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Changes of enzyme activities related to oxidative stress in rice plants inoculated with random mutants of a *Pseudomonas fluorescens* strain able to improve plant fitness upon biotic and abiotic conditions

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Abstract. The *Pseudomonas fluorescens* strain used in this work (Aur 6) has demonstrated its ability to improve fitness of different plant species upon biotic and abiotic stress conditions. Random mutants of this strain were constructed with the Tn5 transposon technology, and biological tests to evaluate loss of salt protection were conducted with all the mutants (104 mutants) on rice seedlings. Mutant 33 showed an evident reduction in its ability to protect plants upon salt stress challenge, whereas mutant 19 was more effective than the wild type. Enzymes related with oxidative stress were studied in both mutants and wild type. Enzyme activities were decreased with mutant 33 with regard to wild type, whereas mutant 19 did not produce important changes suggesting involvement of redox balance associated to the observed modifications in these antioxidant enzymes as one of the probable mechanisms used by these strains. Data of malondialdehyde (MDA) were consistent with this fact. Mutants also affected accumulation of proline, the most common osmolyte in plants. A second experiment to evaluate the ability of both mutants and wild type to stimulate growth on tomato plants was conducted, as this feature was previously demonstrated by wild type. Similar results were obtained in growth of both species, suggesting that mutations of both mutants are related with the capacities of the wild type to stimulate growth. To reveal mutated genes, both mutants were mapped. Three mutated genes were found in mutant 33. A gene related with a general secretion pathway protein D, a gene related with a putative two-component system sensor kinase (ColS), and a gene related with flagellar motor switch protein (FliG). In mutant 19, two mutated genes were found. One gene related with heavy metal efflux pump Czca family, and other gene of 16s rRNA.

Additional keywords: antioxidant enzymes, PGPR, reactive oxygen species, rice, ROS, salt stress.

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Introduction

Due to their sessile nature, plants are continually exposed to stress situations – both biotic and abiotic. As a consequence, the survival of plants depends on their ability to rapidly adjust their physiology, development and growth to escape or mitigate the impacts of stress. All plants are able to perceive and respond to stress signals such as drought, heat, salinity, herbivory, and pathogens (Hirt 2009; de Zelicourt *et al*. 2013). Some responses are common to various stresses, including production of certain proteins and adjustment of primary metabolism (de Zelicourt *et al*. 2013).

Soil salinity is one of the major abiotic stresses. Salt stress affects plant growth and productivity altering their physiology mainly reducing nutrient uptake (Singh *et al*. 2011). Salt stress is also linked to an oxidative stress as a consequence of the generation of reactive oxygen species (ROS), such as superoxide ion, hydrogen peroxide and hydroxyl radicals, which are detrimental to plant survival. Salt-stressed plants display a complex enzyme oxidative defence strategy involved in scavenging of these ROS in response to oxidative stress (Kohler *et al*. 2009).

Plants interact during their growth with microorganisms that live in the soil, some of them beneficial micro-organisms called 'plant growth promoting rhizobacteria' (PGPR) (Lugtenberg and Kamilova 2009) or 'plant health promoting rhizobacteria' (PHPR) (Hayat *et al*. 2010). These terms include bacteria able

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to enhance plant growth, either by indirect mechanisms such as improving plant nutrition solubilising and mineralising nutrients (de Freitas *et al*. 1997; Richardson *et al*. 2009; Friesen *et al*. 2011), or by direct mechanisms such as producing plant growth regulators (Gutiérrez Mañero *et al*. 2001; Lucas García *et al*. 2003; Lucas García *et al*. 2004*b*), producing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase to reduce the level of ethylene in the root, increasing root length and growth (Penrose and Glick 2003), preventing successful invasion of pathogenic microorganisms triggering plant's defensive metabolism (Cattelan *et al*. 1999; Pal *et al*. 2001; Ramos-Solano *et al*. 2010*b*; Zhang *et al*. 2004), or enhancing resistance to drought (Alvarez *et al*. 1996), salinity, waterlogging (Saleem *et al*. 2007) and oxidative stress (Štajner *et al*. 1995, 1997), among others. PGPR belong to several different bacterial genera, including *Rhizobium*, *Bacillus*, *Pseudomonas* and *Burkholderia* among others.

Some PGPR can trigger the plant's defensive metabolism systemically, a phenomenon called induced systemic resistance (ISR) (van Loon *et al*. 1998). This systemic induction of the plant defences keeps it on alert for any situation of stress. This physiological status of the plant has been termed 'priming' (Conrath 2011). Frequently, this status produces redirection of carbon sources to defensive metabolism which may compromise plant growth (van Hulten *et al*. 2006). Primed plants show faster and/or stronger activation of defence responses when subsequently challenged by microbes, insects, or abiotic stress (as salt stress), and this is frequently linked to development of local and systemic immunity and stress tolerance (Conrath *et al*. 2006). The ISR is a plant-mediated mechanism associated to priming, and it is widely accepted that transduction signals generated in response to different stress situations are common.

The strain used in this work (*Pseudomonas fluorescens* denoted as Aur 6) has demonstrated capacity to induce changes in the physiological status of different plants, being able to increase growth and provoke priming, improving resistance against biotic and abiotic stress in rice and tomato among other plant species (see 'Materials and methods'). The objective of this work was to unravel the mechanisms involved in protection against salt stress and to stimulate plant growth in the chosen strain. To achieve this objective, random mutants were constructed, and biological tests to determine the loss of capacity to protect against salt stress challenge were carried out with all mutants in rice. To try to determine the mechanisms involved, activities of enzymes related with oxidative stress, oxidative stress level and proline accumulation were studied under the influence of the two mutants affected; these were also assayed in tomato to evaluate effects on growth. Subsequently, the mutated genes were mapped to attempt to relate these genes with the loss of the mutant ability to alter the physiological status of the plant against stress.

Materials and methods

Strain used

The strain used was Aur 6. This strain was isolated from the rhizosphere of *Lupinus hispanicus* (Gutiérrez Mañero *et al*. 2003). It was identified by 16s DNA sequencing as *Pseudomonas fluorescens* (GenBank accession number HM486749) and was deposited in the Spanish Culture Type Bank (CECT 5398). Aur 6 is able to produce auxin-like compounds (1.48 μg mL^{-1} IAA-like), is also able to solubilise phosphate and degrade 1-aminocyclo- propane-1 carboxylic acid (ACC) (Gutiérrez Mañero *et al*. 2003). It has shown a growth promoting effect on *Lupinus* sp. (Lucas García *et al*. 2003), tomato and pepper (Cezón *et al*. 2003), pine and holm-oak tree (Lucas García *et al*. 2004) and has shown ability to induce systemic resistance against *Pseudomonas syringae* DC3000 in *Arabidopsis thaliana* (L.) Heynh. (Ramos Solano *et al*. 2008), and against salt stress (Barriuso *et al*. 2008). In addition, it has also demonstrated biocontrol ability against *Xanthomonas campestris* in tomato, alone and in combination with other bacterial strains (Domenech *et al*. 2006), and in rice against *Pyricularia oryzae* (Lucas *et al*. 2009). This strain is sensitive to kanamycin and resistant to nitrofurantoin.

Bacterial strains, plasmid, transposon and mutagenesis

Aur 6 strain described above was used as recipient. *Escherichia coli* S17–1 pir carrying pUTmini-Tn5 Km2 transposon (Biomedal) was used as conjugal transposon donors. *E. coli* S17–1 pir was grown in LB medium and incubated overnight at 32°C. Aur 6 was cultured in nutrient broth overnight at 28°C. Cultures were incubated in an orbital shaker at 300 rpm 24 h. Both donor and recipient cultures were centrifuged at 9000*g* for 5 min and then washed twice with magnesium sulfate 10 mM. The pellets were suspended in 100 μL of magnesium sulfate 10 mM, and mixed at a ratio of 1 : 1. Drops of 20 μL were placed on Petri dishes containing Nutritive agar and were incubated overnight at 30°C. After incubation, the colonies from each drop were transferred to a microfuge tube with 200 μL magnesium sulfate 10 mM and were suspended by vortexing. The appropriate dilutions were made and inoculated onto nutritive agar containing kanamycin (50 mg mL^{-1}) and nitrofurantoin (50 mg mL^{-1}). After 2 days, 104 mutants were picked onto new plates of the same composition. Mutants were kept in nutrient broth amended with 15% glycerol tubes at −80°C for further studies.

Inoculum preparation

To produce the inoculum, each mutant and the wild type were grown in 100 mL nutritive broth (DIFCO) in a 250 mL Erlenmeyer flask on a shaker (125 rpm) at 28°C for 24 h. The culture was centrifuged (350*g* for 10 min), washed with sterile water and pellets were suspended in sterile $MgSO_4$ 10 mM to achieve 10^8 colony forming units (cfu) mL^{-1}. The enumeration and calculations were conducted following the 'drop method' (Hoben and Somasegran 1982).

Screening bioassay on rice: plant growth and protection against salt stress

To verify the loss of the capacity to protect plants against salt stress of the different mutants with regard to wild type and effects on growth, biological assays were done. These assays were done in 24 well trays, using six wells per treatment. Treatments were the 104 mutants plus four comparative treatments (Table 1): plants non-inoculated and not stressed with salt (control: C) plants non-inoculated and stressed with salt (control-salt: CS), plants inoculated with wild type and not

Table 1. Abbreviations for comparative treatments used to compare the effects of the mutants

Comparative treatments	Abbreviation
Plants non-inoculated and non-stressed with salt	C
Plants non-inoculated and stressed with salt	CS
Plants inoculated with wild type Aur 6	WTC
Plants inoculated with wild type, Aur 6 and stressed with salt	WTS

stressed (wild type-control: WTC), plants inoculated with wild type and stressed with salt (wild type-salt: WTS). In total 648 wells (27 trays) were used in the experiment.

Wells were filled with 50 mL of agar-agar (0.6%) containing Hoagland plant nutrient solution (Sigma) in the concentration designed by manufacturer (0.16%). Seeds of *Oryza sativa* L. var. Thaibonnet were surface sterilised with ethanol 70% during 2 min. Afterwards, seeds were shaken in a 5% hypoclorite solution containing Tween 20 at 0.6% for 20 min. Seeds were pre-germinated in agar-agar plates (1.2%). Three days after germination, two seedlings were sown in each well. Trays were maintained in a culture chamber Sanyo MLR350H with a light-dark cycle of 15–19 h, temperature of 33−28°C.

Inoculum was prepared as described above. Each mutant was inoculated in six wells (replicates). Seedlings were grown as described above for 4 days. Strains were inoculated on the agar of each well in a volume to reach 10^8 cfu mL^{-1} of substrate. Four days after inoculation, a non-destructive measurement of shoot length (mm) from the substrate to the tip of the plant was taken (L0). After that, salt stress challenge was delivered watering all salt treatments (plants inoculated with each mutant and the comparative treatments WTS and CS) with a concentrated NaCl solution to reach 60 mM in the substrate. One week after, shoot length was measured again (L1). Also, in this time FW (mg) was measured in all plants of each treatment, and the number of curled and discoloured leaves were counted.

First, the difference in length (L1-L0) between the wild type-control (WTC) and the non-inoculated control (C) was calculated to check bacterial ability as PGPR. Then, each mutant was compared with the WTS, calculating the relative change in length induced by the mutant, being the WTS 100%; data is expressed as a percentage (Table 2). Next, the ability of the wild-type strain to protect against salt stress was checked by comparing the number of curled and discoloured leaves in the salt stressed control (CS) to WTS, and then, effects of each mutant was compared with WTS and expressed as an increment (+) or a decrease (–). Statistical significance of data was calculated as described below. These experiments were conducted twice with similar results.

Bioassay with mutants on rice

A new experiment was conducted on rice to determine the mechanism of action involved in growth and protection against salt stress. The experimental set up was the one described for the screening bioassay on rice. Treatments were mutant 33 (which lost the greatest protection capacity with regard to wild-type strain) with salt, plants inoculated with mutant 19 (which exhibited an improved protection capacity with regard to wild type strain) with salt, and the four comparative treatments: plants non-inoculated and not stressed with salt (control: C) plants non-inoculated and stressed with salt (control-salt: CS), plants inoculated with wild type and not stressed (wild type-control: WTC), plants inoculated with wild type and stressed with salt (wild type-salt: WTS.

Plants from each replicate were harvested and pooled and powdered in liquid nitrogen, and the powder obtained was used for all analyses: enzyme activities related to oxidative stress, malondialdehyde (MDA) determination and proline determination.

Enzyme activities related to oxidative stress

Soluble proteins were extracted by resuspending 5–10 mg of powder in 1 mL of potassium phosphate buffer 0.1 M pH 7 containing 2 mM PMSF. Samples were sonicated 10 min and then centrifuged for 10 min at 21 000*g*. The supernatant was divided into aliquots, frozen in liquid nitrogen and stored at −80°C for further analysis. All above operations were conducted at 0−4°C.

To measure the amount of total protein from plant extract, 250 µL of Bradford reagent and 50 µL of sample and BSA dilutions were inoculated in ELISA 96 well plates and incubated for 5–45 min at room temperature and measured using a plate reader at absorbance of 595 nm. A calibration curve was constructed from commercial BSA dilutions expressed in milligrams. The units of protein were expressed as mg μL^{-1}.

Enzyme activities related to oxidative stress in plant extracts were measured with an spectrophotometer: superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), guaiacol peroxidase (GPX, EC 1.11.1.7), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), dehydroascorbate reductase (DHAR; EC 1.8.5.1) and glutathione reductase (GR, EC 1.6.4.2). Except for SOD, units are expressed as µmol mg $protein^{-1}$ min^{-1}.

SOD activity was determined following the specifications of the SOD activity detection kit (SOD Assay Kit-WST, Sigma-Aldrich). With this method, the rate of the reduction with O_2 is linearly related to the xanthine oxidase (XO) activity, and inhibited by SOD. Inhibition activity of SOD can be determined by colourimetric method. The unit used for this activity was: % inhibition µg $protein^{-1}$.

CAT was measured by the method by García-Limones *et al.* (2002). The reaction mixture consisted of 50 mM potassium phosphate buffer, pH 7.0, 20 mM H_2O_2 and 120 µL of enzyme extract in a final volume of 1.2 mL. The reaction was started by adding H_2O_2 and the decrease in A_{240} produced by H_2O_2 breakdown was recorded. Extinction coefficient of 36 mM^{-1} cm^{-1} was used to calculate activity.

APX was measured by the method by García-Limones *et al.* (2002). The reaction mixture consisted of 50 mM potassium phosphate buffer, pH 7.0, 0.25 mM sodium ascorbate, 5 mM H_2O_2 and 100 µL of enzyme extract in a final volume of 1.2 mL. Adding H_2O_2 started the reaction and the oxidation of ascorbate was determined by the decrease in A_{290}. Extinction coefficient of 2.8 mM^{-1} cm^{-1} was used to calculate activity.

GPX was measured by the method by García-Limones *et al.* (2002). The reaction mixture consisted of 100 mM potassium phosphate buffer, pH 6.5, 15 mM guaiacol, 0.05% (v/v) H_2O_2 and 120 µL enzyme extract in a final volume of 1.2 mL. Adding

Table 2. Length variation (increase or decrease %) of rice seedlings inoculated with the mutants

Significant differences compared with plants inoculated with wild type and stressed with salt (WTS) are indicated: *, $P < 0.05$; significant differences compared with plants non-inoculated and stressed with salt (CS) are indicated: #, $P < 0.05$. Numbers in curled and discoloured leaves columns are the differences between mutant and WTS. Positive values indicate more curled or discoloured leaves in mutant treatment, and negative values indicate less curled or discoloured leaves in mutant treatment

Mutant number	Length variation%	Curled leaves	Discoloured leaves	Mutant number	Length variation%	Curled leaves	Discoloured leaves	Mutant number	Length variation%	Curled leaves	Discoloured leaves	Mutant number	Length variation%	Curled leaves	Discoloured leaves
1	–5.43	0	–5	27	–7.23	6	–5	53	–5.74*	–2	–1	79	–10.35*	10	2
2	–8.93	2	–5	28	–7.18	6	–4	54	–4.36	–1	–1	80	–1.16	12	3
3	0.20	5	–5	29	–10.58	5	–5	55	–2.86	4	–2	81	3.45	2	1
4	7.61	4	–5	30	–6.88	3	–2	56	–5.43	3	–2	82	3.17	7	–1
5	8.02	3	2	31	–13.25*	2	2	57	–6.72*	3	4	83	–5.36	11	–1
6	8.57	7	6	32	–16.95*#	4	1	58	–2.38	5	2	84	0.19	11	–1
7	2.73	7	5	33	–17.31*#	6	6	59	–3.18	8	3	85	–0.96	5	0
8	–0.57	1	–3	34	–16.83*#	0	5	60	1.86	3	–2	86	0.81	3	–1
9	11.26*	1	–5	35	–14.25*	2	3	61	–4.56	3	–1	87	1.62	8	2
10	11.68*	1	–5	36	–10.31	0	5	62	–1.38	0	–2	88	–2.03	5	–1
11	2.27	3	–5	37	–9.18	3	–2	63	–2.13	1	–2	89	–6.00	11	3
12	1.39	7	–5	38	–5.35	0	1	64	–6.76*	–2	–2	90	–8.22	11	–1
13	0.06	–1	–5	39	–10.28	–3	–1	65	–1.88	–2	–1	91	–5.75	11	4
14	–1.52	–5	–5	40	–20.10*#	3	0	66	–9.12*	–2	–2	92	–7.45	12	0
15	3.61	1	–5	41	5.89	1	–2	67	–5.72*	0	–2	93	0.66	10	–1
16	–7.40	7	–5	42	5.75	0	–1	68	–0.38	6	0	94	–3.47	10	2
17	10.99*	7	–2	43	3.91	0	1	69	–8.34*	2	–1	95	–3.85	12	0
18	12.11*	5	–3	44	3.78	–1	0	70	–8.89*	2	–2	96	3.87	8	0
19	12.80*	–5	–4	45	3.60	–2	–2	71	–6.87*	1	0	97	–4.20	6	0
20	6.57	7	–1	46	3.26	0	–1	72	–8.50*	3	0	98	–5.14	5	1
21	–2.35	5	–2	47	6.07	–3	0	73	–9.28*	3	2	99	–10.03*	6	2
22	8.46	3	–5	48	6.06	–3	–2	74	–9.67*	5	7	100	–1.33	9	1
23	–1.84	6	–2	49	–8.12*	–2	–1	75	–5.34	9	1	101	–7.99	10	–1
24	2.31	5	0	50	–0.80	–2	–1	76	–7.23*	8	1	102	–2.56	12	0
25	–9.29	7		51	–7.23*	–2	–2	77	–1.39	8	0	103	–9.22*	12	0
26	–7.22	5		52	–5.01	–2	–2	78	–3.17	6	2	104	–11.63*	11	2

H_2O_2 started the reaction and the oxidation of guaiacol was determined by the increase in A_{470}. Extinction coefficient of 26.6 mM^{-1} cm^{-1} was used to calculate activity.

MDHAR activity was measured by the method by Xu *et al.* (2008). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.6), 0.2 mM NADH, 2.5 mM AsA, 1 unit of ascorbate oxidase and 100 µL enzyme extract in a final volume of 1.2 mL Adding enzyme extract started the reaction and the reduction of monodehrydro ascorbate was determined by the decrease in A_{340} Extinction coefficient of 6.22 mM^{-1} cm^{-1} was used to calculate activity.

DHAR activity was measured as described by Xu *et al.* (2008) at 265 nm. The reaction mixture consisted of potassium phosphate buffer 50 mM (pH 7.0), 2.5 mM reduced glutathione, 0.2 mM Dehydroascorbate, 0.1 mM EDTA and 100 µL enzyme extract in a final volume of 1.2 mL. Adding enzyme extract started the reaction and the reduction of dehrydro ascorbate was determined by the decrease in A_{265}. Extinction coefficient of 14 mM^{-1} cm^{-1} was used to calculate activity.

GR was measured using the method by García-Limones *et al*. (2002). The assay mixture consisted of 50 mM potassium phosphate buffer, pH 7.5, 3.5 mM $MgCl_2$, 0.15 mM NADPH, 0.5 mM oxidised glutathione and 180 µL of enzyme extract in a final volume of 1.2 mL. Adding NADPH started the reaction and oxidation of this compound was determined by the increase in A_{340}. Extinction coefficient of 6.2 mM^{-1} cm^{-1} was used to calculate activity.

In all assays the blank consisted of the components of the reaction mixture except for the enzyme extract, which was replaced by an equal volume of the assay buffer. In the case of the GR assay, an additional blank without oxidised glutathione was included in order to account for the presence in the extracts of other enzyme activities able to oxidise NADPH.

Malondialdehyde (MDA) determination

The MDA content was determined using the method by Hu *et al*. (2016) with modifications. Briefly, 0.25 g of powder was mixed with 2 mL of reaction solution containing 0.5% (v/v) thiobarbituric acid (TBA) and 20% (v/v) trichloroacetic acid (TCA). The mixture was heated at 95°C for 30 min, then quickly cooled down to room temperature, treated to eliminate air bubbles, and centrifuged at 12 000*g* for 20 min. Then, absorbance of the supernatant was determined by a spectrophotometer at 532 and 600 nm. The MDA content was calculated using the formula: MDA (nmol g FW^{-1}) = ((OD532 – OD600))/(ε × FW), where ε the extinction coefficient (155 mM^{-1} cm^{-1}).

Proline determination

Proline determination was measured following the procedure proposed by Carillo *et al.* (2008) with modifications. Plant powder (0.25 g) was added to 1 mL of 1% (w/v) solution of ninhydrin in 60% (v/v) of acetic acid. After homogenisation the mix was heated at 95°C for 20 min. After cooling and centrifuging, the formed chromogen was extracted with 3 mL of toluene. After separation of the two phases, the absorbance was read at 520 nm. A calibration curve was constructed from commercial proline dilutions to calculate the concentration in plant tissue (µmol g FW^{-1}).

Bioassay with mutants on tomato seedlings

In order to confirm whether these mutants had actually lost (mutant 33) or improved (mutant 19) their capacity to promote growth in other plants different to rice, a growth promotion experiment without any kind of stress was conducted in tomato plants, as this effect had been previously determined (Domenech *et al.* 2006) and because it is phylogenetically very distant from rice. Five hundred tomato seeds (var. Razymo) were pre-germinated and 200 homogeneous seedlings were transplanted to pots (300 mL) filled with peat (Projar PS Seed Pro 5050) and placed under controlled conditions (14 h/10 h light/dark, 30°C/20°C). Plants were separated into four lots with 25 seedlings in each. Three lots were inoculated with the corresponding strains (wild type Aur 6, mutant 33 and mutant 19) delivered at 10^8 cfu mL^{-1}, and the last one served as non-inoculated control. Two weeks after inoculation length, DW of shoot and roots were measured.

Mutant mapping by inverse PCR

To map the two selected mutants, mutant 33 and mutant 19, the protocol described by Washio *et al*. (2010) was followed. Briefly, chromosomal DNA of each mutant was digested with *Pst*I and a subsequent self-ligation was performed by standard methods (Sambrook and Rusell 2001). The self-ligated DNA was used as template for inverse PCR in combination with two primers complementary to the internal sequence of mini-Tn5 (mTn5f 5′-AAGGTGATCCCGGTGGATGAC-3′; mTn5r 5′-CAATCGG CTGCTCTGATGCCGC-3′). PCR products were gel-purified using the QIAquick Gel Extraction Kit as described in the manufacturer's protocol (Qiagen). Each PCR product was ligated into the pCR 4-TOPO vector (Invitrogen). *Escherichia coli* strain DH5α (Invitrogen) was then transformed with the ligation products. Four colonies per PCR product were randomly picked and suspended in tubes containing 6 mL LB and grown for 24 h. Plasmid DNA extraction was performed with the QIAprep Spin Miniprep kit (Qiagen) and fragment inserts were sequenced on an ABI PRISM 377 DNA Sequencer (Applied Biosystems). Sequence editing was performed using the software Clone Manager Professional Suite ver. 6.0. Sequences were analysed by BLASTN 2.2.6 in the National Centre for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov/, accessed 10 July 2017) database. This process was done with forty clones of each mutant.

Statistical analysis

One-way ANOVA with replicates was used to check the statistical differences in length increases and in the number of curled leaves induced by the mutants as compared with WTS. A different analysis was conducted to compare WTC and C to confirm that Aur 6 was acting as PGPR. When significant differences appeared ($P < 0.05$) a Fisher test was used (Sokal and Rohlf 1980). Also one-way ANOVA was used to check statistical differences in enzyme activities, MDA and proline and in the bioassay on tomato.

Pearson correlation coefficient was used to check the correlation between length and fresh weight.

Principal component analysis (PCA) was conducted out with CANOCO ver. 4.5 software (TerBraak and Šmilauer

1998), with length percentage differences (data from Table 1), FW, curled and discoloured leaves data.

Results

Once the 104 mutants were obtained, the bioassay to screen for affected mutants in rice was carried out. In all experiments, the ability to enhance growth by the wild type was confirmed by comparing the WT to C for growth (83.46 ± 3.04 vs 62.12 ± 2.45 for length and 12.22 ± 0.45 vs 9.09 ± 0.36 for FW), being differences significant. Also, the capacity of Aur 6 to protect against salt was checked by comparing WTS to CS, resulting in significant differences for length (51.33 ± 1.85 vs 35.82 ± 1.), and for fresh weight (10.23 ± 0.32 vs 6.16 ± 0.09). The percentages of increase or decrease in length of the plants inoculated with the mutants compared with WTS are shown in Table 2. Asterisks indicate the mutants that showed significant differences with respect to WTS. Twenty-four out of the 104 mutants analysed presented a significant lost in its protection capacity, with decreases in plant length ranging between –5.74 and –20.10%. We noted that five mutants improved the protection capacity of the wild strain by 10.99 and 12.80%. Among them, mutant 19 was the best, decreasing the number of curled and discoloured leaves and increasing fresh weight (data not shown). In addition, among the 24 mutants indicated above, four of them (32, 33, 34 and 40) worsened significantly the value obtained by the CS (plants non-inoculated and stressed with salt), indicating that these mutants, besides losing their ability to protect, had a negative effect on plants. Mutant 33 caused the greatest decrease in seedling length, and induced the greatest withering symptoms, therefore, lost the protection ability more markedly.

No statistical differences between WTS and mutants fresh weight appeared in any case. On the other hand, the correlation analysis revealed a statistically significant positive correlation between length and fresh weight ($r = 0.96$; $P < 0.01$).

Ordination provided by the principal component analysis (PCA) performed with data from Table 2 appears in Fig. 1. Axis I and II absorbed 65.4% and 26.8% of the variance respectively. Mutants 9, 10, 17, 18 and 19 grouped towards the negative values of axis I, sharing the common feature of an improved protection capacity compared with the wild type. Length variation (increase or decrease) and plant FW are the variables that determine separation of these groups along axis I, which accounts for the greatest variance absorption, and therefore, is more relevant on this ordination. Mutants 31, 32,

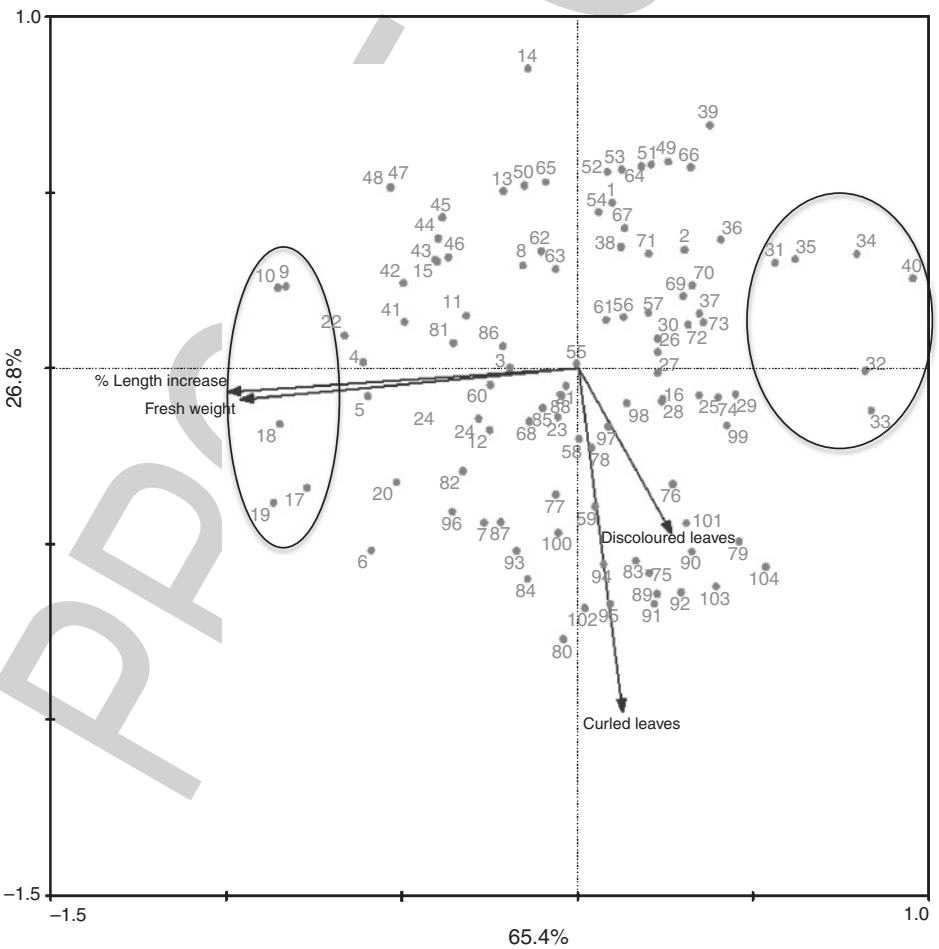

Fig. 1. Principal component analysis (PCA) performed with length percentage differences (data from Table 2), FW, curled and discoloured data. Percentages in axis represent the variance absorbed in each case.

33, 34, 35 and 40 presented a significant loss in their protection capacity and grouped together towards the positive values of axis II. The two clusters mentioned above were marked on the multivariate analysis. The number of curled and discoloured leaves drove the separation along axis II, which is not as relevant since this axis absorbs less variance. However, these criteria were considered for mutant selection among those included in the two groups, and mutants 19 and 33 were selected for their ordination by the weight factors 'curled' leaves and 'discoloured leaves'.

Based on the results presented above, mutant 33 showed the most important loss in its protection capacity, and mutant 19 was the one with the highest increase in its protection capacity. Therefore, these mutants were selected for the rest of the experiments.

In order to determine the mechanisms involved in rice plant defence against salt stress used by the wild type and the mutants, a second experiment on rice seedlings was carried out and the enzyme activities related with scavenging of ROS were determined. Mutant 33, which lost its ability to protect plants rice upon salt stress, induced significant decreases in all enzyme activities with respect to the wild type, except for catalase and DHAR (Fig. 2). Mutant 19 did not induce important changes in these enzyme activities with regard to wild type, it just significantly decreased SOD and increased DHAR (Fig. 2).

Malondialdehyde (MDA), used as marker of oxidative stress, showed significantly higher values in plants inoculated with mutant 33 with regard to plants inoculated with mutant 19 and wild type, with very similar values of the control salt plants CS (Fig. 3*a*). Proline, one of the main osmoprotectants during stress salinity responses, showed significantly lower values in plants inoculated with mutant 33 than those obtained in plants inoculated with mutant 19 and wild type, with very similar values to CS (Fig. 3*b*).

Bioassays were performed on tomato plants to verify the effects of mutants 33 and 19. Data shown in Fig. 4, is length (Fig. 4*a*), DW of roots (Fig. 4*b*), DW of the aerial part (Fig. 4*c*) and total weight (Fig. 4*d*). In all cases mutant 33 presented significantly lower values than the wild strain, and mutant 19 achieved significantly higher values, demonstrating that mutations were related to their effects on plants.

To identify genes affected in each mutant, 40 colonies (clones) of each mutant were mapped. In all cases the pattern of bands on the gel was the same indicating that the transposon insertion was affecting genes indicated below. Some bands that did not appear in all the colonies were also cloned, proving that did not correspond to sequences of genes of the bacteria but to unspecific PCR products.

Three mutated genes were identified in mutant 33. One of them was a gene from a general secretion pathway, protein D. Another was a gene from a putative two-component system, a sensor kinase (ColS), that it is part of the two-component regulatory system named ColR/ColS. The last one was a gene from flagellar motor switch protein FliG, a protein related with the flagelar rotor (Paul *et al*. 2011).

Two mutated genes were identified in mutant 19. One corresponding with gene 16s rRNA and another from heavy metal efflux pump, from the czca family. This protein is part of a chemiosmotic antiporter involved in the efflux of cadmium (Silver and Phung 2005).

Discussion

The ability of some rhizobacteria to improve plant fitness making them more resilient in stressful situations has been known for a long time (Gupta *et al*. 2015). The aim of this study was to unravel some of the mechanisms involved in the effect of bacteria on the plant by comparing loss of effects in mutants. To achieve this goal, random mutagenesis with transposon Tn5 was used on an effective PGPR, followed by subsequent screening of mutants looking for those which had lost the ability to increase growth and to protect against salt stress in rice. Other authors with similar objectives have used these techniques successfully (Llamas *et al*. 2000; Ito *et al*. 2010; Washio *et al*. 2010; Djavaheri *et al*. 2012; Maldonado-González *et al*. 2015). The effectiveness of these mutants was tested also in tomato, as the strain had previously demonstrated growth enhancement in tomato; hence, if this ability was also lost, involvement of mutated genes on biological effect would find greater support. Finally, genes involved in mutations were identified by mapping.

Salinity stress can provoke an excessive generation of reactive oxygen species (ROS) (Tsai *et al*. 2004; Hong *et al*. 2009), producing lipids' oxidation, damage in, for example, proteins, DNA, and chlorophyll. Therefore, plant's antioxidant systems are very important in this stress condition (Han and Lee 2005; Kim *et al*. 2005) and induction of antioxidant enzyme activities by PGPR has been related to an increased tolerance of plants growing under salt stress conditions (Kohler *et al*. 2009). In our work, mutant 33 significantly decreased activities of five out of seven enzymes studied with regard to wild-type-salt treatment (WTS; Fig. 2) and this loss may be involved in the loss of plant protection against salt stress. However, mutant 19 did not modify as many enzyme activities, causing only a significant decrease in SOD and a significant increase in DHAR (Fig. 2). These data were consistent with values of MDA (Fig. 3*a*) which is used as a marker of oxidative stress (AbdElgawad *et al*. 2016); similar values are found in control (C), and in treatments that protect plants, wild type with salt (WTS), and mutant 19, which are significantly lower than those in stressed controls (CS) and mutant 33, which does not protect plants. Probably, other non-antioxidative enzymes responses as tocopherols or polyphenols synthesis are being stimulated (del Río 2015), improving oxidative stress balance found in these treatments, especially in plants inoculated with mutant 19. Furthermore, the improved protection induced by this mutant could be related with a slight non-significant increase of auxin-like compounds (1.48 vs 1.53 $\mu g\ mL^{-1}$) since the rest of putative PGPR traits (phosphate solubilisation and ACC degradation) were not affected in the mutants (data not shown).

Proline content in plants upon salt stress is normally used as a marker of resistance capacity of the plants (Bojórquez-Quintal *et al*. 2014), because is the most common compatible osmolyte in plants. Our results indicated that the ability of mutant 33 to induce proline accumulation was impaired as compared with the wild type-salt treatment (WTS) and to mutant 19 (Fig. 3*b*). All results discussed until now suggest that the mutations induced in mutant 33 are related with its loss of capacity to protect rice plants

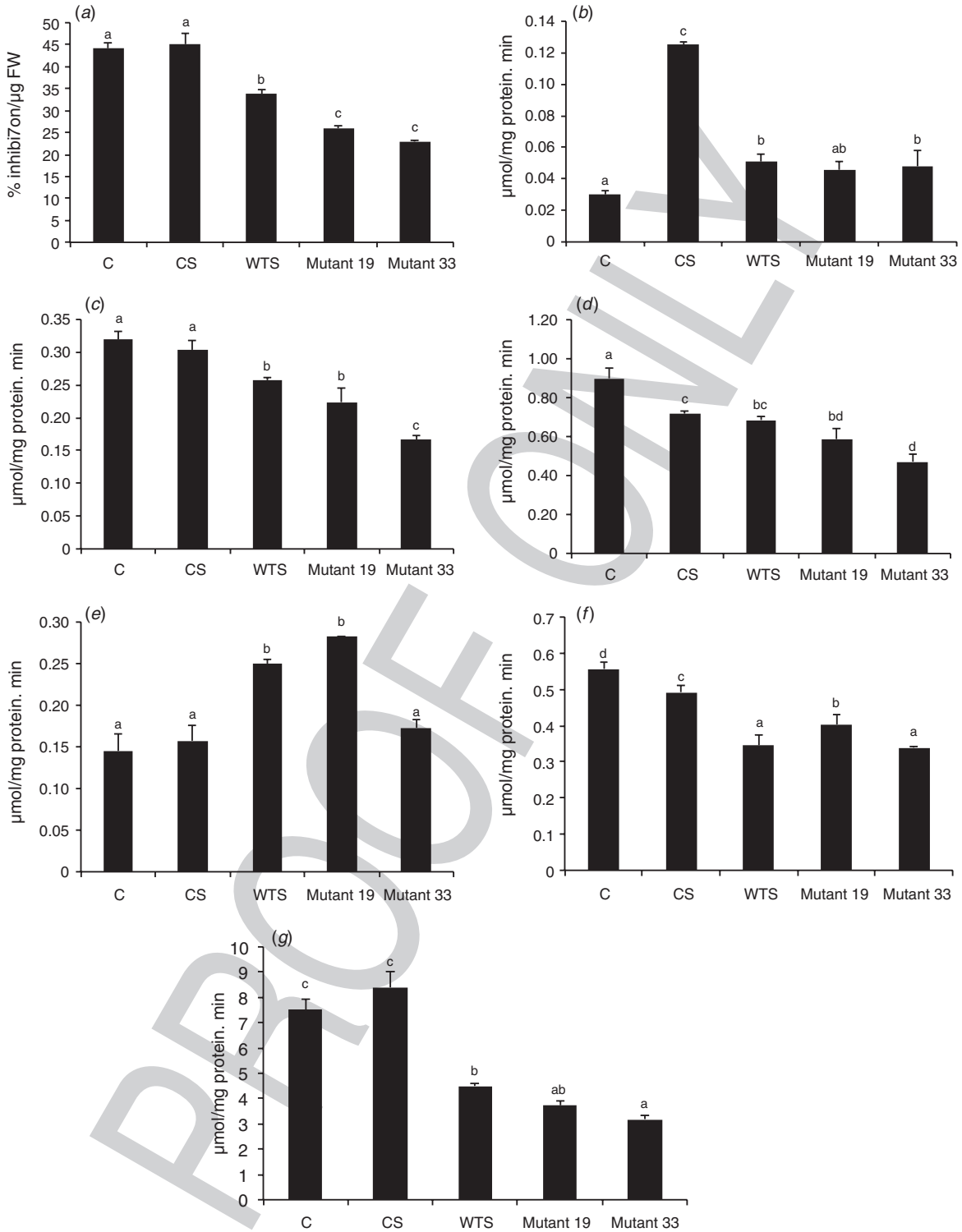

Fig. 2. Enzyme activities related with oxidative stress: (*a*) superoxide dismutase (SOD); (*b*) catalase; (*c*) ascorbate peroxidase (APX); (*d*) guaiacol peroxidase (GPX); (*e*) monodehydroascorbate reductase (MDHAR); (*f*) dehydroascorbate reductase (DHAR) and (*g*) gluthatione reductase (GR). SOD is expressed as % inhibition µg protein^{-1}, all other enzymes are expressed as µmol mg protein $^{-1}$ min^{-1}. Different letters indicate significant differences ($P < 0.05$). Abbreviations: C, control plants (plants non-inoculated and not stressed with salt); CS, plants non-inoculated and stressed with salt; WTS, plants inoculated with wild type and stressed with salt.

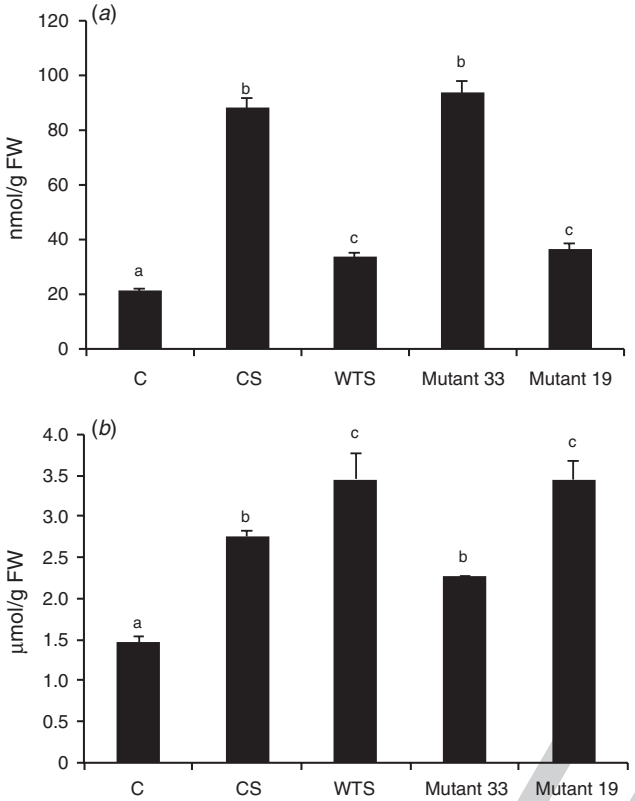

Fig. 3. Concentration of Malondialdehyde (MDA) (*a*), and concentration of proline (*b*) in leaves of rice in the different treatments. Proline was measured as µmol g FW^{-1} and MDA as nmol g FW^{-1}. Different letters indicate significant differences ($P < 0.05$). Abbreviations: C, control plants (plants non-inoculated and not stressed with salt); CS, plants non-inoculated and stressed with salt; WTS, plants inoculated with wild type and stressed with salt.

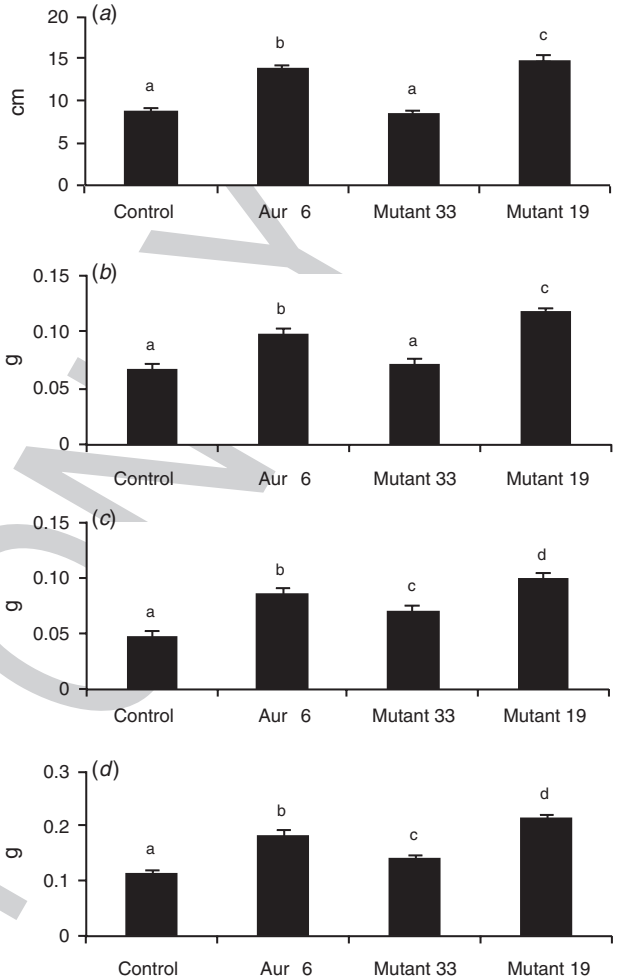

Fig. 4. Length (*a*), shoot DW (*b*), root DW (*c*), and total DW (*d*) of tomato seedlings inoculated with wild type strain (Aur 6), mutant 33 and mutant 19. Different letters indicate significant differences ($P < 0.05$).

upon salt stress. Likewise, it appears that mutations induced in mutant 19 do improve plant fitness.

In order to confirm that strain mutations were involved in effects on plant and it was not limited to rice, mutants were tested in tomato, since the strain had previously reported ability to enhance tomato growth (Domenech *et al*. 2006). This experiment confirmed that genes that were mutated by transposon insertion should be involved in the PGPR metabolic capabilities needed to exert its beneficial effect, since results are consistent with the ones obtained in rice (Fig. 4).

In mutant 33, which had lost the ability to induce protection three mutated genes were identified. One of them was a gene from a general secretion pathway, protein D. In Gram-negative bacteria the general secretion pathway is responsible for extracellular secretion of a high number of different proteins and protein D is involved in the second step of secretion, in the translocation through the outer membrane (Sandkvist *et al*. 1999; Korotkov *et al*. 2012) forming in the outer membrane a large oligomeric ring of 12–18 subunits (Bitter *et al*. 1998). It seems that protein D is the pore through which bacterial secreted proteins are translocated (Kazmierczak *et al*. 1994; Linderoth *et al*. 1996). PGPR elicitation capacity is due to metabolites released by bacteria (De Vleesschauwer and Höfte 2009; Wiesel *et al*. 2014). Protein D is a member of the secretin family that includes proteins required for type II, III and IV secretion (Genin and Boucher 1994). The type II secretion system appears to be typically associated with organisms that colonise surfaces, and in most cases these organisms do not invade cells (Sandkvist 2001), as most of the PGPR.

Another of the genes mutated in the mutant 33 was identified as a gene from a putative, sensor kinase (ColS). ColS it is part of the two-component regulatory system named ColR/ColS. This system is involved in different physiological processes as virulence, bacterial growth in plant, biofilm formation, lipopolysaccharide (LPS) production, catalase activity, and environmental stress resistance (Yan and Wang 2011). It is worth mentioning that lipopolysaccharides can trigger physiological pathways related with plant innate immunity (Erbs and Newman 2012), some of which are also related with plant resistance against abiotic stress situations. Moreover, the involvement of ColS in competitive root tip colonisation has been demonstrated (Dekkers *et al*. 1998) and it has been suggested to be related to the slower uptake of root exudates components (De Weert *et al*. 2006). Therefore, having

this system compromised by the mutation, the ability of the PGPR to exert an effect on the plant, is limited since its ability to colonise root and be competitive in this ecosystem is impaired (Dutta and Podile 2010).

The last gene mutated, identified in mutant 33 was the flagellar motor switch protein FliG. This protein together with FliM and FliN form the flagellar motor switch complex, which plays a central role in bacterial motility and chemotaxis (Kihara *et al*. 2000; Paul *et al*. 2011). FliG is directly associated with the motor switch ring (Francis *et al*. 1992), and it is the component that interacts with the Mot proteins to develop torque (Irikura *et al*. 1993; Lloyd *et al*. 1996). Therefore, it is clear that the mutant 33 had its mobility greatly reduced, which undoubtedly hinders its ability to colonise the root.

Mutant 19 not only didn't lose its ability to protect plants, but even overcame the effect of the wild strain. 16s rRNA was one of the genes mutated identified in this mutant. This mutation should not affect the physiological ability of the strain to improve plant growth, since it has been shown that the number of copies of this gene is high in most bacteria, especially in Gammaproteobacteria, (Větrovský and Baldrian 2013), as Aur 6 is. However, another mutated gene was detected in this mutant: a heavy metal efflux pump from the Czca family. The CzcA protein is a member of the resistance-nodulation-cell division (RND) permease superfamily, and it is part of the CzcCB2A complex, which is very important to heavy metal resistance in many Gram-negative bacteria. This complex works as an active cation efflux mechanism driven by cation/proton antiport (Goldberg *et al*. 1999; Moraleda-Muñoz *et al*. 2010). According to Silver and Phung (2005), Czca proteins collect the cadmium cations of the periplasmic space, which have been previously removed from the cytoplasm by a P-type ATPase, and exchanged by protons. Somehow, the malfunction of this permease enhances the ability of the strain (Aur 6) to improve fitness of rice plants against abiotic stress, and in tomato plants under normal conditions.

Conclusions

In the present work we have demonstrated the ability of *Pseudomonas fluorescens* Aur 6 to protect rice plants against salt stress situations, and its probable relationship with changes in enzymatic mechanisms related to oxidative stress, while improving the ability of the plant to accumulate compatible osmolytes such as proline. We have identified several mutated genes responsible for the loss of effectiveness of PGPR to improve plant fitness upon abiotic stress challenge in rice and tomato. They are genes involved in protein transport from the cytoplasm, and associated with colonisation ability. Moreover, and perhaps more surprisingly, we have identified a mutated gene that increases the capacity of the PGPR to enhance the fitness of the plant with regard to the wild type under the stress conditions tested.

Conflicts of interest

The authors declare no conflicts of interest.

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References

AbdElgawad H, Zinta G, Hegab MM, Pandey R, Asard H, Abuelsoud W (2016) High salinity induces different oxidative stress and antioxidant responses in maize seedlings organs. *Frontiers in Plant Science* **7**, 145–156. doi:10.3389/fpls.2016.00276

Alvarez MI, Sueldo RJ, Barassi CA (1996) Effect of *Azospirillum* on coleoptile growth in wheat seedlings under water stress. *Cereal Research Communications* **24**, 101–107.

Barriuso J, Solano B, Gutiérrez Mañero F (2008) Protection against pathogen and salt stress by four plant growth-promoting rhizobacteria isolated from *Pinus* sp. on *Arabidopsis thaliana*. *Phytopathology* **98**(6), 666–672. doi:10.1094/PHYTO-98-6-0666

Bitter W, Koster M, Latijnhouwers M, Cock H, Tommassen J (1998) Formation of oligomeric rings by XcpQ and PilQ, which are involved in protein transport across the outer membrane of *Pseudomonas aeruginosa*. *Molecular Microbiology* **27**, 209–219. doi:10.1046/j.1365-2958.1998.00677.x

Bojórquez-Quintal E, Velarde-Buendía A, Ku-González A, Carillo-Pech M, Ortega-Camacho D, Echevarría-Machado I (2014) Mechanisms of salt tolerance in habanero pepper plants (*Capsicum chinense* Jacq.): proline accumulation, ions dynamics and sodium root-shoot partition and compartmentation. *Frontiers in Plant Science* **5**, 605–618.

Carillo P, Mastrolonardo G, Nacca F, Parisi D, Verlotta A, Fuggi A (2008) Nitrogen metabolism in durum wheat under salinity: accumulation of proline and glycine betaine. *Functional Plant Biology* **35**(5), 412–426. doi:10.1071/FP08108

Cattelan AJ, Hartel PG, Fuhrmann JJ (1999) Screening for plant growth-promoting rhizobacteria to promote early soybean growth. *Soil Science Society of America Journal* **63**(6), 1670–1680. doi:10.2136/sssaj1999.6361670x

Cezón R, Mañero FG, Probanza A, Ramos B, Lucas García JA (2003) Effects of two plant growth-promoting rhizobacteria on the germination and growth of pepper seedlings (*Capsicum Annum*) cv. Roxy. *Archives of Agronomy and Soil Science* **49**(6), 593–603. doi:10.1080/03650340310001620123

Conrath U (2011) Molecular aspects of defence priming. *Trends in Plant Science* **16**, 524–531. doi:10.1016/j.tplants.2011.06.004

Conrath U, Beckers GJM, Flors V, García-Agustín P, Jakab G, Mauch F, Newman MA (2006) Priming: getting ready for battle. *Molecular Plant-Microbe Interactions* **19**(10), 1062–1071. doi:10.1094/MPMI-19-1062

de Freitas JR, Banerjee MR, Germida JJ (1997) Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (*Brassica napus* L.). *Biology and Fertility of Soils* **24**(4), 358–364. doi:10.1007/s003740050258

De Vleesschauwer D, Höfte M (2009) Rhizobacteria-induced systemic resistance. *Advances in Botanical Research* **51**, 223–281. doi:10.1016/S0065-2296(09)51006-3

De Weert S, Dekkers LC, Bitter W, Tuinman S, Wijfjes AHM, Van Boxtel R, Lugtenberg BJJ (2006) The two-component colR/S system of *Pseudomonas fluorescens* WCS365 plays a role in rhizosphere competence through maintaining the structure and function of the outer membrane. *FEMS Microbiology Ecology* **58**(2), 205–213. doi:10.1111/j.1574-6941.2006.00158.x

de Zelicourt A, Al-Yousif M, Hirt H (2013) Rhizosphere microbes as essential partners for plant stress tolerance. *Molecular Plant Pathology* **6**(2), 242–245.

Dekkers LC, Bloemendaal CJ, de Weger LA, Wijffelman CA, Spaink HP, Lugtenberg BJ (1998) A two-component system plays an important role in the root-colonizing ability of *Pseudomonas fluorescens* strain

WCS365. *Molecular Plant-Microbe Interactions* **11**(1), 45–56. doi:10.1094/MPMI.1998.11.1.45

del Río LA (2015) ROS and RNS in plant physiology: an overview. *Journal of Experimental Botany* **66**(10), 2827–2837. doi:10.1093/jxb/erv099

Djavaheri M, Mercado-Blanco J, Versluis C, Meyer JM, van Loon LC, Bakker PAHM (2012) Iron-regulated metabolites produced by *Pseudomonas fluorescens* WCS374r are not required for eliciting induced systemic resistance against *Pseudomonas syringae* pv. tomato in *Arabidopsis*. *MicrobiologyOpen* **1**(3), 311–325. doi:10.1002/mbo3.32

Domenech J, Reddy MS, Kloepper JW, Ramos B, Gutierrez-Mañero J (2006) Combined application of the biological product LS213 with *Bacillus*, *Pseudomonas* or *Chryseobacterium* for growth promotion and biological control of soil-borne diseases in pepper and tomato. *BioControl* **51**(2), 245–258. doi:10.1007/s10526-005-2940-z

Dutta S, Podile AR (2010) Plant growth promoting rhizobacteria (PGPR): the bugs to debug the root zone. *Critical Reviews in Microbiology* **36**(3), 232–244. doi:10.3109/10408411003766806

Erbs G, Newman MA (2012) The role of lipopolysaccharide and peptidoglycan, two glycosylated bacterial microbe-associated molecular patterns (MAMPs), in plant innate immunity. *Molecular Plant Pathology* **13**(1), 95–104. doi:10.1111/j.1364-3703.2011.00730.x

Francis NR, Irikura VM, Yamaguchi S, DeRosier DJ, Macnab RM (1992) Localization of the *Salmonella typhimurium* flagellar switch protein FliG to the cytoplasmic M-ring face of the basal body. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 6304–6308. doi:10.1073/pnas.89.14.6304

Friesen ML, Porter SS, Stark SC, von Wettberg EJ, Sachs JL, Martinez-Romero E (2011) Microbially mediated plant functional traits. *Annual Review of Ecology Evolution and Systematics* **42**(1), 23–46. doi:10.1146/annurev-ecolsys-102710-145039

García-Limones C, Hervás A, Navas-Cortés JA, Jiménez-Díaz RM, Tena M (2002) Induction of an antioxidant enzyme system and other oxidative stress markers associated with compatible and incompatible interactions between chickpea (*Cicer arietinum* L.) and *Fusarium oxysporum* f. sp. *ciceris*. *Physiological and Molecular Plant Pathology* **61**(6), 325–337. doi:10.1006/pmpp.2003.0445

Genin S, Boucher CA (1994) A superfamily of proteins involved in different secretion pathways in gram-negative bacteria: modular structure and specificity of the *N*-terminal domain. *Molecular & General Genetics* **243**, 112–118. doi:10.1007/BF00283883

Goldberg M, Pribyl T, Juhnke S, Nies DH (1999) Energetics and topology of CzcA, a cation/proton antiporter of the resistance-nodulation-cell division protein family. *The Journal of Biological Chemistry* **274**(37), 26065–26070. doi:10.1074/jbc.274.37.26065

Gupta G, Parihar SS, Ahirwar NK, Snehi SK, Singh V (2015) Plant growth promoting rhizobacteria (PGPR): current and future prospects for development of sustainable agriculture. *Journal of Microbial & Biochemical Technology* **7**(2), 96–102.

Gutiérrez Mañero FJ, Ramos-Solano B, Probanza A, Mehouachi J, Tadeo FR, Talon M (2001) The plant-growth-promoting rhizobacteria *Bacillus pumilus* and *Bacillus licheniformis* produce high amounts of physiologically active gibberellins. *Physiologia Plantarum* **111**(2), 206–211. doi:10.1034/j.1399-3054.2001.1110211.x

Gutiérrez Mañero F, Probanza A, Ramos B, Colon Flores J, Lucas Garcia JA (2003) Effects of culture filtrates of rhizobacteria isolated from wild lupine on germination, growth, and biological nitrogen fixation of lupine seedlings. *Journal of Plant Nutrition* **26**(5), 1101–1115. doi:10.1081/PLN-120020078

Han HS, Lee KD (2005) Plant growth-promoting rhizobacteria: effect on antioxidant status, photo- synthesis, mineral uptake and growth of lettuce under soil salinity. *Research Journal of Agriculture and Biological Sciences* **1**, 210–215.

Hayat R, Ali S, Amara U, Khalid R, Ahmed I (2010) Soil beneficial bacteria and their role in plant growth promotion: a review. *Annals of Microbiology* **60**(4), 579–598. doi:10.1007/s13213-010-0117-1

Hirt H (2009) 'Plant stress biology: from genomics to systems biology.' (Wiley-Blackwell: Hoboken, NJ, USA)

Hoben HJ, Somasegran P (1982) Comparison of the pour spread and drop plate methods for enumeration of *Rhizobium* ssp. in inoculants made from pre-sterilized peat. *Applied and Environmental Microbiology* **44**, 1246–1247.

Hong CY, Chao YY, Yang MY, Cheng SY, Cho SC (2009) NaCl-induced expression of glutathione reductase in roots of rice (*Oryza sativa* L.) seedlings is mediated through hydrogen peroxide but not abscisic acid. *Plant and Soil* **320**(1–2), 103–115. doi:10.1007/s11104-008-9874-z

Hu Z, Fan J, Chen K, Amombo E, Chen L, Fu J (2016) Effects of ethylene on photosystem II and antioxidant enzyme activity in Bermuda grass under low temperature. *Photosynthesis Research* **128**(1), 59–72. doi:10.1007/s11120-015-0199-5

Irikura VM, Kihara M, Yamaguchi S, Sockett H, Macnab RM (1993) *Salmonella typhimurium* fliG and fliN mutations causing defects in assembly, rotation, and switching of the flagellar motor. *Journal of Bacteriology* **175**, 802–810. doi:10.1128/jb.175.3.802-810.1993

Ito M, Kim YG, Tsuji H, Kiwaki M, Nomoto K, Tanaka R, Okada N, Danbara H (2010) A practical random mutagenesis system for probiotic *Lactobacillus casei* using Tn5 transposition complexes. *Journal of Applied Microbiology* **109**(2), 657–666.

Kazmierczak BI, Mielke DL, Russel M, Model P (1994) pIV, a filamentous phage protein that mediates phage export across the bacterial cell envelope, forms a multimer. *Journal of Molecular Biology* **238**, 187–198. doi:10.1006/jmbi.1994.1280

Kihara M, Miller GU, Macnab RM (2000) Deletion analysis of the flagellar switch protein FliG of *Salmonella*. *Journal of Bacteriology* **182**(11), 3022–3028. doi:10.1128/JB.182.11.3022-3028.2000

Kim SY, Lim JH, Park MR, Kim YJ, Park T, Seo YW, Choi KG, Yun SJ (2005) Enhanced antioxidant enzymes are associated with reduced hydrogen peroxide in barley roots under saline stress. *Journal of Biochemistry and Molecular Biology* **38**, 218–224.

Kohler J, Hernández JA, Caravaca F, Roldán A (2009) Induction of antioxidant enzymes is involved in the greater effectiveness of a PGPR versus AM fungi with respect to increasing the tolerance of lettuce to severe salt stress. *Environmental and Experimental Botany* **65**(2–3), 245–252. doi:10.1016/j.envexpbot.2008.09.008

Korotkov KV, Sandkvist M, Hol WGJ (2012) The type II secretion system: biogenesis, molecular architecture and mechanism. *Nature Reviews. Microbiology* **10**(5), 336–351.

Linderoth NA, Model P, Russel M (1996) Essential role of a sodium dodecyl sulfate-resistant protein IV multimer in assembly-export of filamentous phage. *Journal of Bacteriology* **178**, 1962–1970. doi:10.1128/jb.178.7.1962-1970.1996

Llamas I, Argandoña M, Quesada E, del Moral A (2000) Transposon mutagenesis in *Halomonas eurihalina*. *Research in Microbiology* **151** (1), 13–18. doi:10.1016/S0923-2508(00)00132-7

Lloyd SA, Tang H, Wang X, Billings S, Blair DF (1996) Torque generation in the flagellar motor of *Escherichia coli*: evidence of a direct role for FliG but not FliM or FliN. *Journal of Bacteriology* **178**, 223–231. doi:10.1128/jb.178.1.223-231.1996

Lucas JA, Ramos Solano B, Montes F, Ojeda J, Megias M, Gutierrez Mañero FJ (2009) Use of two PGPR strains in the integrated management of blast disease in rice (*Oryza sativa*) in Southern Spain. *Field Crops Research* **114**(3), 404–410. doi:10.1016/j.fcr.2009.09.013

Lucas García JA, Schloter M, Durkaya T, Hartmann A, Gutiérrez Mañero FJ (2003) Colonization of pepper roots by a plant growth promoting *Pseudomonas fluorescens* strain. *Biology and Fertility of Soils* **37**(6), 381–385.

Lucas García JA, Domenech J, Santamaría C, Camacho M, Daza A, Gutierrez-Mañero FJ (2004) Growth of forest plants (pine and holm oak) inoculated with rhizobacteria: relationship with microbial community structure and biological activity of its rhizosphere. *Environmental and Experimental Botany* **52**(3), 239–251. doi:10.1016/j.envexpbot.2004.02.003

Lucas García JA, Probanza A, Ramos B, Colón Flores J, Gutiérrez Mañero FJ (2004*b*) Effects of plant growth promoting rhizobacteria (PGPRs) on the biological nitrogen fixation, nodulation, and growth of *Lupinus albus* l. cv. Multolupa. *Engineering in Life Sciences* **4**(1), 71–77. doi:10.1002/elsc.200400013

Lugtenberg B, Kamilova F (2009) Plant-growth-promoting rhizobacteria. *Annual Review of Microbiology* **63**, 541–556. doi:10.1146/annurev.micro.62.081307.162918

Maldonado-González MM, Bakker PAHM, Prieto P, Mercado-Blanco J (2015) *Arabidopsis thaliana* as a tool to identify traits involved in *Verticillium dahliae* biocontrol by the olive root endophyte *Pseudomonas fluorescens* PICF7. *Frontiers in Microbiology* **6**, 266. doi:10.3389/fmicb.2015.00266

Moraleda-Muñoz A, Pérez J, Extremera AL, Muñoz-Dorado J (2010) Differential regulation of six heavy metal efflux systems in the response of *Myxococcus xanthus* to copper. *Applied and Environmental Microbiology* **76**(18), 6069–6076. doi:10.1128/AEM.00753-10

Pal KK, Tilak KV, Saxena AK, Dey R, Singh CS (2001) Suppression of maize root diseases caused by *Macrophomina phaseolina*, *Fusarium moniliforme* and *Fusarium graminearum* by plant growth promoting rhizobacteria. *Microbiological Research* **156**(3), 209–223. doi:10.1078/0944-5013-00103

Paul K, Brunstetter D, Titen S, Blair DF (2011) A molecular mechanism of direction switching in the flagellar motor of *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **108**(41), 17171–17176. doi:10.1073/pnas.1110111108

Penrose D, Glick B (2003) Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. *Physiologia Plantarum* **118**(1), 10–15. doi:10.1034/j.1399-3054.2003.00086.x

Ramos Solano B, Barriuso Maicas J, Pereyra De La Iglesia M, Domenech J, Gutiérrez Mañero FJ (2008) Systemic disease protection elicited by plant growth promoting rhizobacteria strains: relationship between metabolic responses, systemic disease protection, and biotic elicitors. *Phytopathology* **98**(4), 451–457. doi:10.1094/PHYTO-98-4-0451

Ramos-Solano B, Lucas García JA, Garcia-Villaraco A, Algar E, Garcia-Cristobal J, Gutierrez Mañero FJ (2010*b*) Siderophore and chitinase producing isolates from the rhizosphere of *Nicotiana glauca* Graham enhance growth and induce systemic resistance in *Solanum lycopersicum* L. *Plant and Soil* **334**(1–2), 189–197. doi:10.1007/s11104-010-0371-9

Richardson AE, Barea JM, Mcneill AM, Prigent-Combaret C (2009) Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by micro-organisms. *Plant and Soil* **321**(1–2), 305–339. doi:10.1007/s11104-009-9895-2

Saleem M, Arshad M, Hussain S, Bhatti AS (2007) Perspective of plant growth promoting rhizobacteria (PGPR) containing ACC deaminase in stress agriculture. *Journal of Industrial Microbiology & Biotechnology* **34**(10), 635–648. doi:10.1007/s10295-007-0240-6

Sambrook J, Russel DW (2001) 'Molecular Cloning: A Laboratory Manual. 3rd Edition.' (Cold Spring Harbor Laboratory Press: New York)

Sandkvist M (2001) Type II secretion and pathogenesis. *Infection and Immunity* **69**(6), 3523–3535. doi:10.1128/IAI.69.6.3523-3535.2001

Sandkvist MM, Hough LPL, Bagdasarian MMM, Bagdasarian MM (1999) Direct interaction of the EpsL and EpsM proteins of the general secretion apparatus in *Vibrio cholerae*. *Journal of Bacteriology* **181** (10), 3129–3135.

Silver S, Phung LT (2005) A bacterial view of the periodic table: genes and proteins for toxic inorganic ions. *Journal of Industrial Microbiology & Biotechnology* **32**(11–12), 587–605. doi:10.1007/s10295-005-0019-6

Singh JS, Pandey VC, Singh DP (2011) Efficient soil microorganisms: a new dimension for sustain- able agriculture and environmental development. *Agriculture, Ecosystems & Environment* **140**, 339–353. doi:10.1016/j.agee.2011.01.017

Sokal RR, Rohlf FJ (1980) 'Introducción a la bioestadística.' (SA Reverté: Barcelona, Spain)

Štajner D, Gasaić O, Matković B, Varga SZI (1995) Metolachlor effect on antioxidants enzyme activities and pigments content in seeds and young leaves of wheat (*Triticum aestivum* L.). *Agricoltura Mediterranea* **125**, 267–273.

Štajner D, Kevrešan S, Gašić O, Mimica-Dukić N, Zongli H (1997) Nitrogen and *Azotobacter chroococcum* engance oxidative stress tolerance in sugar beet. *Biologia Plantarum* **39**, 441–445. doi:10.1023/A:1001000830977

TerBraak CFJ, Šmilauer P (1998) 'CANOCO reference manual and user's guide to Canoco for Windows. Software for canonical community ordination (ver. 4)'. (Centre for Biometry: Wageningen, Germany)

Tsai YC, Hong CY, Liu LF, Kao CH (2004) Relative importance of Na^+ and Cl^- in NaCl-induced antioxidant systems in roots of rice seedlings. *Physiologia Plantarum* **122**, 86–94. doi:10.1111/j.1399-3054.2004.00387.x

van Hulten M, Pelser M, van Loon LC, Pieterse CMJ, Ton J (2006) Costs and benefits of priming for defense in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America* **103**(14), 5602–5607. doi:10.1073/pnas.0510213103

van Loon LC, Bakker PAHM, Pieterse CMJ (1998) Systemic resistance induced by rhizosphere bacteria. *Annual Review of Phytopathology* **36**(1), 453–483. doi:10.1146/annurev.phyto.36.1.453

Větrovský T, Baldrian P (2013) The variability of the 16S rRNA gene in bacterial genomes and Its consequences for bacterial community analyses. *PLoS One* **8**(2), e57923. doi:10.1371/journal.pone.0057923

Washio K, Lim SP, Roongsawang N, Morikawa M (2010) Identification and characterization of the genes responsible for the production of the cyclic lipopeptide arthrofactin by *Pseudomonas* sp. MIS38. *Bioscience, Biotechnology, and Biochemistry* **74**(5), 992–999. doi:10.1271/bbb.90860

Wiesel L, Newton AC, Elliott I, Booty D, Gilroy EM, Birch PRJ, Hein I (2014) Molecular effects of resistance elicitors from biological origin and their potential for crop protection. *Frontiers in Plant Science* **5**, 655. doi:10.3389/fpls.2014.00655

Xu C, Natarajan S, Sullivan JH (2008) Impact of solar ultraviolet-B radiation on the antioxidant defense system in soybean lines differing in flavonoid contents. *Environmental and Experimental Botany* **63**(1–3), 39–48. doi:10.1016/j.envexpbot.2007.10.029

Yan Q, Wang N (2011) The ColR/ColS two-component system plays multiple roles in the pathogenicity of the citrus canker pathogen *Xanthomonas citri* subsp. *citri*. *Journal of Bacteriology* **193**(7), 1590–1599. doi:10.1128/JB.01415-10

Zhang S, Reddy M, Kloepper J (2004) Tobacco growth enhancement and blue mold disease protection by rhizobacteria: relationship between plant growth promotion and systemic disease protection by PGPR strain 90-166. *Plant and Soil* **262**(1–2), 277–288. doi:10.1023/B:PLSO.0000037048.26437.fa