013).

To evaluate the contribution of the *pehA* locus to the bacterial fitness enhancement conferred by pCSSH1, we generated the null *pehA* mutant cosmid (pCSSH3).

Competition experiments between strains carrying pCSSH1 or pCSSH3 were conducted with the result that strains carrying pCSSH3 were almost entirely outcompeted. In addition, cosmid pCSSH3 did not confer an advantage and gave a comparable result to pLAFR3 strongly suggesting that *pehA* was the locus responsible for enhancing *P. putida* fitness in pCSSH1. We later confirmed this suggestion using plasmid pMIR185 which carries only the *pehA* gene. This plasmid is a derivative of pBBR1MCS-5 that has a higher copy number per cell than pCSSH1 (Antoine & Locht, 1992). Inclusion of pMIR85 in *P. putida* caused an extraordinary improvement in colonization fitness. The use of plasmid-based systems containing extra copies of certain genes to improve root colonization has been previously reported. Examples of this are riboflavin-synthesis genes of *Sinorhizobium meliloti* (Yang et al., 2002) and the site-specific recombinase *sss* of *P. fluorescens* WCS365 (Dekkers et al., 2000). The finding that a higher dosage of *pehA* provided by a stable cosmid/plasmid, without antibiotic selective pressure, may increase the bacterial fitness in the rhizosphere of monocotyledonous, and also dicotyledonous plants, with agronomic interest, like maize and alfalfa, suggests that *pehA* has potential to be applied in biotechnological processes. This is of particular importance since efficient rhizosphere colonization is often a prerequisite for bacteria to exert successful biological control (Chin-A-Woeng et al., 2000; Lugtenberg & Kamilova, 2009).

Following microbe recognition plants release a large amount of H_2O_2 (Barloy-Hubler et al., 2004) and other ROS in a process known as oxidative burst (Lamb & Dixon, 1997; Torres, 2010). Specially, young root parts such as the radical apices constitute the most active zone of ROS production (Liszkay et al., 2004) and therefore oxidative stress caused to bacteria is more severe in these locations. An increase in the *pehA* dosage provided by different vectors contributed to a better colonization of the complete root system (almost 30 fold) and the improvement was especially noticeable at the root tips (nearly 900 fold). In addition, the increase in the dose of this protein enhanced in two orders of magnitude resistance against the oxidative stressor H_2O_2 in vitro. Given that the ability to cope with oxidative stress was revealed to be important for the survival of *P. putida* in the rhizosphere (Matilla et al., 2007), an explanation for the fitness enhancement observed might be the peroxidase activity of *PehA*. In fact, the competitive advantage observed for the null *pehA* mutant in the maize root tip colonization experiments with heme-reconstituted PehA-purified fragments highlighted the importance of the enzymatic activity of *PehA* as the mechanism which improves rhizosphere colonization. In spite of the increased ability in the tip colonization capacity caused by the addition of exogenous heme-PehA peptides, we did not observe complementation in our competition experiments between *PehA*-overexpressing and *PehA* non-overexpressing strains, a result which is compatible with the evidences presented above on *PehA* remaining attached to the bacterial surface. Similarly, complementation of mutants in the cell-surface anchored adhesines LapA and LapF was not observed when incubated in competition with the wild-type strain (Martínez-Gil et al., 2010; Yousef-Coronado et al., 2008). Thus, we propose that the secretion of the peroxidase *PehA* to the extracellular milieu through the type I secretion system encoded by *pehBCD*, whose expression was induced by H_2O_2 (Figure S9A), may help the bacteria to cope with the plant-imposed oxidative stress and confer 'autoprotection' to *P. putida* under the oxidative stress conditions that prevail in the rhizosphere and especially in the root tips.

A higher dosage of *pehA* also increased bacterial fitness in co-culture under laboratory conditions; although to a much lesser extent (at least one order of magnitude) than in the rhizosphere, indicating that while this effect is not rhizospheric-specific, it is magnified in this niche. Interestingly, the fitness advantage provided by pCSSH1 in culture medium was initiated in the stationary phase of growth coinciding with the expression of *peh* genes (Figure S9B). The presence of pCSSH1 was also responsible for a 25% decrease in the number of dead cells after 72 h of culture. It is known that oxidative damage increases in stationary phase cultures (Dukan & Nystrom, 1998). By inactivating or overexpressing a superoxide dismutase a correlation between ROS levels and protein aggregation was observed and a connection between the amount of aggregated proteins and dead cells was established (Maisonneuve et al., 2008). Thus, the accumulation of damaged proteins in the stationary phase can lead to cell death in starved bacteria (Navarro Llorens et al., 2010). We propose that *PehA* reduces death of KT2440 in the stationary phase by protecting cells against oxidative damage.

The finding that a higher dose of *pehA* greatly increased the fitness of *P. putida* KT2440 in the rhizosphere of monocotyledonous and dicotyledonous plants prompted us to investigate whether the presence of extra-copies of this gene might also increase biocontrol ability of this bacterial strain in plants of agronomic interest. The potential of this strain to protect Arabidopsis against *P. syringae* pv. *tomato* through ISR has already been shown (Matilla et al., 2010). Rice blast caused by *M. oryzae* has been used as a model of host–parasite interaction (TeBeest et al., 2007) and its bacterial-mediated protection has been investigated in studies of ISR elicitation (Balmer et al., 2013). KT2440 colonized efficiently the roots of rice (not shown). In the present work, we demonstrated that *P. putida* KT2440 from the rhizosphere notably protects rice plants against the disease caused by the foliar pathogen *M. oryzae*. Given the absence of direct antagonism of the bacteria against

the fungus (our unpublished results) and their physical separation, we conclude that this protection was established by systemic resistance. The null *pehA* mutant and the strain overexpressing *pehA* were both able to protect rice plants although the protection level was 20% higher with the strain overexpressing *pehA*, indicating that PehA dose positively influences ISR. The treatment involving the strain with the highest *pehA* copy number was the most effective considering both the number of susceptible-type lesions and their severity and the results obtained with this treatment were similar to those obtained with the positive chemical control agent BTH.

Some bacterial ISR traits are effective only for specific plant-pathogen systems (Doornbos et al., 2012). As a relevant example, the pyocyanin secreted by *P. aeruginosa* 7NSK2 induces systemic resistance in rice against *M. oryzae*, whereas it increases susceptibility to the pathogen *R. solani* (De Vleesschauwer et al., 2006). *pehA* was shown to be an ISR determinant in Arabidopsis against bacterial disease (Matilla et al., 2010). In addition, we have confirmed here that its increased dosage caused an improvement upon fungal infection control in rice although a null *pehA* mutant still exhibited control potential. These results are compatible with *P. putida* presenting more than one determinant of ISR elicitation against rice blast; one being *pehA*. Whether *P. putida* has several ISR determinants with additive effects is currently unknown. This phenomenon, however, has been observed in other biocontrol bacteria (De Vleesschauwer & Höfte, 2009).

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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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