Major cereal crops benefit from biological nitrogen fixation when inoculated with the nitrogen-fixing bacterium *Pseudomonas protegens* Pf-5 X940

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Summary

A main goal of biological nitrogen fixation research has been to expand the nitrogen-fixing ability to major cereal crops. In this work, we demonstrate the use of the efficient nitrogen-fixing rhizobacterium *Pseudomonas protegens* Pf-5 X940 as a chassis to engineer the transfer of nitrogen fixed by BNF to maize and wheat under non-gnotobiotic conditions. Inoculation of maize and wheat with Pf-5 X940 largely improved nitrogen content and biomass accumulation in both vegetative and reproductive tissues, and this beneficial effect was positively associated with high nitrogen fixation rates in roots. ^{15}N isotope dilution analysis showed that maize and wheat plants obtained substantial amounts of fixed nitrogen from the atmosphere. Pf-5 X940-GFP-tagged cells were always reisolated from the maize and wheat root surface but never from the inner root tissues. Confocal laser scanning microscopy confirmed root surface colonization of Pf-5 X940-GFP in wheat plants, and microcolonies were mostly visualized at the junctions between epidermal root cells. Genetic analysis using biofilm formation-related *Pseudomonas* mutants confirmed the relevance of bacterial root adhesion in the increase in nitrogen content, biomass accumulation and nitrogen fixation rates in wheat roots. To our knowledge, this is the first report of robust BNF in major cereal crops.

Introduction

Nitrogen is essential to all living systems. Although dinitrogen is the most abundant gas in the Earth's atmosphere (78%), most organisms are not known to use this molecular form. Consequently, these organisms depend on fixed nitrogen for their nutritional requirements. Biological nitrogen fixation (BNF), i.e. the ATP-dependent reduction of dinitrogen to bioavailable ammonia, is widespread among prokaryotes but completely absent in multicellular organisms (Dos Santos *et al.*, 2012). However, BNF can directly benefit eukaryotes, including plants, animals and fungi, through symbiotic and endophytic associations with nitrogen-fixing bacteria and archaea, the best known example being the legume-rhizobium symbiosis (Oldroyd *et al.*, 2011).

Due to the limited bioavailability of nitrogen in the form of ammonium and nitrates, and the dependence of plant growth on these elements, nitrogen fertilizer application has been a crucial input for the high crop yields that drive modern agriculture (Galloway *et al.*, 2008). Unfortunately, a vast amount of non-renewable fossil fuel is required for the production and delivery of nitrogen fertilizers (Erisman *et al.*, 2007). In the last decade, the price of nitrogen

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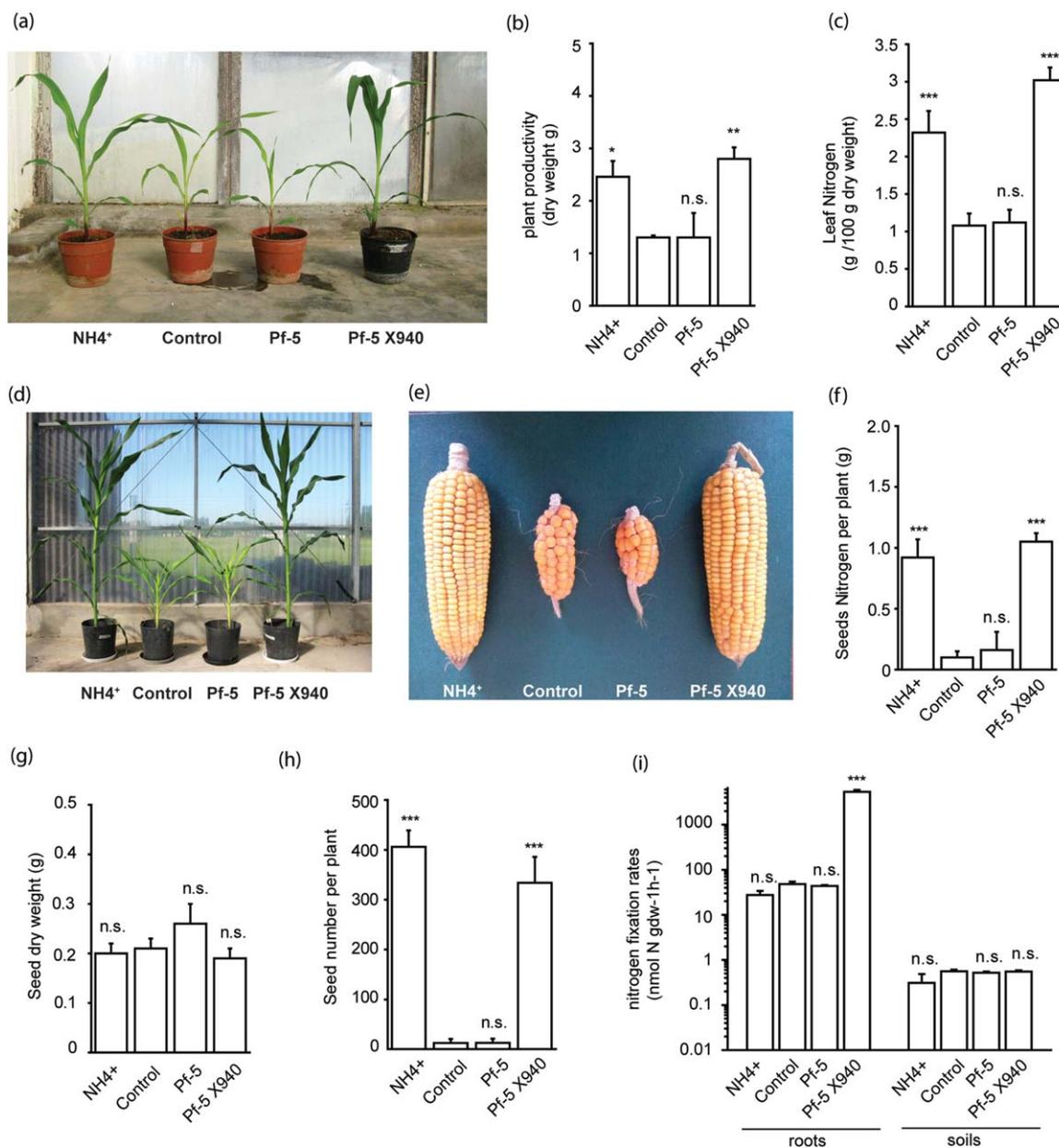
Fig. 1. Effects of the inoculation with Pf-5 X940 on maize productivity and nitrogen content. The biomass and nitrogen content of maize plants was analysed after exposure to four differential treatments: supplementation with ammonium (NH_4^+), no supplementation (Control), inoculation with the non-nitrogen fixing wild-type strain (Pf-5), and inoculation with the isogenic nitrogen-fixing strain (Pf-5 X940).

A. Representative images of two-month-old maize plants in 1-L pots.
B. Dry weight of two-month-old maize plants in 1-L pots.
C. Total nitrogen content in leaves from two-month-old maize plants in 1-L pots.
D. Representative images of maize plants in 10-L pots.
E. Representative images of corns from maize plants in 10-L pots.
F. Total nitrogen content in seeds from maize plants in 10-L pots.
G. Dry weight of seeds from maize plants in 10-L pots.
H. Seed production of maize plants in 10-L pots.
I. Nitrogen fixation rates within root and soil compartments after 2 months of treatment with the inoculants.
The statistical analysis was carried out with ANOVA followed by Dunnett's contrast test (***$P < 0.0001$, **$P < 0.001$*, $P < 0.05$, n.s. not significant). All values are means + SEM ($n = 24$).
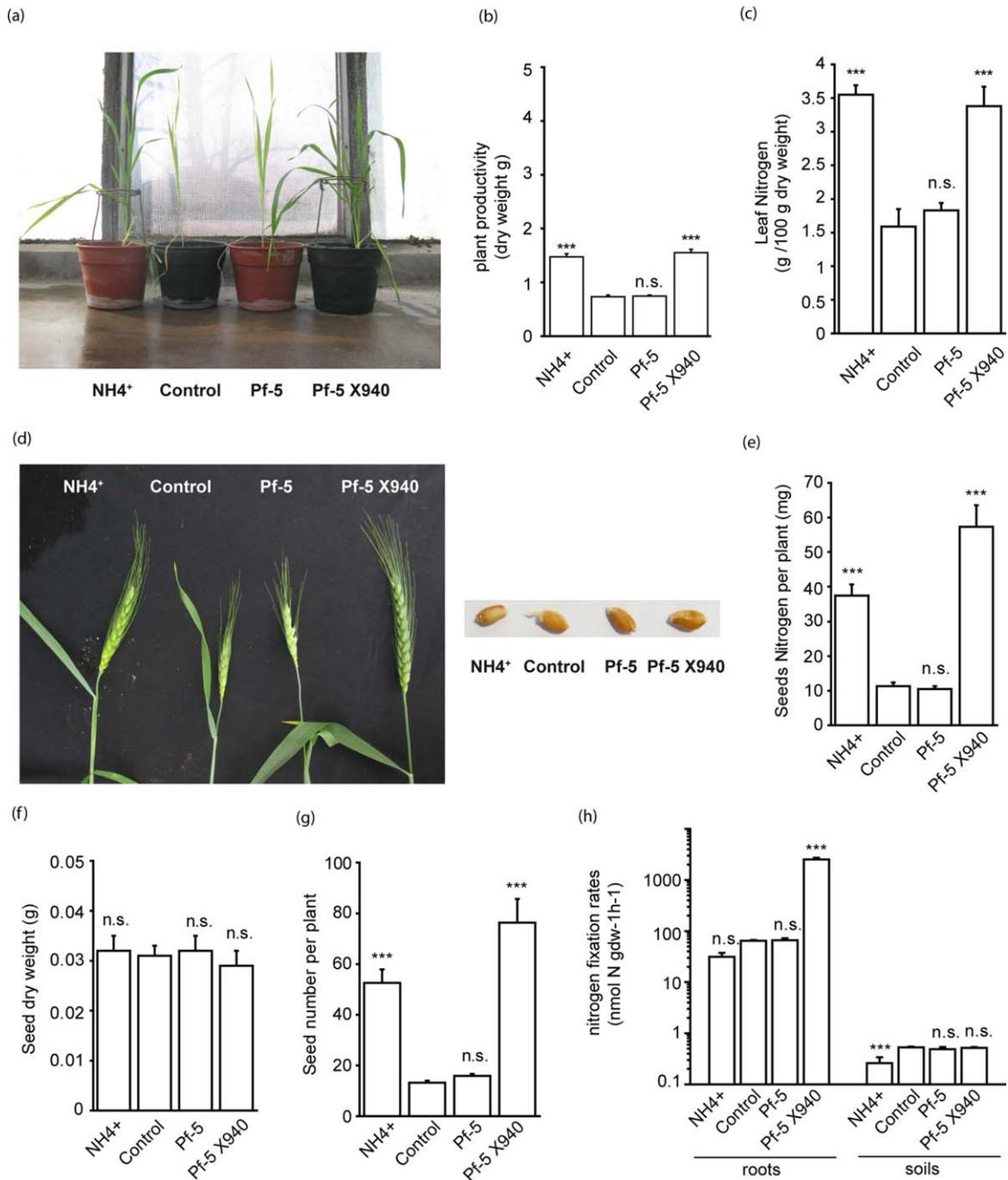
Fig. 2. Effects of the inoculation with Pf-5 X940 on wheat productivity and nitrogen content. The biomass and nitrogen content of wheat plants was analysed after exposure to four differential treatments: supplementation with ammonium (NH_4^+), no supplementation (Control), inoculation with the non-nitrogen fixing wild-type strain (Pf-5), and inoculation with the isogenic nitrogen-fixing strain (Pf-5 X940).
A. Representative images of two-month-old wheat plants.
B. Dry weight of two-month-old wheat plants.
C. Total nitrogen content in leaves from two-month-old wheat plants.
D. Representative images of wheat spikes (left) and seeds (right).
E. Total nitrogen content in seeds from wheat plants.
F. Dry weight of seeds from wheat plants.
G. Seed production of wheat plants.
H. Nitrogen fixation rates within root and soil compartments after 2 months of treatment with the inoculants.
The statistical analysis was carried out with ANOVA followed by Dunnett's contrast test (***$P < 0.0001$, n.s. not significant). All values are means + SEM ($n = 24$).
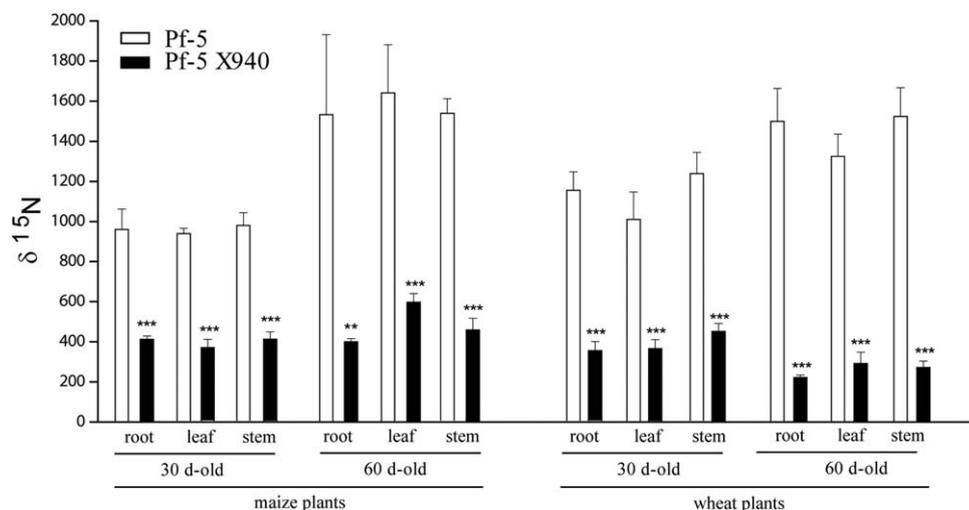
Fig. 3. Quantification of biological nitrogen fixation in strain Pf-5X940-inoculated maize and wheat plants. For the evaluation of BNF in maize and wheat plants inoculated with Pf-5 or Pf-5X940 using the isotope dilution assay, the ^{15}N atmospheric excess was analysed in different tissues after 1 or 2 months of treatment with the inoculants. All values are means + SEM ($n = 20$). Statistically significant differences between δ^{15}N levels are indicated by asterisks (Student's *t*-test, **$P < 0.01$, ***$P < 0.0001$).

fertilizers has increased more than 300%, mainly due to rising fossil fuel costs, partially related to the dwindling petroleum reserves (USDA, 2014). Additionally, nitrogen fertilizer application results in high emission of greenhouse gases, which constitute a key factor in climate change. Chemical nitrogen delivery is a largely inefficient process because around half of the nitrogen fertilizer applied is lost to leaching, resulting in significant health and environmental problems (Olivares *et al.*, 2013). Therefore, replacing chemical nitrogen fixation, both totally or partially, by BNF can attenuate our dependence on fossil fuels thereby generating a new perspective of agricultural sustainability (Farrar *et al.*, 2014).

Since the 1970s, when the first transfer of the nitrogenase complex was carried out, specifically the horizontal transfer of the *nif* cluster from the facultative anaerobe *Klebsiella pneumoniae* to *Escherichia coli* (Dixon and Postgate, 1971), scientists have speculated about the possibility of engineering cereal crops that could be self-supported by BNF (Venkateshwaran, 2015). There is a renewed interest in engineering these types of major crops for BNF and three general approaches have been recently proposed: (i) expressing heterologous nitrogenase in plant cells, (ii) engineering the symbiotic nodule into non-nodulating plants, and (iii) using plant-associative diazotrophs to fix nitrogen for the benefit of their non-legume host plants (Santi *et al.*, 2013; Oldroyd and Dixon, 2014; Geddes *et al.*, 2015). However, despite the remarkable advances in synthetic biology (Temme *et al.*, 2012; Wang *et al.*, 2013a; Wang *et al.*, 2013b; Yang *et al.*, 2014), this progress is not automatically extrapolated to any bacteria, mainly because most plant-colonizing microbes and plants themselves are aerobic organisms and nitrogenase is oxygen-sensitive (Stueken *et al.*, 2015). In addition, accessory factors for the protection of nitrogenase against oxygen are not yet understood (Geddes *et al.*, 2015). Engi-

neering bacterial competitiveness, persistence in the rhizosphere and ammonium release are also key steps for an efficient translation of the fixed nitrogen to plants (Geddes *et al.*, 2015).

Recently, we have transferred a genomic island (X940) with demonstrated nitrogenase activity from the polyhydroxybutyrate (PHB) – producing strain *Pseudomonas stutzeri* A1501 to the aerobic root-associated beneficial bacterium *Pseudomonas protegens* Pf-5. The nitrogen-fixing strain *P. protegens* Pf-5 X940 showed unrepressed expression of both nitrogenase structural and biosynthetic genes in association with the presence of high levels of nitrogenase activity (Setten *et al.*, 2013). In addition, we have demonstrated that heterologous PHB production can regulate heterologous nitrogenase activity in *P. protegens* Pf-5 X940 (Pascuan *et al.*, 2015). Interestingly, we have also shown that inoculation of different flowering plant species with Pf-5 X940 increases plant growth under sterile hydroponic growth-chamber conditions and that this beneficial effect could be attributed to the excretion of a high amount of ammonium to the medium (Setten *et al.*, 2013). In the present study, we demonstrate the use of the plant-colonizing bacterium Pf-5 X940 to support the nitrogen requirements of maize and wheat under non-gnotobiotic soil greenhouse conditions.

Results and discussion

Effects of inoculation with Pf-5 X940 on maize and wheat productivity and nitrogen content

To analyse the impact of the ammonium-excreting strain Pf-5 X940 on major cereal crops, we assessed the effect of the inoculation of this bacterium on the productivity and nitrogen content of maize and wheat plants by using an experimental system with non-gnotobiotic agronomic soil under greenhouse conditions. Plants were exposed to four

(a)

(b)

		Presence of GFP colonies	
		Pf-5-ChrGFP	Pf-5 X940-ChrGFP
Leaves	Phyllosphere	−	−
	Endophyte	−	−
Root	Rhizosphere	+	+
	Endophyte	−	−

− Absence
+ Presence

(c)

(d)

Maize

Wheat

···●··· Pf-5-ChrGFP
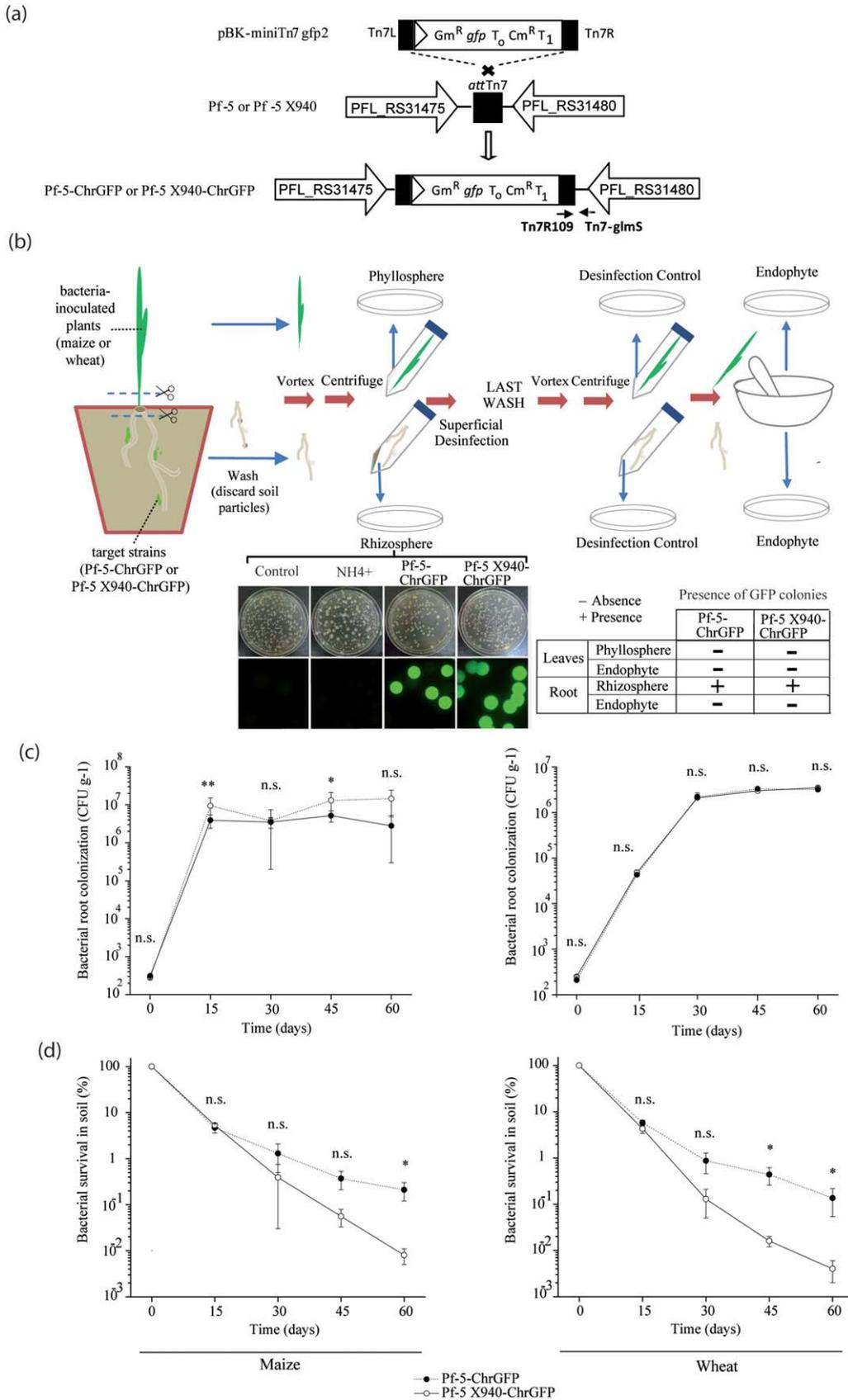—○— Pf-5 X940-ChrGFP

Fig. 4. Tagging of bacterial strains with chromosomal GFP markers and localization patterns of strain Pf-5 X940 in maize and wheat plants.
A. GFP genomic tagging of *Pseudomonas protegens* strains (Pf-5-ChrGFP and Pf5 X940-ChrGFP) by a chromosomal insertion at a predictable neutral *att* site downstream of the *glmS* gene (PFL_RS31480) was obtained by using the *gfp* delivery vector pBK-miniTn7-gfp2. Gm^R, gentamicin resistance; Cm^R, chloramphenicol resistance; mob+, mobilizable plasmid; To and T1, transcriptional terminators. Tn7R109 and Tn7-glmS primers were used to confirm the correct GFP cassette insertion downstream of the *glmS* gene.
B. Scheme showing the procedure designed to re-isolate strains Pf-5-ChrGFP and Pf5 X940-ChrGFP from different plant localizations (phyllosphere, rhizosphere, and endosphere) after 2 months of bacterial inoculation. A detection limit of 1×10^{-1} CFU of GFP-tagged-derived *Pseudomonas* strains/g of tissue was used. Each experiment was performed at least eight times and typical results are presented.
C. Colonization of Pf-5-ChrGFP and its nitrogen-fixing isogenic strain Pf5 X940-ChrGFP in root samples. The amount of bacteria in soil samples was determined in maize (left panel) and wheat (right panel) inoculation experiments under non-gnotobiotic soil greenhouse conditions. The statistical analysis was carried out with Student's *t*-test (**$P < 0.01$,*$P < 0.05$, n.s. not significant). All values are means + SEM ($n = 16$).
D. Survival of Pf-5-ChrGFP and its nitrogen-fixing isogenic strain Pf5 X940-ChrGFP in soil samples. The amount of bacteria in soil samples was determined in maize (left panel) and wheat wheat (right panel) inoculation experiments under non-gnotobiotic soil greenhouse conditions. One hundred percent survival was considered to be the number of bacteria at time zero (2×10^6 CFU g^{-1} soil). The statistical analysis was carried out with Student's *t*-test (*$P < 0.05$, n.s. not significant). All values are means + SEM ($n = 16$).

differential treatments: supplementation with fixed nitrogen (NH4$^+$), no supplementation (control), and inoculation either with the non-fixing wild-type strain Pf-5 or its isogenic nitrogen-fixing strain Pf-5 X940. Similarly to that observed in field conditions in the absence of added nitrogen fertilization (Witcombe *et al.*, 2008), the untreated plants and Pf-5-treated plants had a significantly lower biomass and nitrogen content than nitrogen-supplemented plants in both vegetative and reproductive tissues (Figs 1A–H and 2A–G). More importantly, this phenotype was completely reversed by inoculation with Pf-5 X940 (Figs 1A–H and 2A–G). Maize plants inoculated with Pf-5 X940 had increases of 115% in biomass accumulation (Fig. 1A–D), of 170% in nitrogen content of leaves (Fig. 1C), of 556% in nitrogen content in seeds (Fig. 1F) and of 2489% in terms of seed yield (Fig. 1E, G and H), with respect to plants inoculated with the parental isogenic strain Pf-5. Similarly, wheat plants inoculated with Pf-5 X940 showed increases of 112% in biomass accumulation (Fig. 2A and B), of 85% in nitrogen content of leaves (Fig. 2c), of 379% in seed yield (Fig. 2D, F and G), and of 473% in seed nitrogen (Fig. 2E), compared with plants inoculated with strain Pf-5. Similar to Pf-5 X940-treated Arabidopsis plants growing under sterile hydroponic growth-chamber conditions (Setten *et al.*, 2013), the phenotypic reversion of nitrogen deficiency in maize and wheat plants by Pf-5 X940 was positively associated with an increase in the inorganic nitrogen levels in non-gnotobiotic soil (Fig. S1). In addition, soil microbial activity was also increased in Pf-5 X940-treated maize and wheat plants (Fig. S2), which could be attributed to increasing amounts of root exudates (Welsh, 2000). Finally, the nitrogen fixation rates associated with isolated roots and soil without roots from the rhizosphere of the Pf-5 X940-treated maize and wheat plants were significantly increased only in isolated roots when exposed to Pf-5 X940 (Figs 1I and 2H), showing that nitrogen fixation is active in this organ.

Quantification of BNF in Pf-5 X940-treated maize and wheat plants

Substantial levels of BNF have been well documented in non-legume plant species such as elephant grasses (de Morais *et al.*, 2011), oil palms (Zakry *et al.*, 2012) and poplar trees (Knoth *et al.*, 2014) inoculated with either nitrogen-fixing single-strain or multi-strain consortia. In this study, we performed stable-isotope dilution analysis of inoculated plants, grown in greenhouse conditions with ^{15}N-supplemented soil, to measure the level of nitrogen fixation associated with Pf-5 X940-treated maize and wheat plants in comparison with plants inoculated with the non-fixing wild-type strain Pf-5 (Fig. 3). One month after inoculation, the δ^{15}N value was significantly lower in root, leaf and stem tissues of Pf-5 X940-treated maize and wheat plants than in those of Pf-5-treated plants (Fig. 3), suggesting that nitrogen fixation is active since the early stages of plant growth. Two months after inoculation, and relative to control Pf-5-treated maize and wheat plants, the nitrogen derived from gaseous nitrogen (%Ndfa) in different Pf-5 X940-treated maize and wheat plant organs was 74% and 85% for roots, 63% and 78% for leaves and 70% and 82% for stems respectively (Fig. 3). These results provide further evidence that maize and wheat plants are incorporating the nitrogen provided by BNF under non-gnotobiotic soil greenhouse conditions.

Localization pattern of Pf-5 X940 in maize and wheat plants

Although *P. protegens* Pf-5 was isolated from the cotton rhizosphere and is traditionally described as a root-colonizing strain (Loper *et al.*, 2007), no empirical evidence on the capacity of Pf-5 to colonize plant tissues has yet been reported. Additionally, the ability of Pf-5 X940 to fix nitrogen could change its plant-localization pattern with respect to the parent strain Pf-5. In this context, standard
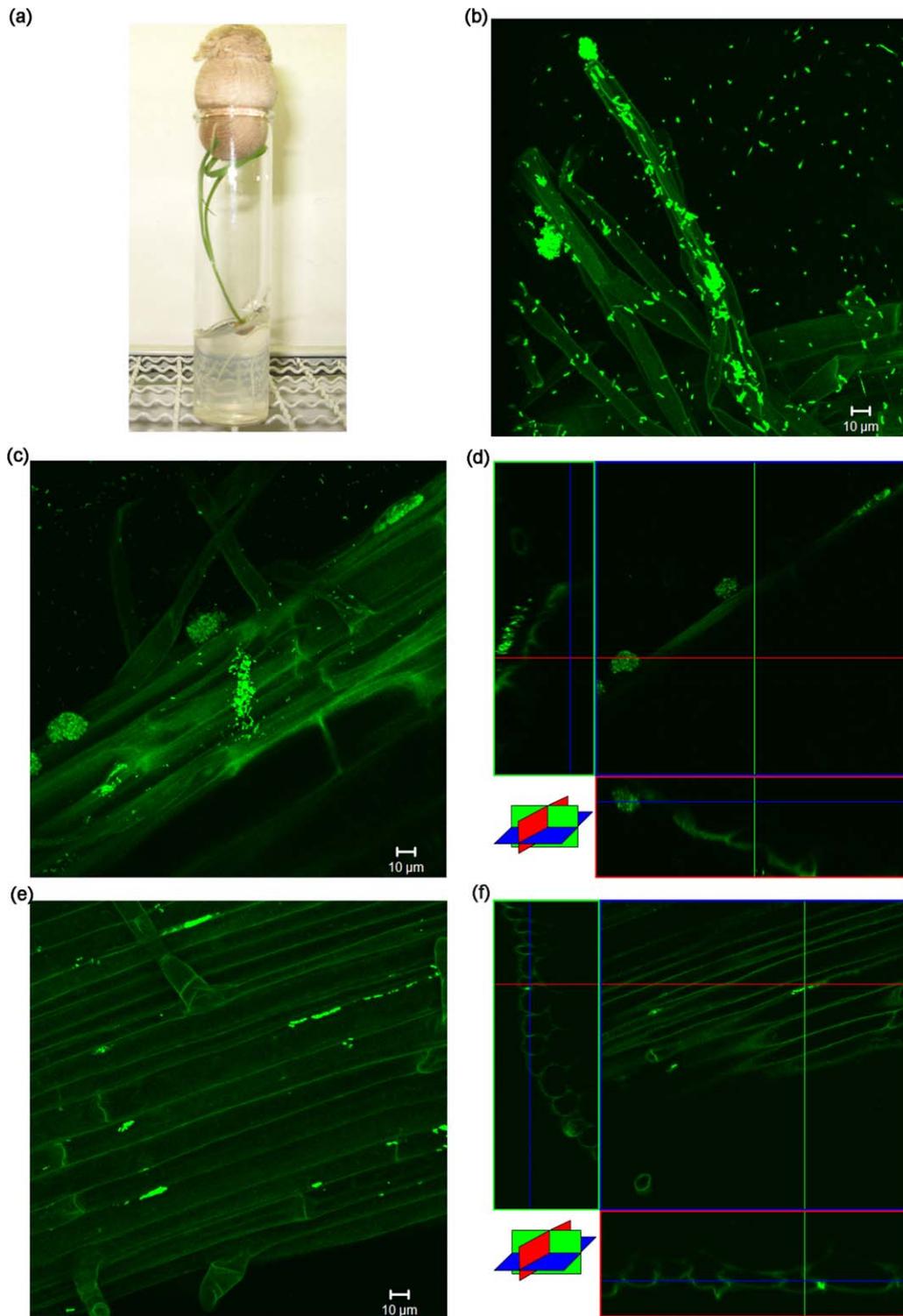
selective plating studies in minimal medium containing both chloramphenicol and octanoate as selective agents, coupled with fluorescence detection of *Pseudomonas* Pf-5 strains (Pf-5-ChrGFP and Pf5 X940-ChrGFP) carrying a stable and non-transmissible chromosomal single copy of the green fluorescent protein (GFP) (Fig. 4A), were carried out. The localization patterns of Pf-5-ChrGFP and Pf5 X940-ChrGFP in maize and wheat plants grown in non-

Fig. 5. Visualization under confocal laser scanning microscopy for fluorescent-tagged bacterium Pf5 X940-pMP4655GFP colonization on the surface of wheat roots.
Wheat plants were removed from the medium 7 days after bacterization with Pf5 X940-pMP4655GFP and root samples were visualized under confocal laser scanning microscopy (CLSM). The colonization pattern of GFP-tagged bacteria was similar during the following 21 days (data not shown). The size bar represents 10 μm in all panels.
A. Representative image of axenic wheat plants used for the analysis of bacterial colonization.
B. CLSM image showing Pf5 X940-pMP4655GFP microcolonies on root hairs.
C. CLSM image showing Pf5 X940-pMP4655GFP microcolonies on the surface of a lateral root.
D. Orthogonal planes of image c, showing Pf5 X940-pMP4655GFP microcolonies on the root surface.
E. CLSM image showing dispersed and large microcolonies of Pf5 X940-pMP4655GFP in a lateral root.
F. Orthogonal planes of image e, showing a representative microcolony of Pf5 X940-pMP4655GFP at the epidermal junction cells.

gnotobiotic soil under greenhouse conditions were examined in the phyllosphere, rhizosphere and endosphere (Fig. 4B). Two months after inoculation, Pf-5- and Pf5 X940-tagged cells were found colonizing the rhizoplane of maize and wheat plants but never as endophytes or epiphytes of the phyllosphere or within root tissues (Fig. 4B). The inability of Pf-5-ChrGFP and Pf5 X940-ChrGFP to internally colonize the roots could also impair the colonization of the vascular tissue and further prevent bacteria spread towards the phyllosphere. The long-term stable population size of Pf5 X940-ChrGFP on maize and wheat root surfaces along our experimental timeframe (i.e., 2 months) reached an average of 2.1×10^6 CFU g^{-1} and 1.1×10^7 CFU g^{-1} of root, respectively, which was not significantly different from that of the control Pf-5-ChrGFP strain (Fig. 4C). In contrast, survival of both Pf-5-ChrGFP and Pf5 X940-ChrGFP decreased rapidly in the soil following inoculation, and population sizes of Pf5 X940-ChrGFP were lower than those of Pf-5-ChrGFP, 60 days after inoculation of maize and wheat (Fig. 4D). Thus, our results may imply that the genetic modification for nitrogen fixation decreases bacterial survival in the rhizosphere in the long term, probably due to the expected fitness cost derived from the acquisition of nitrogen fixation genes (Pascuan *et al.*, 2015), but does not seem to impact upon the ability of the nitrogen-fixing bacteria to colonize the root surface.

Colonization pattern of Pf-5 X940 on the wheat root surface

By using confocal laser scanning microscopy (CLSM), we monitored the colonization pattern of Pf-5-pMP4655GFP and Pf-5 X940-pMP4655GFP expressing an enhanced GFP encoded by a multiple-copy vector on wheat roots under axenic conditions (Fig. 5A). After 7 days, Pf-5 X940-pMP4655GFP cells were localized on both root hairs and lateral roots, forming dispersed and large microcolonies (Fig. 5B–D). Fluorescent-tagged bacteria were always visualized on the root surface but never within root tissues (Fig. 5C–D). The root surface colonized with Pf-5 X940-pMP4655GFP clearly displayed the most abundant numbers of microcolonies at the junctions between epidermal plant root cells (Fig. 5E–F), where high concentrations of

root exudates are thought to leak from the root (Chin-A-Woeng *et al.*, 1997; Peters and Szilagyi, 2006). In addition, CLSM imagery also showed that the colonization pattern of Pf-5-pMP4655GFP cells was similar to that displayed by its derivative strain Pf-5 X940-pMP4655GFP (Fig. S3). The efficient root colonization by Pf-5 X940, and the abundance of this strain at the junctions between epidermal root cells could explain the nutrient availability to support the high energy demands of nitrogen fixation.

Effects of Pf-5 X940-root adhesion deficiency on wheat productivity and nitrogen content

Adhesion is a universal root colonization fitness factor for plant beneficial bacteria, including *Pseudomonas* (Barahona *et al.*, 2010). In *Pseudomonas fluorescens* strains, large adhesion protein (lapA) is a critical component for biofilm formation, and is specifically required for the initial step of bacterial adhesion (Hinsa *et al.*, 2003; Newell *et al.*, 2009; Duque *et al.*, 2013). Here, we analysed the relevance of bacterial adhesion on the beneficial effects promoted by Pf-5 X940 in wheat plants using lapA mutant strains (Fig. 6). As expected for biofilm formation-related mutants, lapA mutant strains (Pf-5 lapA::KmR and Pf-5 X940 lapA::KmR) were unable to colonize wheat roots, whereas wild-type lapA strains (Pf-5 and Pf-5 X940) and lapA mutant strains complemented with a wild-type copy of the lapA gene (Pf-5 lapA::KmR+lapA and Pf-5 X940 lapA::KmR+lapA) showed efficient wheat root colonization (Fig. 6A). In addition, lapA mutation seems not to have affected bacterial survival in the soil compartment, which is consistent with a mutation in a structural gene that has no pleiotropic effects (Fig. 6B). In contrast to that observed in Pf-5 X940-treated plants, inoculation of wheat with Pf-5 X940 lapA::KmR did not improve nitrogen content (Fig. 6C), biomass accumulation (Fig. 6D) or nitrogen fixation rates in roots (Fig. 6E), suggesting that root adhesion is a critical factor for the wheat benefit from BNF when inoculated with nitrogen-fixing *Pseudomonas*. Complementation of the lapA mutation restored the beneficial effects in wheat plants produced by Pf-5 X940 (Fig. 6C–E), confirming the importance of the close plant-microbe physical interaction for effective BNF.
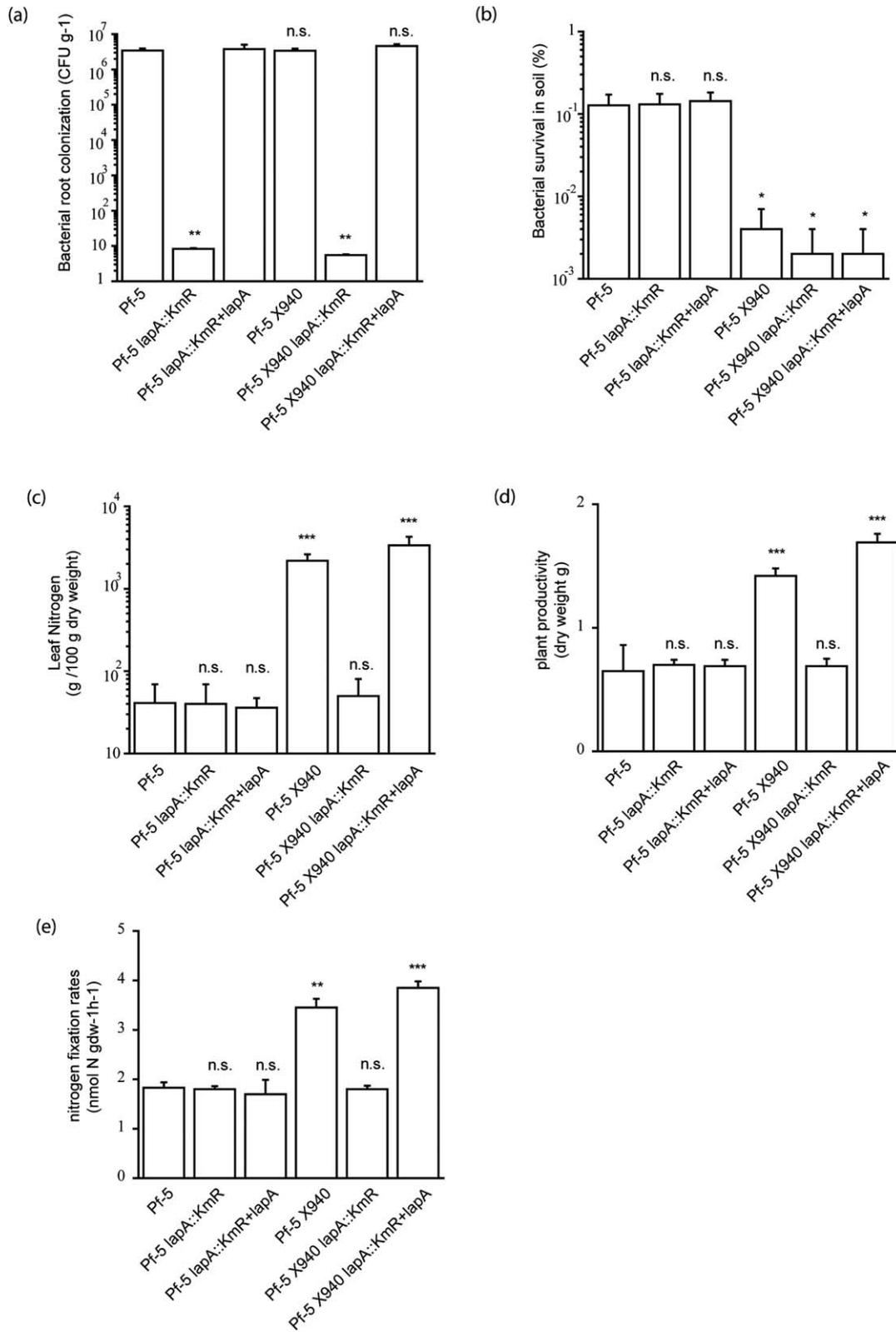
Fig. 6. Effects of Pf-5 X940-root adhesion deficiency on wheat productivity and nitrogen content. Bacterial root colonization, survival of bacteria in soil compartment, plant productivity, leaf nitrogen content and nitrogen fixation rates in roots in 2-month-old wheat plants treated with different bacterial inoculants under non-gnotobiotic soil greenhouse conditions were analysed. The statistical analysis was carried out with ANOVA followed by Dunnett's contrast test (***$P < 0.0001$, **$P < 0.001$, *$P < 0.05$, n.s. not significant). All values are means + SEM ($n = 20$).

Conclusions

Previous studies have reported the potential for ammonia-excreting diazotrophic bacteria to supply fixed nitrogen to non-legume plants, including model plant species or minor crops. Our results show that the ammonia-excreting *P. protegens* Pf-5 X940 strain substantially improves the growth and the nitrogen content of maize and wheat under non-gnotobiotic soil greenhouse conditions. This outcome is a consequence of BNF and root colonization by the bacterium is necessary to produce this beneficial effect. Therefore, under tested experimental conditions and for varieties here used, inoculation of major cereal crops with strain Pf-5 X940 can fully replace nitrogen fertilization. The competition with the indigenous microbial communities and the adaptation to different soil oxygen levels could potentially be critical factors affecting BNF in major cereal crops inoculated with strain Pf-5 X940 under field conditions. Related to this, it may be interesting to evaluate whether cereal-endophytic, nitrogen-fixing bacteria, such as *P. fluorescens* PICF7 (Mercado-Blanco *et al.*, 2016) harboring the X940 cosmid, are capable to overcome such potential constraints, thereby expanding the use of nitrogen-fixing inoculants to diverse climates and soils. To our knowledge, this is the first report of robust BNF in major cereal crops.

Experimental procedures

Bacterial strains and plant species

The strains used in this study were the wild type strain *Pseudomonas protegens* Pf-5 (Howell and Stipanovic, 1979) and its derived strains Pf-5 X940 (Setten *et al.*, 2013), Pf-5 lapA::KmR and Pf-5 lapA::Km+lapA (Ayub *et al.*, 2015). The commercial cereal crops (wheat and maize) used were *Triticum aestivum* (Bobwhite 26) and *Zea mays* (Pannar BIOGENE BG6607YR).

Pseudomonas *strains containing the GFP marker within a high-copy-number vector*

Pf-5 and Pf5 X940 competent cells were transformed with the multiple-copy vector pMP4655 to constitutively express the enhanced green fluorescent protein (Bloemberg *et al.*, 2000). Briefly, *Pseudomonas* competent cells were prepared as follows: 500 ml of LB medium was inoculated with 5 ml of an overnight culture and cells were grown under agitation (250 rpm) at 28°C until a 0.5 OD_{600}. Cultures were chilled on ice for 20 min and centrifuged at 4000 × *g* and 4°C for 15 min. Pellets were resuspended in 500 ml of glycerol 10% v v^{-1} previously chilled on ice and centrifuged at 4000 × *g* and 4°C for 15 min. This step was repeated three times, resuspending cells in each step in a smaller volume of 10% v v^{-1} chilled glycerol: 250, 20 and 1 ml. A volume of 40 μl of competent cells was electroporated with the pMP4655 vector (Prieto and Mercado-Blanco, 2008). Transformants were selected by plating on LB agar containing 75 μg ml^{-1} of kanamycin. After incubation at 28°C for 24 to 48 h, the colonies that arose were screened for GFP fluorescence under the microscope and magnifying glass. The resulting GFP-tagged strains were named Pf-5-pMP4655GFP and Pf5 X940-pMP4655GFP.

GFP chromosomal tagging of Pseudomonas *strains*

A single copy of the gene encoding GFP was introduced at a neutral chromosomal site into strains Pf-5 and Pf-5 X940, by using a mini-Tn*7* delivery system (Koch *et al.*, 2001). Briefly, *E. coli* SM10λpir (Miller and Mekalanos, 1988) carrying the Tn*7* transposition helper vector pUX-BF13 (Bao *et al.*, 1991), *E. coli* DH5α carrying the *gfp* delivery vector pBK-miniTn*7*-*gfp*2 (Koch *et al.*, 2001), *E. coli* MT616 carrying the mobilizing plasmid pRK600 (Finan *et al.*, 1986), and the target strains Pf-5 and Pf-5 X940 were combined in a tetraparental mating on nutrient agar and incubated overnight at 37°C. This conjugation mixture was then resuspended in saline solution (0.9% w v^{-1} NaCl) and spread onto 0.5 NE2 medium agar plates (Ayub *et al.*, 2006), where *E. coli* cannot grow, containing 10 μg ml^{-1} gentamicin (for Pf-5) or 10 μg ml^{-1} gentamicin and 25 μg ml^{-1} kanamycin (for Pf-5 X940). After incubation at 28°C for 72–96 h, the colonies that arose were screened for GFP fluorescence under the microscope. Fluorescent and gentamicin-resistant colonies were subjected to colony PCR targeting the *Pseudomonas*-specific *gap-1* gene, and the correct genomic *gfp* integration at the *att*Tn*7* site was confirmed in the colonies positive by PCR with the specific oligonucleotides Tn7R109 and Tn7-glmS (Lambertsen *et al.*, 2004). GFP-tagged-derived strains were called Pf-5-ChrGFP and Pf5 X940-ChrGFP.

Plant productivity and nitrogen determinations

Wheat and maize seeds were sown in 1-l or 10-l pots containing a mixture of soil: vermiculite (1:1), with an average nitrogen content of 21 mg kg^{-1} (inorganic + organic nitrogen). The soil was collected from the Pampean Region, Buenos Aires, Argentina. This soil, under good agricultural practices, contains diverse indigenous microorganisms (Figuerola *et al.*, 2012). For the inoculation assays, bacteria were prepared as follows: Pf-5 and Pf-5 X940 were grown overnight in LB medium at 28°C with shaking (250 rpm), and then centrifuged and resuspended in an equal volume of saline solution (0.9% w v^{-1} NaCl). After that, 1 or 10 ml of the bacterial solution containing 10^8 or 10^9 colony forming units per ml (CFU ml^{-1}) were used to inoculate plants in 1-l or 10-l pots respectively. Bacterial inoculation was performed during sowing, as described below. First, the pots were saturated with tap water, and then, the seeds were sown to a depth of 1 cm. Subsequently, the holes generated by seeding were filled with soil. Finally, the bacterial suspensions were added in the middle of the pots. Nitrogen fertilization positive controls were performed adding 400 mg or 4 g of $(NH_4)_2SO_4$ in the 1-l and 10-l pots respectively. This fertilization was performed twice: on the sowing day and 20 days later. Plants were grown in a greenhouse and irrigated with non-sterile tap water. The greenhouse temperature ranged between 9°C and 18°C for wheat and between 16°C and 25°C for maize, with an average photoperiod of 10 and 14 h for wheat and maize respectively.

Supplemental lighting was used at an intensity of 198 μmol m^{-2} s^{-1}. Maize controlled fecundation was performed by hand pollination. For this, male and female inflorescences were covered with paper bags. Pollen collected from each plant was applied to a silk of a different plant (from the same treatment) every day during the receptive period of the silks (approximately 5 days). To measure dry weight, the aerial part of 2-month-old plants and seeds were dried for 48 h at 60°C. Seed number and seed weight were recorded for each plant. Soil mineral nitrogen was measured as previously described (Setten *et al.*, 2013) and total nitrogen content in plant matter was established by the Kjeldahl method. Briefly, 150 mg of plant dry matter (green tissue or seed) were digested and distilled on VELP units following the company's instructions (VELP Scientifica, Italy). Distilled nitrogen (ammonium) was titrated using 0.005 N H$_2$SO$_4$. Nitrogen determinations were performed in Laboratorios Fox (www.foxlab.com.ar). Dehydrogenase activity in soil samples was analysed by the reduction of triphenyltetrazolium chloride according to Casida *et al.* (1964). The nitrogen fixation process in the roots, stems and leaves (15 mg dry weight) was measured in 10-ml tubes filled with well water sterilized by filtration. Homogenized soil samples without roots were transferred to the pre-weighed containers by using a plastic syringe with the luer tip removed. Then, 2 ml of these soil samples were transferred to 10-ml tubes. The upper space of the containers was exchanged by gaseous nitrogen. Nitrogenase activity was evaluated by acetylene reduction as previously described (Setten *et al.*, 2013). For root-adhesion assays, the cosmid X940 was introduced by transformation into competent cells of the Pf-5-derived lapA mutant (Pf-5 lapA::KmR) and its complemented derived strain (Pf-5 lapA:Km+lapA) (Ayub *et al.*, 2015). Transformants were selected by plating on L agar medium without (NH4)$_2$SO$_4$ according to Setten *et al.* (2013). The recombinant strains containing the cosmid X940 were named Pf-5 X940-lapA::KmR and Pf-5 X940-lapA::KmR+lapA. BNF was evaluated in the lapA mutants by acetylene reduction assays in Erlenmeyer flasks with plastic screw caps under nitrogen-limiting conditions according to Setten *et al.* (2013).

Bacterial localization assays on maize and wheat-soil system

Greenhouse maize and wheat plants were inoculated with *Pseudomonas* strains as described above, but using the GFP-tagged strains Pf-5-ChrGFP and Pf5 X940-ChrGFP. Bacterial persistence and colonization of maize and wheat roots and soil were studied for 2 months after inoculation. First, the aerial green tissues were cut 1 cm above the soil, washed with tap water and placed in 50-ml tubes containing 25 ml of saline solution (0.9% w v^{-1} NaCl). Then, the roots were carefully washed with tap water to remove adhering soil particles, and placed in 50-ml tubes containing 25 ml of saline solution. The tubes were vortexed for 2 min and centrifuged at 4000 × *g* and 4°C for 15 min. The supernatants were removed, leaving 1 ml of saline solution in each tube. For surface colony counts, named phyllosphere or rhizosphere depending on their tissue origin, aliquots were plated in selective minimal medium NL (7.5 mM KH$_2$PO$_4$, 17.22 mM K$_2$HPO$_4$, 3.42 mM NaCl,

7.57 mM (NH$_4$)$_2$SO$_4$, 2 mM MgSO$_4$ 7 H$_2$O, 3.7 μM FeCl$_3$ 6 H$_2$O, 0.1 μM CuCl$_2$ 2 H$_2$O, 0.1 μM ZnSO$_4$ 7 H$_2$O, 0.73 μM MnCl$_2$ H$_2$O, 1 μM CaCl$_2$ 2 H$_2$O, 0.21 μM NaMoO$_4$, 3.4 mM citric acid, 0.25% w v^{-1} octanoate and 30 μg ml^{-1} chloramphenicol, pH = 7) and incubated for two days at 28°C to determine the number of CFU. For surface-sterilization of plant tissues, the tubes were vortexed with a solution 1% v v^{-1} sodium hypochlorite, 0.1% w v^{-1} SDS, 0.2% v v^{-1} Tween-20 for 2 min. The solution was discarded and tissues were washed with sterilized distilled water. Then, a solution 70% v v^{-1} ethanol was added to the tubes and incubated for 3 min. After that, the tissues were washed four times with sterilized distilled water. The tissues were then vortexed in the last wash for 1 min. The tubes were centrifuged at 4000 × *g* and 4°C for 15 min. The supernatants were removed, leaving 1 ml of saline solution. Then, 100 μl was plated in LN medium to determine the number of CFU, as described above, as disinfection control. Experiments showing bacteria in this control were discarded. Tissues were macerated using a mortar on saline solution (1 or 2 ml depending on the amount of tissue) and aliquots were prepared to determine the CFU of endophytes using LN medium as described previously. To analyse the persistence of bacteria outside the host plant, the soil from each pot was vigorously mixed and two 8-g samples were taken: one to analyse the amount of Pf-5-ChrGFP and Pf5 X940-ChrGFP strains and the other to determine soil dry weight. To determine the amount of GFP-tagged bacteria, the soil was mixed with 40 ml of saline solution, vortexed for 2 min and aliquots taken to determine the CFU of bacteria present in the soil as described above. In all the cases, Pf-5-ChrGFP and Pf5 X940-ChrGFP counts were performed analyzing the number of GFP-expressing colonies using a fluorescence magnifying glass. The phyllosphere, rhizosphere and endophytic compartments were operationally defined as schematically shown in Fig. 4B.

Visualization of bacterial root-colonization by CLSM

Wheat seeds were surface-sterilized using 2% v v^{-1} sodium hypochlorite for 3 min, 70% v v^{-1} ethanol for 3 min and washing three times with sterilized distilled water. Seeds were incubated on petri dishes with 1% agar water for 24 h before inoculation. Pf-5-pMP4655GFP and Pf5 X940-pMP4655GFP were cultured overnight, centrifuged at 4000 × *g* for 15 min, washed with saline solution (0.9% w v^{-1} NaCl) and diluted to an OD$_{600}$ 0.15. Seeds were incubated with the bacterial suspension for 10 min and placed on filter papers for 2 min to remove the excess bacterial suspension. Then, seeds were transferred to sterile cotton-stoppered glass tubes with 0.4% w v^{-1} agar-water medium. Plants were grown for 21 days in a growth chamber, with a 16 h light/8 h dark cycle and a constant temperature of 25°C. After 21 days, roots were washed with saline solution, cut in small pieces (around 1 cm) within 2–8 cm of the root tip. The root sections were observed by scanning different focal planes of the surface using a C-Apochromat 40X/1.2 objective from a laser scanning confocal microscope (LSM 510 Pascal, MicroImaging, Oberkochen, Germany), using a 488 nm argon laser excitation and

500 nm long pass emission filter. Representative projections of CSLM images are shown.

^{15}N isotope dilution analysis

An isotope dilution strategy (McAulie *et al.*, 1958), consisting in treating the 1-l pots containing the maize and wheat-soil system with 36 mg of $NH4_2(SO_4)$ with 10 atom % ^{15}N excess on the sowing day, was used to measure the incorporation of nitrogen derived from BNF into maize and wheat under greenhouse conditions as described above with slight modifications. Pf-5- and Pf5 X940-treated plants growing in ^{15}N-supplemented soil for one or 2 months were harvested to evaluate the quantity of nitrogen obtained by BNF. Whole plants were washed with distilled water and then different tissues were separated and dried at 67°C for 4 days. The tissues were weighed and finely ground and 6-µg samples were taken to analyse the nitrogen content by using continuous-flow mass spectrometer ANCA-SL (Europa Scientific). Stable isotope ratios were reported as $\delta^{15}N$, a value given in parts per thousand (‰). The nitrogen derived from gaseous nitrogen (%Ndfa) in wheat plants inoculated with Pf-5 X940 was measured using the following calculation: %Ndfa = 100 [1 − ($\delta^{15}N$ Pf-5 X940-treated plants/Pf-5-treated plants)].

Acknowledgements

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Effects of the inoculation with Pf-5 X940 on the levels of inorganic nitrogen in soil. Two months after inoculation, nitrate and ammonium compounds were measured in the soils. The statistical analysis was carried out with ANOVA followed by Dunnett's contrast test (**$P < 0.001$, *$P < 0.05$, n.s. not significant). All values are means + SEM ($n = 16$).

Fig. S2. Effects of the inoculation with Pf-5 X940 on the levels of microbial activity in soil. Two months after inoculation, dehydrogenase activities were measured in the soils. The statistical analysis was carried out with ANOVA followed by Dunnett's contrast test (***$P < 0.0001$, n.s. not significant). All values are means + SEM ($n = 16$).

Fig. S3. Visualization under confocal laser scanning microscopy of colonization by the fluorescent-tagged bacterium Pf5-pMP4655GFP on the surface of wheat roots. Picture A, C and D show CLSM images of Pf-5 GFP-tagged cells during root surface colonization, and picture B shows orthogonal planes of the confocal image presented in picture A.