

Micro-scale water potential gradients visualized in soil around plant root tips using microbiosensors

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ABSTRACT

Water availability and movement in soil are critical determinants of resource availability to, and interactions among, members of the soil community. However, it has been impossible to observe gradients in soil water potential empirically at millimetre spatial scales. Here we describe progress towards that goal using output from two microbial biosensors, *Pantoea agglomerans* BRT98/pPProGreen and *Pseudomonas putida* KT2442/pPProGreen, engineered with a reporter system based on the osmotically sensitive *proU* promoter from *Escherichia coli*. The *proU*-GFP construct in both microbiosensors produced green fluorescent protein (GFP) as a function total water potential in non-sterile soil. Controlled experiments in liquid culture showed that dramatically different microbiosensor growth rates (resulting from exposure to different salts as osmolytes) did not alter the GFP output as a function of water potential in either sensor, but *P. agglomerans*' GFP levels at a given water potential were strongly influenced by the type of carbon (energy) source available to the microbes. In non-sterile rhizosphere soil along *Zea mays* L. roots, though GFP expression was quite variable, microbiosensors reported statistically significantly more negative soil water potentials as a function of axial distance from root tips, reflecting the gradient in soil water potential hypothesized to develop during transpiration.

Key-words: *Zea mays*; bioreporter; rhizosphere.

INTRODUCTION

The distribution and availability of water in soil influences diverse processes including nutrient availability for plant growth, decomposition of organic matter, activity of micro- and mesofauna, and microbial activity. Current tools commonly used to measure water availability (water potential) in soil, such as thermocouple psychrometres, tensiometres and time domain reflectometry, integrate water potential on

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gross spatial scales but do not provide information at the very small scales in which microbes, plant roots and root hairs operate. Mathematical models of gradients in water potentials around roots (i.e. 'single-root' models, Hillel 1998) have been developed in agronomic, plant physiological, soil science and environmental engineering literatures for several decades because of the importance of those gradients in influencing e.g. diffusion, bulk flow, and thus distributions of nutrients and other solutes around plant roots (e.g. Tinker & Nye 2000). But those predicted gradients at small scales around plant roots remain unobserved for lack of a water potential sensing tool that can operate at such scales (Hillel 1998). Here we describe development of microbial biosensors that report the water potentials they experience in their soil environment using a plasmid-borne fusion of the *proU* promoter from *Escherichia coli* and the reporter gene *gfp*.

Microbiosensors have been used previously in soil to detect diverse compounds such as sugars (Jaeger *et al.* 1999; Bringhurst, Cardon & Gage 2001), nitrate (DeAngelis *et al.* 2005), amino acids (Jaeger *et al.* 1999), and contaminants (Casavant *et al.* 2003), as well as to indicate microbial activity and growth (Ramos, Molbak & Molin 2000). Microbiosensors are living bacterial cells into which fusions of a promoter of interest and a reporter gene driven by that promoter are inserted. The outputs produced by microbiosensors are varied and have individual strengths and weaknesses (Hansen & Sørensen 2001; Killham & Yeomans 2001; Leveau & Lindow 2002; Cardon & Gage 2006; Gage *et al.* 2008). For example, production of ice-nucleating proteins (by *inaZ* reporter genes) can be coupled into amplification systems and thus produce very sensitive output, but the destructive harvest of soils is required for quantification of *InaZ* (Jaeger *et al.* 1999; DeAngelis *et al.* 2005). Fluorescence from green fluorescent protein (GFP) and light from *lux* gene products can be quantified non-destructively using a microscope or a camera lens coupled to a sensitive charge-coupled device (CCD) camera, respectively (Bringhurst *et al.* 2001; Darwent *et al.* 2003; DeAngelis *et al.* 2005), but signal is not amplified (Leveau & Lindow 2002). Beyond such challenges with the reporting mechanism itself, microbiosensors are living organisms with their own specific environmental and resource preferences, preferences that influence metabolism, growth and division rates and thus,

potentially, concentrations of reporter molecule per cell (Leveau & Lindow 2001). An ideal microbiosensor should report faithfully on the condition of interest no matter the state of other environmental conditions and resources; how closely living microbiosensors conform to this high standard is rarely tested yet it shapes the kind of information that can be gathered using them (Gage *et al.* 2008).

Two microorganisms, *Pantoea agglomerans* BRT98/pPProGreen (Axtell & Beattie 2002) and *Pseudomonas putida* KT2442/pPProGreen (newly developed here) were engineered with a reporter system known to be sensitive to osmotic potential (one component of total water potential). Within the reporter system, expression of a *gfp* gene, and thus production of GFP, was linked to the promoter from the *proU* operon of *E. coli*; genes in this operon are known to be upregulated when the cells experience osmotic stress (May *et al.* 1989). Previous work has demonstrated that promoter (P_{proU}) activity in *E. coli* and *Salmonella enterica* (May *et al.* 1989; Herbst, Kneip & Bremer 1994; Lucht & Bremer 1994), and engineered into *P. agglomerans* BRT98 and *Pseudomonas syringae* (Axtell & Beattie 2002), scales with increasing osmotic stress in the environment. We explored the expression of *proU-gfp* in each of our two microbiosensors in liquid culture, examining their sensitivity to the type and concentration of osmolyte present as well as the type of carbon (energy) source available. We also tested whether the *proU-gfp* construct in *P. agglomerans* BRT98 and *P. putida* KT2442 produced GFP as a function of total water potential in soil, which has both osmotic and matric components.

Ultimately, the ability to detect gradients of water availability in soil, and particularly in the rhizosphere, is of special interest because water availability has a direct influence on microbial activity and mobility, on the flow of soil solution and diffusion of dissolved nutrients, and on capture of nutrients by plant roots. After the testing described earlier, we examined whether the microbiosensor *P. agglomerans* BRT98/pPProGreen deployed in non-sterile soil could detect a drawdown in soil water potential around living *Zea mays* L. (corn) roots during transpiration. Gradients in water potential are known to develop within plant root axes during transpiration (e.g. Landsberg & Fowkes 1978; Hsiao & Xu 2000; Zwieniecki, Thompson & Holbrook 2002), and we examined whether *proU-gfp* expression from the microbiosensors indicated less negative rhizosphere soil water potential near the root tip, and more negative water potential in soil along the maturing root axis, during transpiration. We also examined whether the pattern in production of GFP around roots disappeared when transpiration was not occurring. Such small-scale gradients in soil water potential have been impossible to observe *in situ* using other measurement techniques (Hillel 1998).

MATERIALS AND METHODS

Strains and plasmids

P. agglomerans (BRT98) (Marcell & Beattie 2002) and *E. coli* DH5 α (both obtained from G. Beattie) and the soil

bacterium *P. putida* (KT2442) (obtained from C. Arango Pinedo) were used in the study. Strains BRT98 and KT2442 are both rifampin resistant.

The plasmid pPProGreen (Axtell & Beattie 2002) contains a fusion of the *proU* promoter to *gfp*(F64L-S65T) (Tsien 1998). The positive control plasmid pPNptGreen (Axtell & Beattie 2002) contains a fusion of the *nptII* promoter (insensitive to osmotic or water potential) to *gfp*(F64L-S65T). (The *nptII* promoter drives the expression of three antibiotic resistance genes in the transposon Tn5.) Both plasmids were obtained from G. Beattie, as were *P. agglomerans* strains BRT98/pPNptGreen and BRT98/pPProGreen. To create the *P. putida* biosensor and control strains, respectively, pPProGreen and pPNptGreen were moved from *E. coli* DH5 α into *P. putida* KT2442 by triparental mating using *E. coli* helper strain MT616/pRK600. Both pPProGreen and pPNptGreen conferred resistance to the antibiotic kanamycin, which was used at 50 $\mu\text{g mL}^{-1}$ kanamycin in all growth media.

Microbiosensor response to varied water potential, osmolytes, and carbon sources in liquid culture

The four engineered strains (*P. putida*/pPProGreen, *P. putida*/pPNptGreen, *P. agglomerans*/pPProGreen, and *P. agglomerans*/pPNptGreen) were streaked on separate Luria-Bertani agar plates supplemented with kanamycin, and colonies were picked for various tests in liquid culture.

Sensitivity of new strains *P. putida*/pPProGreen (biosensor) and *P. putida*/pPNptGreen (control) to water potential in solution

Colonies were picked from plates and grown in 10 mL of medium across a range of osmotic potentials, in triplicate 125 mL flasks, with shaking, at 30 °C. Medium in flasks was $\frac{1}{2}$ 21C medium (Halverson & Firestone 2000) plus kanamycin, supplemented with sodium chloride (NaCl) calculated using the Van't Hoff equation to produce a range of osmotic potentials: -0.15, -0.4, -0.6, -1.1 and -1.5 MPa. (The osmotic potential of the medium without supplemental NaCl was -0.15 MPa. NaCl was added to produce 0, 54, 97, 206 and 295 mM NaCl to generate the varied water potentials.) Water potentials of media were confirmed using screen-caged thermocouple psychrometers immersed for at least 8 h in media at 25 °C (Wescor, Logan, UT, USA, connected to a CR7 datalogger, Campbell Scientific, Logan, UT, USA). Growth was monitored by measuring optical density of 100 μL subsamples of each culture at 595 nm (OD_{595}) in a 96-well plate using a Bio-Rad 550 plate reader (BioRad, Hercules, CA, USA). During mid-exponential growth phase (OD_{595} of 0.05 to 0.2), the triplicate cultures at each water potential were sampled for determining GFP levels. A 100 μL sample of bacteria was fixed in 1 mL of $\frac{1}{2}$ 21C media with 0.5% formalin and the geometric mean fluorescence per cell determined by dual laser flow

cytometry (Becton-Dickinson FacsCalibur, San Jose, CA, USA, abbreviated as FACS hereafter) standardized with Inspeck Green 505/515, 6 μm calibration beads (Molecular Probes, Eugene, OR, USA; we determined that fixation caused a repeatable 40% loss of fluorescence, so all samples were fixed to provide the necessary flexibility of timing between harvest and analysis demanded by the multi-user, multi-department FACS facility.) The 488 nm argon laser line was used for excitation of GFP, coupled with a 530/30 nm bandpass emission filter. Size-related forward scatter data also gathered by the FACS was used by WinMDI 2.8 software (Windows Multiple Document Interface for Flow Cytometry software, J. Trotter, The Scripps Research Institute, La Jolla, CA, USA) to gate (select) fluorescence data from only bacteria in the stream, thus avoiding mixing data from bacteria with other smaller, non-living particles in solution. Data for >100 000 bacteria per flask sample were collected and WinMDI 2.8 was used to calculate the geometric mean of fluorescence per bacterial cell in each flask sample.

To test whether the new biosensor *P. putida*/pPProGreen could detect water potential more broadly defined than salt-based osmotic potential in solution, we also examined GFP production by *P. putida*/pPProGreen and control *P. putida*/pPNptGreen in triplicate solutions of $\frac{1}{2}$ 21C + kanamycin supplemented with the nonpermeating solute polyethylene glycol (PEG-8000, Axtell & Beattie 2002). Following Michel (1983), we used PEG-8000 to generate matric potentials as for the NaCl supplement experiment described earlier, and we used the same growth conditions and analysis techniques. Concentrations of 0, 4, 15, 26 and 33% w/v PEG 8000 in $\frac{1}{2}$ 21C media were used to generate water potentials of -0.15, -0.17, -0.40, -0.94 and -1.42 MPa.

Expression of GFP by P. putida/pPProGreen and P. agglomerans/pPProGreen in response to osmotic potential generated by two different osmolytes, and supported by two different carbon sources

To test whether the GFP expression at a given osmotic potential was influenced by osmolyte type or by the carbon (energy) source available, *P. putida*/pPProGreen and *P. agglomerans*/pPProGreen were inoculated into separate 250 mL Erlenmeyer flasks containing 20 mL of $\frac{1}{2}$ 21C medium amended with KCl or NaCl to produce osmotic potentials of -0.5, -1.0 and -1.5 MPa. Carbon sources were either glucose or succinate at 0.15% (w/v), which supported different growth rates at the same water potentials. The contribution of carbon source to the overall osmotic potential of the growth media was taken into account. Each treatment was replicated three times. Bacterial cultures were maintained at mid-exponential growth by periodic dilution for 18 h, growth rate (based on OD_{595}) was documented for each treatment and strain, and fluorescence measurements were collected hourly by FACS for the final 8 h of the experiment and averaged for each flask.

Microbiosensor response in soil held at controlled water potentials

Tests detailed earlier were in liquid culture. To examine whether GFP fluorescence from *P. putida*/pPProGreen and *P. agglomerans*/pPProGreen microbiosensors in soil scaled with soil water potential, we constructed sealed vials for controlling soil samples at known water potentials. For each of the biosensors, triplicate vials were constructed at each of 13 water potentials between -0.15 and -4 MPa generated by NaCl in water. A small air space was left in the top of each vial, a mesh platform was suspended in that air space on top of an o-ring wedged in the vial, and 40 mg of sandy loam held in a small plastic cup made from the top of an eppendorf tube was placed on the mesh platform (the sandy loam was natural loam steam pasteurized for use in the University of Connecticut greenhouses and derived from common Connecticut acidic soils). Three replicates of soil suspended over pure water were also constructed for each biosensor. Vials were sealed with rubber septum caps, held at constant 25 °C, and soil water potential was allowed to equilibrate for at least three days with the range of controlled water potentials in the vial atmospheres. Microbiosensors grown overnight in $\frac{1}{2}$ 21C medium were pelleted and then resuspended in that medium plus 0.5% glucose and 0.08 M NaCl at an OD_{595} of 0.24, measured as described previously in a BioRad 550 plate reader. Eight microliters of bacterial suspension were deposited in the soil in each vial using a syringe needle inserted through the septum cap. After equilibration for at least 12 h, bacteria were extracted from soil in each vial by vortexing the soil in 1 mL of $\frac{1}{2}$ 21C medium, filtering the solution through a nylon filter of 10 μm pore size (Osmonics, Minnetonka, MN, USA), and then fixing with 0.5% formalin. Fluorescence detected through the 530/30 nm bandpass filter, from more than 3000 bacteria per vial sample, was analysed by FACS and WinMDI software as described earlier, using gating based on forward scatter to isolate particles in the flow cytometry stream that were the size of bacteria.

Tests of *P. agglomerans*/pPProGreen (biosensor) and *P. agglomerans*/pPNptGreen (control) prior to use in rhizosphere soil

Expression of GFP by P. agglomerans/pPProGreen at different growth stages

Based on results from the experiments with varied osmolytes and carbon sources in liquid culture, and the tests of sensitivity of the two microbiosensors to controlled soil water potentials described earlier, *P. agglomerans*/pPProGreen was chosen (because of its much higher fluorescence per cell) for use in rhizosphere soil around plant roots (see Discussion for further rationale). However, in the rhizosphere environment, it is likely that growth rates might vary and that bacterial populations would not remain in exponential growth throughout an experiment. We therefore tested whether GFP fluorescence from *P. agglomerans*/

pPProGreen cells, at known osmotic potentials, changed depending on the growth stage of the bacteria.

P. agglomerans/pPProGreen was inoculated into replicate 200 mL Erlenmeyer flasks containing 20 mL of $\frac{1}{2}$ 21C medium amended with NaCl to produce osmotic potentials of -0.15 , -0.5 and -1.0 MPa, with glucose as a carbon source. Four replicate flasks ($n = 4$) were used for -0.15 and -0.5 MPa; five ($n = 5$) were used for -1.0 MPa because GFP production is more variable at the more negative water potentials when it is calculated by dividing culture fluorescence by culture OD. The relatively high variation in the low water potential samples likely comes about from dividing the relatively high culture fluorescence by a low OD, the measurement of which is variable because of the limited instrument sensitivity at low ODs. The flasks were maintained at 30°C with shaking. In order to detect whether the growth stage of the inoculum at time zero might influence the expression of GFP as the bacteria regrew through various growth stages (exponential to stationary phases), the cultures used to inoculate experimental flasks at time zero were grown to three different stages (mid-exponential, stationary phase for a short time, stationary phase for a longer period) prior to inoculation in three separate experiments. At time zero, inoculating bacteria were diluted into new media and the OD₅₉₅ and GFP fluorescence that developed in flasks over time was tracked over ten hours using 100 μL subsamples removed to a 96-well plate and analysed in a Biotek Synergy HT (Winooski, VT) plate reader. GFP fluorescence for all samples was normalized by dividing by OD₅₉₅ at each sampling point. GFP fluorescence per cell was also measured from two flasks per water potential using FACS as described for previous experiments, but because of time constraints, only during one experiment.

Check of the sensitivity of the P. agglomerans/pPNptGreen (positive control) strain to osmotic potential in solution

Finally, to support use of *P. agglomerans*/pPProGreen in the rhizosphere of transpiring plants, two kinds of controls in soil microcosms were necessary for interpretation of microbiosensor GFP output in soils around roots – a plant and a microbial control (see the Methods section). The plant control was simply having some plants that were not transpiring, as well as some that were. The microbial control tested whether the microbial strain *P. agglomerans* was capable of being active and producing GFP in soil under the conditions of the rhizosphere experiment. The microbial control strain *P. agglomerans*/pPNptGreen has the same background metabolism as the *P. agglomerans*/pPProGreen biosensor, but the *nptIII* promoter drives GFP production constitutively at a high level, a state similar to the status of the microbiosensor *P. agglomerans*/pPProGreen at the most negative soil water potentials. Prior to using the control and microbiosensor strains in soil, however, it was necessary to reconfirm that GFP content in the control strain *P. agglomerans*/pPNptGreen did not scale strongly with osmotic potential (following Wright & Beattie 2004). In an

experiment parallel to that described earlier for *P. putida*/pPProGreen (biosensor) and *P. putida*/pPNptGreen (control), both *P. agglomerans* strains were inoculated into 250 mL Erlenmeyer flasks, containing 20 mL of $\frac{1}{2}$ 21C medium amended with 0.15% (w/v) glucose and with KCl or NaCl to produce osmotic potentials of -0.5 , -1.0 and -1.5 MPa. Each treatment was replicated three times for each bacterial strain. Cultures were maintained at mid-exponential growth for approximately 18 h, and fluorescence measurements were collected by FACS at 8, 12, and 17 h time points and averaged for each flask.

Microbiosensor and control reports in soil around corn root tips

Z. mays (L.) was grown from seed in soil in glass fronted microcosms (40 cm tall \times 22 cm wide \times 1 cm deep, back made of 1/8 in. PVC sheet, sides made of thick neoprene foam strips) in the greenhouse at University of Connecticut with 15 h day length (maintained during winter using high pressure sodium fixtures). Soil was a mix of unpasteurized loam, sand and peat in equal proportions, moistened, but not soaked, before filling the microcosms. Microcosms were filled with soil from the top with frequent tamping to yield a homogeneously packed soil column. Roots were encouraged to grow through soil against the glass side of the microcosm by tipping the microcosm approximately 30 degrees from vertical. Glass was covered with aluminum foil to prevent exposure of roots to light during the approximately 2 weeks of plant growth. Plants were always used before the corn primary root tip reached the base of the microcosm.

In preparation for soil inoculation, *P. agglomerans*/pPProGreen and the control *P. agglomerans*/pPNptGreen were grown separately in 20 mL of NaCl- and glucose-amended $\frac{1}{2}$ 21C media (0.3% glucose by weight, and 0.08 M NaCl), in 250 mL flasks with shaking at 30°C . Cells were harvested during late exponential growth (OD 0.4) and diluted to 0.04 OD in fresh medium. When plants were between 13 and 16 d old, the glass was removed from the front of the microcosms in very early morning and approximately 5 mL of bacteria/media were misted evenly across the exposed surface using a 25 mL reagent sprayer (Kontes, Vineland, NJ, USA). Microbiosensors were misted onto soil just prior to treatments, rather than mixed into soil when seeds were planted, because these were non-sterile soils with active food webs, and microbiosensor populations would potentially have been grazed down dramatically over the course of 2–3 weeks of plant growth prior to our treatments (e.g. Bringhurst *et al.* 2001).

Six microcosms were inoculated with the *P. agglomerans*/pPProGreen biosensor, and three with the control strain *P. agglomerans*/pPNptGreen. Each microcosm was reassembled and returned to the greenhouse, where three of the plants inoculated with biosensors, as well as the three plants inoculated with the control strain, were encouraged to transpire by applying a gentle breeze with a fan over 15 h in the light. Control plants in the other three microcosms

inoculated with the biosensor strain were discouraged from transpiring by placing a pre-moistened plastic bag over the shoot of the corn (no wilting nor overheating of the corn occurred; these experiments were conducted during winter of 2006 when heat load and sun angle were low in the greenhouse). At the end of 15 h, the microcosms were removed from the greenhouse and biosensor fluorescence was examined on an inverted Nikon TE 300 epifluorescence microscope (Nikon, Melville, NY) *in situ* in the microcosm soil. The microscope was equipped with a 465–495 nm excitation and 515–555 nm emission filter set; fluorescence was imaged at 40× magnification using a 12-bit Retiga EX CCD camera (1036 × 1360 pixels, QImaging, Burnaby, BC, Canada) and Openlab software (PerkinElmer, Waltham, MA, USA) on a Macintosh G4. Measured fluorescence was standardized across microcosms using Inspeck Green 505/515, 6 μm calibration beads (Molecular Probes). On average, for each microcosm, 45 images of rhizosphere soil, with field of view approximately 220 μm by 210 μm, were captured, using a 0.6 N.A., extra long-working distance 40× objective (Nikon, Melville, NY, USA; 2.7 mm working distance), along a main root between 0 and 80 mm from the root tip and within 1.4 mm of the root surface. GFP fluorescence from bacteria in focus in images (e.g. Fig. 5a) was quantified using National Institutes of Health (NIH) Image (a public domain image processing and analysis program developed at the NIH). Approximately 12 bacteria were in focus per image.

Root development has been extensively studied in corn, and the bulk of water-conducting xylem elements are known to mature and open only at a distance from the root tip. To help interpret any gradients in rhizosphere water potential observable with the microbiosensors, several root sections were cut by hand after all non-destructive fluorescence images had been taken. Sections were stained with berberine hemisulfate (0.1%) and counterstained with aniline blue (0.5% w/v) to detect suberin (e.g. in mature casparian strip), lignin (e.g. in secondary walls of mature metaxylem) and callose, using fluorescence microscopy (Brundrett, Enstone & Peterson 1988). Young, undifferentiated root sections were also embedded in methacrylate, cross-sectioned and examined with light microscopy.

Statistics

Results from liquid culture experiments were analysed by analysis of variance (ANOVA) using PROC GLM in SAS software (SAS Institute Inc., Cary, NC, USA) with carbon type, water potential and osmolyte as fixed effects. *Post hoc* comparisons were made using the Tukey test. Regression exploring GFP production per cell as a function of controlled soil water potential in vial experiments was also analysed using PROC GLM. For microcosm experiments, biosensor fluorescence along roots was tested for normality using the Shapiro–Wilks test, and homogeneity of variance using the Brown–Forsythe’s test. Analysis of covariance (ANCOVA) was carried out with root replicate as the grouping variable and distances along the root designated as the

covariate. Results of all *F* tests were considered significant at 95% confidence if $P < 0.05$ for a given *F* statistic. When ANCOVA demonstrated no significant difference between the three replicates, data from all three microcosms were pooled. As a conservative starting point, we tested whether a simple linear regression relating GFP fluorescence per cell to distance from the tip along the root axis was significant, and if the slope was significantly different from zero.

RESULTS

P. putida/pProGreen response to varied water potential

In both PEG and NaCl treatments, absolute fluorescence of the positive control strain *P. putida*/pPNptGreen (in which the *nptII* promoter was constitutively active) was approximately 5-fold higher than that produced by the microbiosensor *P. putida*/pProGreen, even at the most negative osmotic potential used in the experiment. Given this difference, we plot the GFP fluorescence induction ratio rather than raw fluorescence values to facilitate comparison of the induction of GFP production in reporter and control strains at different water potentials. This ratio is calculated as the average GFP fluorescence per cell at each osmotic potential divided by the average GFP fluorescence per cell from bacteria experiencing $\frac{1}{2}$ 21C medium alone at -0.15 MPa (Fig. 1). *P. putida*/pProGreen produced GFP as a linear function of osmotic potential generated by NaCl ($R^2 = 0.96$, $P < 0.001$) and by PEG-8000 ($R^2 = 0.98$, $P < 0.001$), but induction of fluorescence in NaCl was approximately double that in PEG at any potential (Fig. 1). GFP fluorescence was not induced by increasing concentrations of NaCl in the control strain *P. putida*/pPNptGreen ($R^2 = 0.20$, $P = 0.09$; Fig. 1). Increasing concentrations of PEG-8000 in growth media resulted in a very slight, but significant,

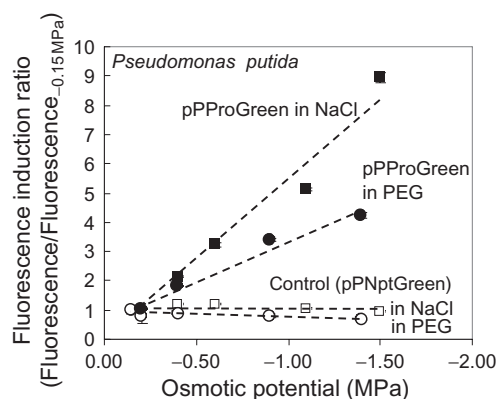


Figure 1. Mean (± 1 SE, $n = 3$) induction of GFP fluorescence in microbiosensor *P. putida*/pProGreen and control strain *P. putida*/pPNptGreen as a function of water potential controlled osmotically (NaCl) or with PEG8000. Values are expressed relative to fluorescence of microbiosensor or control strains in -0.15 MPa osmotic solution. PEG, polyethylene glycol.

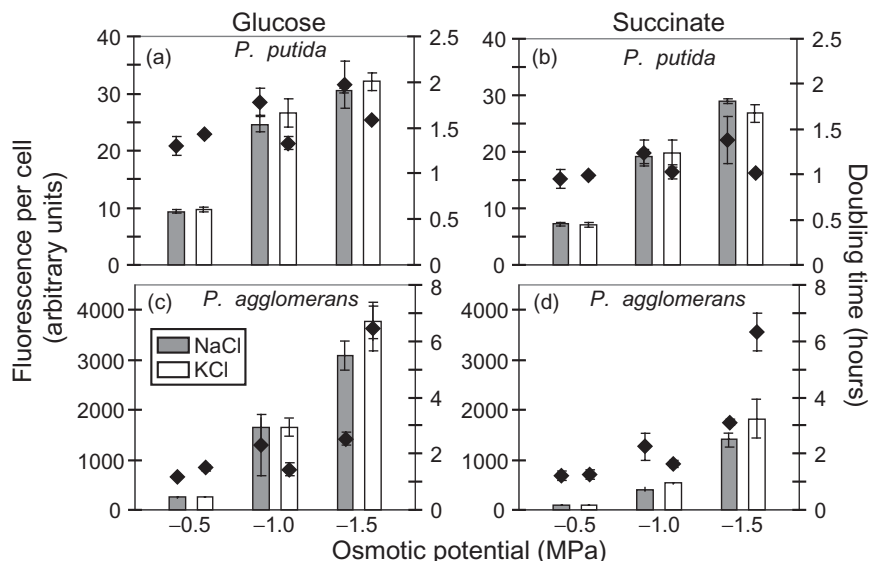


Figure 2. Mean (± 1 SE, $n = 3$) GFP fluorescence (bars) from *P. putida*/pPProGreen (a,b) and *P. agglomerans*/pPProGreen (c,d) grown in media set at three osmotic potentials produced with either NaCl or KCl, and grown using either glucose (a,c) or succinate (b,d) as a carbon source. Diamond symbols designate the mean (± 1 SE, $n = 3$) doubling time of the bacterial biosensors, as indicated on the right-hand Y axis.

decrease in GFP in *P. putida*/pPNptGreen ($R^2 = 0.39$, $P < 0.01$; Fig. 1).

Microbiosensor expression under varied water potentials, osmolytes, and carbon sources

GFP fluorescence per cell from the *P. agglomerans*/pPProGreen biosensor was 25–200 fold more intense than fluorescence from the *P. putida*/pPProGreen biosensor (Fig. 2a,b versus Fig. 2c,d). GFP fluorescence in *P. putida*/pPProGreen scaled with osmotic potential in liquid culture, with a small effect of carbon source (glucose versus succinate) but with no effect of osmolyte type (KCl versus NaCl) (Fig. 2a,b; Table 1). GFP fluorescence in *P. agglomerans*/pPProGreen also scaled with osmotic potential, with a strong effect of carbon source (Fig. 2c,d; Table 1). Osmolyte type had no statistically significant effect on GFP fluorescence per *P. agglomerans*/pPProGreen cell at any osmotic potential (Fig. 2c,d; Table 1), even though doubling times were nearly two times longer in high concentrations of KCl compared with NaCl (Fig. 2c,d, diamond symbols).

Microbiosensor expression in soil at controlled water potentials

Given the dramatic differences in absolute GFP fluorescence from the two biosensors illustrated in Fig. 2, comparison of induction of GFP fluorescence from the biosensors in soil in vials as a function of soil water potential is reported as fluorescence induction ratios in Fig. 3 (similar to Fig. 1). For this figure, the ratio is calculated as the average GFP fluorescence per cell at each soil water potential divided by the average GFP fluorescence per cell from bacteria in soil suspended above pure water. Both *P. putida*/pPProGreen and *P. agglomerans*/pPProGreen produced GFP fluorescence that scaled linearly with total soil water potential from 0 to at least -2 MPa. In *P. putida*/pPProGreen, GFP fluorescence could be expressed as a linear function of water potential from 0 to -4.0 MPa with a slope significantly different from zero ($R^2 = 0.52$, $P < 0.001$; Fig. 3). The GFP expression by *P. agglomerans*/pPProGreen appeared to scale linearly between 0 and -2 MPa ($R^2 = 0.33$, $P < 0.0001$), but the response was variable and appeared to saturate at higher water potentials (Fig. 3).

	<i>P. putida</i> /pPProGreen		<i>P. agglomerans</i> /pPProGreen	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Ψ	186.30	<0.001	143.78	<0.001
C	17.88	0.001	84.32	<0.001
Osm	0.15	0.704	3.35	0.080
$\Psi \times C$	1.44	0.257	18.05	<0.001
$\Psi \times \text{Osm}$	0.23	0.794	2.39	0.113
C \times Osm	1.03	0.319	0.05	0.830
$\Psi \times C \times \text{Osm}$	0.28	0.756	0.27	0.767

Statistically significant results at $P < 0.05$ are shown in bold.

Table 1. Results of analysis of variance exploring green fluorescent protein production by two microbiosensors, supported by two different carbon (C) sources, in response to a range of water potentials (Ψ) generated by two osmolytes (Osm)

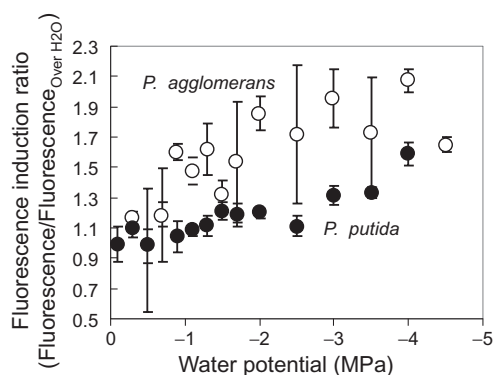


Figure 3. Mean (± 1 SE, $n = 3$) GFP induction in *P. putida*/pPProGreen (closed circles) and *P. agglomerans*/pPProGreen (open circles) in soils as a function of experimentally controlled soil water potentials. Values are expressed relative to fluorescence of microbiosensors in soil equilibrated within headspace above pure water.

Production of GFP by *P. agglomerans*/pPProGreen at different growth stages, and response of control *P. agglomerans*/pPNptGreen to osmotic potential in liquid culture

P. agglomerans/pPProGreen was chosen for rhizosphere experiments because its fluorescence was brighter (Fig. 2) and its induction was stronger (Fig. 3) than that in *P. putida*/pPProGreen. Prior to use of *P. agglomerans*/pPProGreen in rhizosphere soil, two tests in liquid culture were conducted, one to test whether growth stage of bacteria (exponential or stationary) affected the GFP signal, and a second to reconfirm that the positive control *P. agglomerans*/pPNptGreen did not respond strongly to water potential.

Growth stage experiment

For ease of viewing, trajectories of OD₅₉₅ tracking bacterial growth in individual flasks are connected in Fig. 4a, but individual points only (without connection lines for individual flasks) are shown for GFP fluorescence per cell in Fig. 4b. (In the three separate experiments, the growth status of cells at time zero inoculation did not affect GFP per cell that developed during cell growth.) GFP fluorescence from populations of *P. agglomerans*/pPProGreen cells at stationary phase and during batch culture was not affected by growth stage (Fig. 4b). As in Fig. 2c,d, GFP per cell was more variable at more negative water potentials. Subsamples of cells removed from plate reader wells and analysed by FACS also showed consistent patterns of GFP fluorescence per cell during culture growth (Fig. 4c).

P. putida/pPNptGreen sensitivity experiment

GFP fluorescence from the positive (constitutive) control strain *P. agglomerans*/pPNptGreen was not strongly

induced by negative osmotic potential in solution (Fig. 5b, repeating observations by Wright & Beattie 2004, and echoing results for *P. putida*/pPNptGreen in Fig. 1). Though there was a statistically significant increase in fluorescence per cell in the control strain as salt concentrations in solution (and thus water potentials) increased (ANOVA, $P < 0.001$), this increase was very small (induction ratios changed approximately twofold from -0.5 to -1.5 MPa). The induction of the *P. agglomerans*/pPProGreen microbiosensor was, in comparison, a 15-fold increase in GFP fluorescence upon shifting from -0.5 to -1.5 MPa (Fig. 5b). Results in Fig. 5b are expressed as the average GFP fluorescence per cell at each osmotic potential divided by the average GFP fluorescence per cell from bacteria experiencing $1/2$ 21C medium alone at -0.15 MPa.

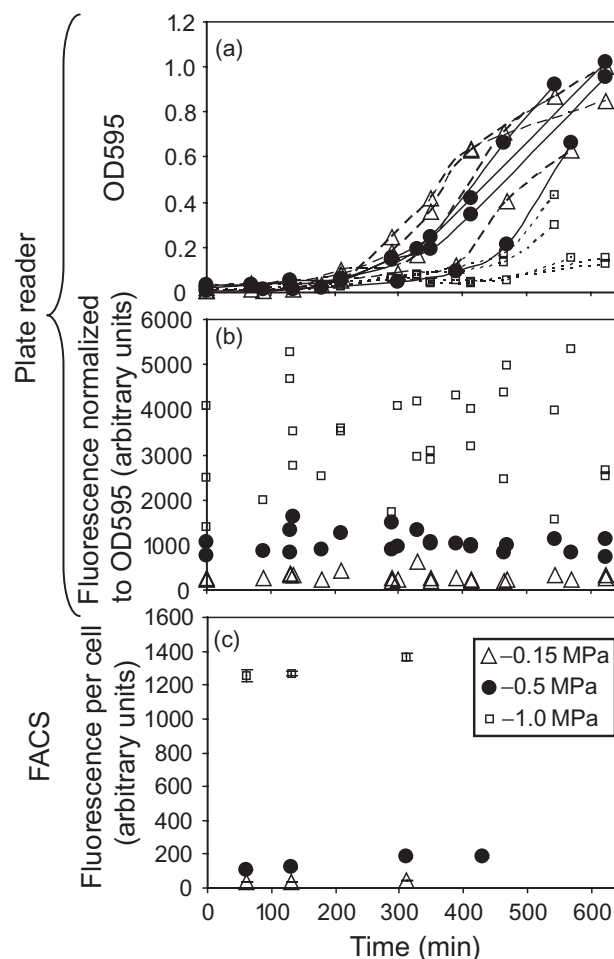


Figure 4. Mean (± 1 SE) OD₅₉₅ (a) and GFP fluorescence normalized to OD₅₉₅ (b) measured by plate reader from repeated subsamples of liquid cultures of *P. agglomerans*/pPProGreen growing through exponential to stationary phase at three osmotic potentials. (c) GFP fluorescence per cell measured by flow cytometry (FACS) from the same cultures.

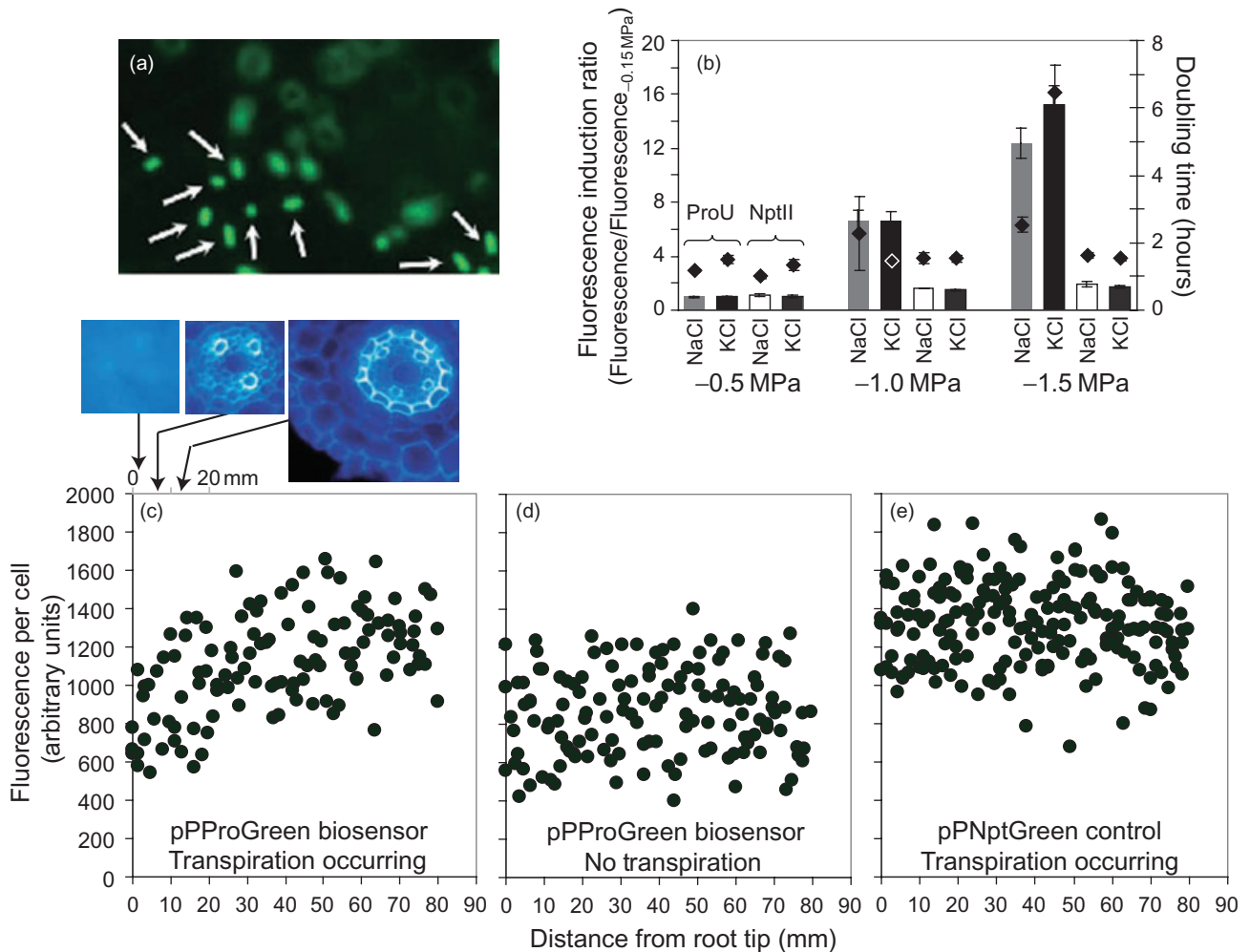


Figure 5. (a) Portion of one image taken of rhizosphere bacteria in soil showing green fluorescent protein (GFP) fluorescence from bacteria in focus (white arrows) and out of focus. (b) Induction of GFP fluorescence (mean \pm 1 SE, $n = 3$) from *P. agglomerans*/pPPProGreen and positive control *P. agglomerans*/pPNptGreen at three osmotic potentials produced with either NaCl (ProU \square , NptII \square) or KCl (ProU \blacksquare , NptII \blacksquare) in solution. Diamond symbols designate the mean doubling times (\pm 1 SE, $n = 3$), as indicated on the right-hand y-axis. (c,d) GFP fluorescence from water potential-sensing *P. agglomerans*/pPPProGreen cells in soil around roots of corn plants that were (c), or were not (d), transpiring. Inset figures at the top of panel (c) show stained cross-sections of root indicating deposition of suberin, lignin and/or callose. (e) GFP fluorescence from the positive control *P. agglomerans*/pPNptGreen cells in soil around roots of corn plants that were transpiring.

Microbiosensor expression in the rhizosphere around root tips of *Z. mays*

Approximately 45 images were captured with a CCD camera at a resolution of $0.16 \mu\text{m}/\text{pixel}$ along one root per microcosm from the tip up to 80 mm along the root axis. A portion of one image is reproduced in Fig. 5a, and arrows indicate bacteria considered in focus. All measures of fluorescence were standardized using Inspeck Green 505/515, $6 \mu\text{m}$ calibration beads. Each point in Fig. 5c–e is the average fluorescence from all in-focus bacteria in an image. Panels in Fig. 5c–e contain all \sim 45 points taken from each of the 3 plants experiencing the treatments: (Fig. 5c) *P. agglomerans*/pPPProGreen in soil around roots of transpiring *Z. mays*, (Fig. 5d) *P. agglomerans*/pPPProGreen in soil around roots of *Z. mays* that were not transpiring, and

(Fig. 5e) positive control *P. agglomerans*/pPNptGreen in soil around roots of transpiring *Z. mays*.

When corn was transpiring, GFP fluorescence from *P. agglomerans*/pPPProGreen in soil around corn roots increased as a function of distance along the root axis from the tip (Fig. 5c). ANCOVA of GFP fluorescence from *P. agglomerans*/pPPProGreen microbiosensors in the rhizosphere of three separate roots (each on a separate transpiring plant in a separate microcosm) revealed that the slope and intercept of linear regressions relating fluorescence to distance from the root tip were statistically indistinguishable among the three roots ($P = 0.635$ and $P = 0.07$ for slope and intercept, respectively). Values from the three root replicates treated with microbiosensors were thus pooled into one data set of approximately 135 points (Fig. 5c). The positive slope of the regression was significantly different from

zero ($P < 0.0001$, $R^2 = 0.28$), indicating that GFP fluorescence per cell was increasing significantly as a function of distance from the root tip. Staining of root tissue cross-sections with berberine hemisulfate and aniline blue highlighted the forming casparian strip (rightmost inset panel above Fig. 5c) and several maturing metaxylem vessel elements ~15 mm back from the tip.

When corn was not transpiring, GFP fluorescence from *P. agglomerans*/pPProGreen in soil around corn roots stayed low and constant as a function of distance along the root axis from the tip (Fig. 5d). ANCOVA of GFP fluorescence again revealed that the slope and intercept of linear regressions relating fluorescence to distance from the root tip were statistically indistinguishable among the three tested roots ($P = 0.851$ and $P = 0.184$ for slope and intercept, respectively). In this treatment, the slope of GFP fluorescence per cell as a function of distance from the root tip (determined from the combined data set, Fig. 5d) was not significantly different from zero ($P = 0.5032$).

When corn was transpiring and the positive control strain *P. agglomerans*/pPNptGreen was used in soil around corn roots (Fig. 5e), the intercepts of regressions relating fluorescence to distance from the root tip for the three plants differed slightly (but significantly, $P < 0.001$), but slopes did not ($P = 0.83$), and slopes for all three roots were indistinguishable from zero ($P > 0.4$ for all roots). As expected for a functioning, constitutive, positive control, GFP fluorescence remained high and bright at all distances from the tip.

DISCUSSION

The microbiosensors described here provide the first empirical glimpse of small-scale gradients in soil water potential around plant roots. *P. agglomerans*/pPProGreen in the rhizosphere of *Z. mays* roots revealed more negative soil water potentials as a function of axial distance from corn root tips when transpiration was encouraged over 15 h in the greenhouse (Fig. 5c). When transpiration was discouraged (Fig. 5d), GFP fluorescence per rhizosphere biosensor was low, similar to that seen around the meristem of transpiring roots, and it was constant along the root axis, consistent with the plants' not extracting water from rhizosphere soil when the transpiration stream was not active. It should be noted that the roots analysed in these experiments were optically accessible through the glass front of the growth chamber and had part of their surface appressed against the glass. This likely altered root and bacterial physiology to some extent. Nevertheless, the roots and their associated microbial biosensors gave results congruent with the hypothesis that transpiration effects on water potential can be detected using microbiosensors.

A number of papers have explored development of conducting tissue in corn roots. Root tissue near the root tip does not have mature xylem conduits (e.g. Peterson & Steudle 1993), so water potentials of root tips are not drawn down as deeply as water is transpired from leaves. However, behind the root tip, where metaxylem elements have matured and become conductive, transpirational demand

for water can draw down root water potential dramatically (e.g. Landsberg & Fowkes 1978; Frensch & Steudle 1989; Doussan, Vercambre & Pages 1998; Hsiao & Xu 2000), and thus draw water from rhizosphere soil. Where along the root axis this transition to conductive, mature xylem occurs likely varies with a number of factors such as root growth rate, soil type, root age and type (e.g. Wang, McCully & Canny 1994). In our sampling, the appearance of maturing metaxylem with lignified cell walls at 15 mm behind the tip (Fig. 5) is congruent with previous anatomical work by, for example, Frensch & Steudle (1989), who reported that corn roots have only two mature early metaxylem vessels per cross-section 20 mm from the root tip, and none at 10 mm (Wang *et al.* 1994 note, however, that lignification of maturing metaxylem elements does not necessarily mean the vessels are completely open and conductive). We did not aim to use the biosensors in this study to determine exactly where along the corn root axis water uptake commenced. Rather, our goal was to use transpiring corn plants to draw down rhizosphere water potential to test whether these microbiosensors could detect changing water potentials in nonsterile soil. The relatively simple pattern in GFP fluorescence we observed using *P. agglomerans*/pPProGreen, suggesting least negative rhizosphere water potentials near the tip and increasingly more negative water potentials along the root axis, is consistent with these observations of tissue development. The hint of a steeper water potential gradient along the distance 0–40 mm relative to 40–80 mm is intriguing and not yet explained.

Leveau & Lindow (2001) developed a theoretical model describing influences beyond promoter activity on production of reporter molecules such as GFP by microbiosensors, and they suggested that a higher bacterial growth rate (a shorter doubling time) could dilute the GFP pool per microbiosensor cell. Following this reasoning, a microbiosensor growth rate differential, with more rapid growth of microbiosensors near the root tip where exudation of labile sugars is highest, might produce the pattern observed in Fig. 5c. However, we show in Fig. 2c,d that the microbiosensor's GFP expression is insensitive to bacterial growth rate, across a range of water potentials. This is most easily seen in panels c and d of Fig. 2, at -1.5 MPa, where doubling times (the diamond symbols) are vastly different when bacteria are growing in the NaCl versus KCl solutions (both at -1.5 MPa), but the GFP expression in response to water potential is statistically indistinguishable (Table 1). The growth rate-dependent dilution of GFP molecules predicted by Leveau and Lindow's model should occur if the model's underlying assumptions are met, but, these assumptions include, for example, that promoter firing rate is constant across growth rates. As Leveau & Lindow (2001) point out, this assumption is not necessarily warranted. Fortunately, in *P. agglomerans*/pPProGreen microbiosensors, the dilution of GFP pools during more rapid cell divisions is compensated for by an as-yet unidentified process (perhaps more rapid *proU* promoter firing at higher growth rates), producing, in the end, a faithful GFP report of water potential even when cells are growing at different rates (Fig. 2).

For our practical purposes, this characteristic is essential and a highlight of this work, as bacterial growth rates likely vary in the rhizosphere.

One concern with these microbiosensors is that as currently engineered, live microbiosensor cells at the least negative water potentials in microcosm soil may not be visible, as GFP production per cell will be low. DeAngelis *et al.* (2005) approached this problem by engineering microbiosensors to have both constitutive production of red fluorescent protein (for identification of bacteria in soil) and inducible production of GFP (linked to a promoter of interest). This strategy potentially requires a double draw on cellular resources, to make red and sometimes GFP, resources that otherwise could be used for growth.

Rather than possibly tax the microbes metabolically by engineering them to support constitutive production of red fluorescent protein, instead we considered whether the pattern in Fig. 5c would likely result from an inability to see dark cells. Clearly cells with fluorescence of 400 or above are visible, as that is approximately the lowest fluorescence value reported in Fig. 5d. Figure 5c also demonstrates that average brightness of cells with fluorescence above 400 units clearly increases as a function of a distance from the root tip. And, the high fluorescence values at larger distances from the tip result not from the average of some very high and a range of lower (down to 400) values, but, instead, result from a general increase in the fluorescence of in-focus cells in images. Along the entire root axis that we examined, the variation in microbiosensor fluorescence per cell within individual images is similar to that observed around the tip; the absolute values of GFP fluorescence simply shift upward. For the pattern of increasing fluorescence as a function of distance from the root tip to be misleading, and not represent the average pattern in the entire pool of microbiosensors along the root, it would be necessary for the proportion of 'dark' (undetected) cells in the microbiosensor population to be increasing as a function of distance from the root tip, meaning that the fluorescence in the population of microbiosensors would have to become strongly bimodal at locations farther from the tip. Such strong bimodal expression, suggesting very different water potentials near one another at very small spatial scales in this moist soil, would not be consistent with the well-accepted soil physical assumption that at small distances, water potentials equilibrate via water films and vapour.

Beyond the demonstration that these microbiosensors can reveal a water potential gradient in the rhizosphere of transpiring roots, another important conclusion can be drawn from these experiments. The idea that microbiosensors in soil can be quantitative is attractive, and it may at first seem that liquid culture data (Figs 1 & 2) and/or controlled soil water potential data (Fig. 3) should serve to calibrate the signal observed in Fig. 5. However, one of the most important messages of this paper is the demonstration that because microbiosensors are living organisms with resource preferences and species-specific environmental sensitivities, microbiosensor reports should not be considered perfectly quantitative (Gage *et al.* 2008).

To illustrate why exact calibration of the GFP signal in the rhizosphere is not possible, we purposefully chose two very different microbes – *P. agglomerans* and *P. putida* – to genetically engineer with the same plasmid. These organisms have different resource preferences and environmental sensitivities. For example, the two carbon sources (glucose and succinate) used for *P. agglomerans*/pPProGreen microbiosensor tests in liquid culture resulted in a noticeable alteration in the *scaling* of the GFP report as a function of water potential. Though GFP fluorescence scaled positively with osmotic potential in both glucose (Fig. 2c) and succinate (Fig. 2d) (and was insensitive to the type of osmolyte), the GFP fluorescence at each osmotic potential in succinate culture was substantially depressed relative to that in glucose (Fig. 2c,d). This depression of GFP fluorescence in succinate, relative to glucose, was not driven by a change in growth rate on succinate versus glucose as a carbon source; doubling times matched in the glucose and succinate treatments (e.g. at -1.5 MPa, doubling times were ~ 6 h in KCl and ~ 2.5 – 3 h in NaCl, Fig. 2c,d, diamond symbols).

In contrast to this behaviour of *P. agglomerans*/pPProGreen, the same plasmid, in the *Pseudomonas putida* background, resulted in GFP production by *P. putida*/pPProGreen that was not nearly as sensitive to carbon resource type (Fig. 2a,b). However, the *P. putida*/pPProGreen signal was much more dim than the signal from *P. agglomerans*/pPProGreen (Fig. 2), making *P. putida*/pPProGreen much more difficult to see in soil, and leading us to choose *P. agglomerans*/pPProGreen for rhizosphere experiments. This differential brightness may reflect that *P. agglomerans* is more closely related to *E. coli*, the source of the *proU* promoter; the *proU* promoter may not function optimally in more distantly related *P. putida*.

These results suggest that if *P. agglomerans*/pPProGreen experiences an environmental gradient in energy resource type, that gradient may confound the water potential signal. Taking into account this known limitation in our *P. agglomerans*/pPProGreen microbiosensors, we added them to soil with their own small glucose supply. It is not likely that a gradient in rhizosphere carbon source would have driven the clear signal in Fig. 5c; if anything, one would expect that there would be more simple sugar at the root tip than back along the root length, and that pattern would not likely result in the GFP signal per cell becoming brighter back along the root axis.

Such contrasting strengths and weaknesses among bacterial species used as microbiosensors, and the effects of resources and conditions on expression of reporter genes, deserve more attention within microbiosensor research (Cardon & Gage 2006; Gage *et al.* 2008). It is impossible to test every resource, every condition, or every molecule that a microbe might encounter in a complex natural environment in order to quantify, and correct for, how that resource/condition/molecule affects expression in response to the parameter of interest. Instead, microbiosensors used in complex environments should be viewed as qualitative indicators, for example giving indications of

gradients (e.g. Fig. 5), and they should be used under conditions where in the researchers' best judgment they will not give spurious results. Ideally, they could be used in pairs or groups so that the limitations of one type of microbe can be balanced with the strengths of another. For example, combining these water potential sensors with others available to report, e.g. bacterial growth rates or presence of sugars (Bringhurst *et al.* 2001; Rosado & Gage 2002; Herron 2007) would further strengthen interpretation of patterns of GFP fluorescence.

Related also to questions of biosensor design and interpretation of biosensor expression, the stable version of GFP has a half-life estimated at >24 h (Andersen *et al.* 1998), suggesting at first glance that persistence of GFP in cells would prevent dynamic reports of diel variation in water potential. However, the use of stable GFP is not necessarily incompatible with detection of diel fluctuations in stimulus. The GFP level per cell still can change dynamically as promoter activity waxes and wanes because carbon from roots is available for microbial growth and division; the division itself dilutes pools of mature GFP molecules per cell, and, for example if water potential is increasing, reduced promoter activity does not replenish the pools in daughter pools. Thus, because of active cell division in the rhizosphere, though any individual GFP molecule has a relatively long lifetime, and though GFP levels at a given water potential are independent of bacterial growth rate, GFP levels per cell can vary dynamically as transcription increases or decreases in response to changing water potential as long as cells continue to divide.

The microbiosensors described here offer a new view of gradients in total soil water potential at spatial scales where previously measurements could not be made. The GFP signal related to water potential is complex and must be interpreted conservatively, but the ability to link empirical measurements of spatial and temporal gradients in soil water potential with long-standing mathematical modeling of soil water potential gradients (rhizosphere and otherwise) opens a new window into understanding of the soil physical microenvironment and its effects on communities of organisms that thrive there.

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