Plants form symbiotic associations with endophytic bacteria within tissues of leaves, stems, and roots. It is unclear whether or how plants obtain nitrogen from these endophytic bacteria. Here we present evidence showing nitrogen flow from endophytic bacteria to plants in a process that appears to involve oxidative degradation of bacteria. In our experiments we employed *Agave tequilana* and its seed-transmitted endophyte *Bacillus tequilensis* to elucidate organic nitrogen transfer from $^{15}$N-labeled bacteria to plants. *Bacillus tequilensis* cells grown in a minimal medium with $^{15}$NH$_4$Cl as the nitrogen source were watered onto plants growing in sand. We traced incorporation of $^{15}$N into tryptophan, deoxynucleosides and pheophytin derived from chlorophyll $a$. Probes for hydrogen peroxide show its presence during degradation of bacteria in plant tissues, supporting involvement of reactive oxygen in the degradation process. In another experiment to assess nitrogen absorbed as a result of endophytic colonization of plants we demonstrated that endophytic bacteria potentially transfer more nitrogen to plants and stimulate greater biomass in plants than heat-killed bacteria that do not colonize plants but instead degrade in the soil. Findings presented here support the hypothesis that some plants under nutrient limitation may degrade and obtain nitrogen from endophytic microbes.
molecules or mineralization of dead bacterial cells\(^2\). Whether N is actually transferred from endophytic bacteria to plant host has proven difficult to resolve. The question of the mechanism of transfer of N from endophyte to host plant is an important gap in our knowledge. One possible mechanism for N transfer to host is that plants may scavenge organic nitrogen by oxidation and degradation of endophytic bacteria or their proteins using reactive oxygen species (ROS) to lyse cells and denature proteins. This mechanism has been termed ‘oxidative nitrogen scavenging’ \(^4\).\(^{23}\). The experiments reported here were done to evaluate whether \(^15\)N incorporated into bacterial endophyte biomolecules such as proteins and nucleic acids could be traced into plant molecules; and whether the transfer process involves evidence of oxidative degradation of microbes.

**Results**

**\(^{15}\)N\(_2\) gas assimilation into seedlings.** Shoots of seedlings grown in \(^{15}\)N\(_2\)-enriched air showed higher \(\delta^{15}\)N vs air content (54.93 ± 5.65 \(\delta^{15}\)N vs (%); mean ± standard error of mean) than shoots from seedlings grown in non-enriched air (3.07 ± 1.12 \(\delta^{15}\)N vs (%)). This large difference in the \(^{15}\)N/\(^{14}\)N ratios between the two treatments shows that N was fixed in the seedling tissues.

**Bacillus tequilensis** endophyte. For use in our tests to evaluate \(^{15}\)N transfer/translocation from bacterial endophyte to plant, we isolated an endophyte from seeds, seedlings and young plantlets (bulbillos) of *Agave tequilana* Weber, and identified it as endospore-forming *Bacillus tequilensis* (accession number KF792125) \(^2\) (Fig. 1) using sequence data. *Bacillus tequilensis* was present in both roots (Fig. 1C) and shoots of the host plant.

**Visualization of bacteria in plant tissues.** In soil infection experiments using *B. tequilensis* we observed intracellular colonization by the bacterium into meristems and root epidermal cells of seedlings (Fig. 1A and 1B) and young plantlets. We further observed oxidation of bacterial cells within and on the surface of root epidermal cells (Fig. 1B and 1D) \(^2\).\(^3\).

**Experiment 1**

**\(^{15}\)N tracking experiments.** A significant difference (p<0.05) was observed in \(^{15}\)N-labeled tryptophan (\(^{15}\)N-Trp) concentration between the controls (\(H_2O\) and unlabeled *B. tequilensis*, \(^{15}\)N-*Bteq*) and plants inoculated with \(^{15}\)N-labeled *B. tequilensis* (\(^{15}\)N-*Bteq*). **\(^{15}\)N-labeled Trp in the \(^{15}\)N-*Bteq* treated plants was about 16-fold higher (21.05 ± 5.47 ng/mg) than \(^{15}\)N-*Bteq* treated (1.32 ± 0.40 ng/mg) and \(H_2O\) treated plants (1.36 ng/mg) (Fig. 2, and Supplementary Fig. S5). Further quantification of \(^{15}\)N-Trp demonstrated that plants supplemented with \(^{15}\)NH\(_4\)Cl had higher levels than plants supplemented with \(H_2O\) or \(^{14}\)NH\(_4\)Cl (Supplementary Fig. S1). We detected tryptophan by high-performance liquid chromatography coupled to mass spectrometry in tandem (HPLC-MS/MS), although this amino acid was not quantified (Supplementary Fig. S4)\(^\text{26–28}\).

Nitrogen incorporation into tissues of *A. tequilana* was also confirmed by HPLC-MS/MS detection of \(^{15}\)N-labeled nucleosides (Fig. 3). Additionally, the same \(^{15}\)N-labeled nucleosides were detected from *A. tequilana* supplemented with \(^{15}\)N-labeled NH\(_4\)Cl (Supplementary Fig. S9). Detection of 2’-deoxynucleosides methylated as 5-methyl-2’-deoxycytidine (\(^{15}\)N-MedC) and N’-methyl-2’-deoxyadenosine (\(^{15}\)N-MeA) was also observed in plants of *A. tequilana* supplemented with \(^{15}\)N-labeled \(B. tequilensis\) or 15N-labeled NH\(_4\)Cl (Supplementary Fig. S10 and 11).

**Pheophytin analysis.** Analysis of unlabeled pheophytin a (Fig. 4B)\(^\text{26,27}\) showed a distribution of isotope peaks in agreement with the theoretical values of this molecular formula (C\(_{55}\)H\(_{74}\)N\(_4\)O\(_5\), [M+H]\(^+\) = 871.5731) containing a base peak with \(m/z\) 871.5739 (Fig. 4B). Incubation of *A. tequilana* with the \(^{15}\)N-labeled *B. tequilensis* resulted in incorporation of \(^{15}\)N into the isotopomers of pheophytin. The relative abundance of the isotopomers \(m/z\) 872.57, 873.57, 874.57, 875.57, 876.57 increase by 16, 48, 106, 128 and 200% respectively (p<0.08; p<0.02; p<0.05; p<0.01 and p<0.003, respectively for \(m/z\) 871.57, 872.57, 873.57, 874.57 and 875.57, comparing \(^{14}\)N and \(^{15}\)N-labeled *B. tequilensis* groups, Fig. 4D and inset 4B and 4C). The \(^{15}\)N uptake to form the isotopomer \(m/z\) 876.57 pheophytin was notably greater, than that of unlabeled leaves, attesting to the incorporation of \(^{15}\)N atoms into the tetrapyrole ring of the pheophytin molecule.

**Experiment 2**

**Biomass increases in plants.** In this experiment we treated plants with live or heat-killed \(^{15}\)N-labeled *Bacillus* (121 °C, 10 min); and...
with MMN solution (see methods), non-endophytic *E. coli* (not labeled) and water as controls. Biomass measurements after two months of treatment showed that the greatest biomass increase was seen in plants treated with living *B. tequilensis*, where mean biomass increase was 1.63 ± 0.28 g (mean ± standard deviation). Plants treated with heat-killed *B. tequilensis* or mineral nutrient solution (MMN) showed biomass increases of 0.83 ± 0.16 g for heat-killed *B. tequilensis*; and 0.52 ± 0.34 g for MMN. Plants treated with non-endophytic *Escherichia coli* showed a biomass increase of 0.71 ± 0.14 g. Plants treated only with water showed the least biomass increase (0.15 ± 0.05 g).

**Comparison of 15N absorption from living verses dead *B. tequilensis*.** To evaluate the extent to which 15N was moving into plant tissues after the death of bacteria in soil or through endophytic colonization, we included in the experiment living and heat-killed *B. tequilensis* that had been labeled with 15N. On analysis we found that incorporation of 15N into nucleosides, 2'-deoxycytidine (15N3-dC) and 2'-deoxyadenosine (15N5-dA) was significantly greater (*p*, 0.05) in plants treated with live *Bacillus* (a.u.: 15N3-dC 1.13 × 10^6 ± 13.93; 15N5-dA 4.50 × 10^5 ± 9.05) than plants treated with the heat-killed *Bacillus* (a.u.: 15N3-dC 3.40 × 10^5 ± 26.51; 15N5-dA 1.10 × 10^5 ± 6.83)28–32.

**Discussion**

The assimilation of 15N2 gas into tissues of *Agave* seedlings is indicative of the presence of nitrogen-fixing microorganisms within seedling tissues. From seedlings, seeds and bulbillos, we consistently isolated *Bacillus tequilensis*; however, plants may also have contained other non-cultured bacteria that could have been responsible for N fixation in plant tissues. We do not consider *B. tequilensis* to be responsible for N2 fixation in plant tissues; however, because of its ease of culturing and its endophytic nature, we employed *B. tequilensis* in experiments to evaluate the hypothesis that nitrogenous nutrients may flow from bacterial endophyte populations to plants. We labeled proteins and nucleic acids of *B. tequilensis* with 15N; then inoculated plants with suspensions of bacteria. Using these bacteria we conducted two experiments. In the first experiment we watered plants weekly with a 4 mL suspension of 80.10^6 CFU mL−1 of 15N-labeled *Bacillus tequilensis* for a six-month period. Our results show that plants incubated with the bacterial endophyte came to contain N that was originally contained within the bacterium. Detection of 15N-labeled tryptophan and 2'-deoxynucleosides suggests that 15N-labeled bacterial endophytes enter into or otherwise associate with plant tissues. Because both bacteria and plants possess tryptophan and 2'-deoxynucleosides, with this data alone we cannot evaluate transfer of nitrogen from bacterium to plant. However, we were able to detect the 15N in pheophytin a, a molecule derived from plant chlorophylls. Because chlorophyll is unique to the plant cells, the presence of the 15N label there is a definitive confirmation of transference of N from bacterium to plant tissues.

A second experiment was conducted to evaluate whether soil absorption from dead bacteria could account for some 15N movement into plant tissues, we developed an experiment in which we
treated plants with live or heat-killed 15N-labeled bacteria (121°C, 10 min); and with MMN solution, non-endophytic E. coli (not labeled) and water as controls. Plants were watered weekly for a two-month period using 4 mL of bacterial suspension at a concentration of 80×10^6 CFU mL\(^{-1}\) or MMN solution. In this experiment we found significantly more incorporation of 15N into 2'-deoxynucleosides in plants treated with live Bacillus than in plants treated with heat-killed Bacillus. We further evaluated the growth of these plants compared with those treated with water, E. coli or mineral nutrient solution. The amount of total acquired biomass was almost double in plants treated with living Bacillus than in plants treated with heat-killed Bacillus and E. coli; and more than 3.3 times that seen in plants treated with mineral solution. Enhanced growth of plants, and incorporation of the 15N label into plants treated with living 15N-labeled bacteria are results that are consistent with a scenario where N is transferred to plants from bacteria within tissues of plants rather than being absorbed primarily from dead bacteria in the soil. The fact that non-endophytic E. coli-treated plants demonstrated a biomass increase half that of living endophyte cultures suggests that efficient movement of N from bacteria to plants is a function of living, plant-colonizing, endophytic bacteria. The results of our experiments suggest that some nitrogen may come from decomposition of microbes in the soil. Decomposition of microbes in the soil could explain biomass increases in plants treated with heat-killed B. tequilensis and non-endophytic E. coli.

Microscopic observations of plant roots treated with the living Bacillus and stained using a reactive oxygen probe showed that degrading bacterial cells were associated with reactive oxygen and were often internalized into plant roots. Our observations are similar to those of Paungfoo-Lonhienne et al., where microbes were shown to enter into root cells of tomato and Arabidopsis where they were degraded. The involvement of reactive oxygen in procurement of organic nutrients from bacteria is consistent with 'oxidative nitrogen scavenging', where reactive oxygen and proteases may be involved in nutrient extraction from bacteria. We did not visualize bacteria or their degradation in shoot tissues; however, this is likely due to failure of the aqueous stains to penetrate into the shoot, rather than their absence from shoots.

In a recent study that evaluated the contribution of organic N in wheat from direct microorganism consumption against nitrate, L-alanine and L-tetraalanine absorption, it was found that plants absorbed nutrients through endocytosis of soil microbes, but the N obtained through this process was up to two orders of magnitude slower than other forms of organic and inorganic N in the soil. Our study examining nutrient transfer from B. tequilensis to Agave suggests a process where endophytic bacterial degradation may supply N for plant growth; however, this mechanism may not be rapid and it could depend on occurrence of N deficiency or other nutrient depletion in soils. Our initial experiment to evaluate movement of 15N into plant molecules lasted six months. We do not know the minimum amount of time or precise plant growth conditions needed to see movement of N from bacterial endophytes to host plants. We propose that efficiency of this mode of nutrition depends on the particular host and microbe association. Agave tequilana is a desert plant adapted to growth under low N soil conditions and B. tequilensis is a native endophytic microbe of that plant. Internal colonization of plant tissues may increase the probability of transference of nutrients from endophytes to host plants. In this respect endophytic microbes that fix N may represent a nutritional resource that may be tapped into when soil N is limiting. Equally important to plants could be N derived from soil microbes that grow and obtain nutrients in the soil then colonize growing plants where they may be degraded. Whether N derived from microbes is a significant source of N for plants is a question that requires additional investigation. While our experiments on N transfer from endophyte to host in Agave are not exhaustive, they do provide further evidence that plants may obtain N through degradation of symbiotic microbes.
Methods

Plant materials. For experiments we used one-yr-old seeds and asexual plantlets (bulbils) derived from cuttings of Agave tequilana Weber that were originally collected on an A. tequilana plantation near Atotonilco el Alto, Jalisco, Mexico at coordinates 20°34’27.71”N, 102°32’00.85”, 1900masl.

*N* gas assimilation experiment with seedlings. An experiment was conducted to evaluate whether endophytic microbes fix nitrogen within intact plant tissues. In this experiment seeds were surface disinfected in 3% sodium hypochlorite for 20 min with constant agitation to remove external bacteria, then rinsed three times using sterile water. Five seeds were plated on 0.7% agarose media in each of eight Petri dishes. Four Petri plates were placed in a 1-liter gas chamber in which the air was enriched with 33 mL of *N*2 gas. The other four Petri dishes were placed in a chamber in which the air was not enriched with *N*2. Both chambers were placed under fluorescent lighting with alternating light/dark periods (10 hr/14 hr) at laboratory ambient temperature for 21 days. After incubation shoots were excised from roots, washed to remove any superficial bacteria, then dried for 1 hr in an oven at 60°C. All shoots from a plate were combined to ensure sufficient material for analysis.

Isolation of bacteria from A. tequilana seeds. Seeds were surface sterilized to remove epiphytic microbes. In this process seeds were immersed in a 3% hypochlorite solution for twenty minutes with constant agitation. The seeds were then rinsed with sterile distilled water. To confirm the disinfection process, aliquots of 15N-labeled *Bacillus tequilensis* cells were grown for 18 hr at 37°C in minimal medium with 15N and unlabeled *Bacillus tequilensis* cells were pelleted by centrifugation (5,000 × g, 20 min, 4°C), and washed three times in sterilized glucose solution (0.08%). Four-mL of labeled or unlabeled bacterial cells (see section above ‘Bacterial growth and 15N labeling’) were adjusted to 1 at OD600 (equivalent to 80-100 CFU mL-1) and used to inoculate each of A. tequilana maintained in sterile sand (30 g/dry wt). Additional treatments included plants watered with a mineral solution (50% MMN) supplemented with isotopic or non-isotopic NH4Cl, and plants watered with sterilized distilled water. The plants were watered once a week with 4 mL of each treatment during a six-month period. All plants were approximately 10–12 cm in height with 1 or 2 open leaves. Plants were grown in glass bottles in a growth chamber at 27°C day/night temperature, 14 h photoperiod.

Analysis of plant for *N*15 incorporation. The central leaf ‘cogollo’ and new leaves of *B. tequilensis*-inoculated plants were taken and the presence of 15N-labeled tryptophan ([15N-tryptophan] or *) was quantified by High-Performance Liquid Chromatography coupled to Mass Spectrometry in tandem (HPLC-MS/MS). To reduce the chances that the 15N-labeled tryptophan was from the living bacterium rather than the host plant, the extraction was carried out seven days after inoculation with 15N-labeled bacteria. The detection and quantification of 15N-tryptophan in *B. tequilensis* was performed by the method of Perkins and Roberts26,27, with some modifications. Briefly, 0.5 g fresh leaves were frozen in liquid N2 and ground to a fine powder using mortar and pestle. For pheophytin extraction equal volumes of 85% acetic acid in 1 mL water, and ethyl ether were added and centrifuged at 10,000 rpm for 10 min. The ether phase was collected and concentrated to dryness at 30°C under vacuum. For pheophytin extraction equal volumes of 85% acetic acid in 1 mL water, and ethyl ether were added and centrifuged at 10,000 rpm for 10 min. The ether phase was collected and concentrated to dryness at 30°C under vacuum. For HPLC/MS analysis by adding methanol to obtain a final sample concentration of 1 mg/mL.

HPLC/MS analysis of tryptophan. Tryptophan samples were analyzed using a Waters 2505 HPLC system (Tokyo, Japan) with two pumps LC-20AD, automatic injector SIL-20A, column oven CTO-20A, UV detector SPD-20A and controller CRMC-10A. A column Phenomenex Luna 5 μm (PP2 150 × 2 mm, 100A particle size) was used and chromatography was performed with a flow of 200 μL/min using acetonitrile:H2O (1:10 formic acid) as mobile phase in a gradient of 0 to 5 min 60% of acetonitrile, from 5 to 30 min 60% to 100% of acetonitrile. The column oven was kept at 40°C, UV detector was recorded at 400 and 600 nm. The mass spectrophotometer was operating in electrospray positive mode, with a nebulization and drying gas at 4 Bar and 8 L/min, respectively. Capillary voltage was set to 5000 V and drying gas temperature in 200°C. Column effluent and quadrupole energy were set to 20 eV and 10 eV, respectively. The molecular formula of pheophytin a is C55H74N4O5 and the base peak was detected at [M+H]+ = 872.5731.

Extraction of DNA from A. tequilana. DNA extraction from A. tequilana leaves was made after six months of treatments as described above. Freshly collected leaves (1 g) were ground to powder in liquid nitrogen using a TissueLyzer system (Tokyo, Japan) with two pumps LC-20AD, automatic injector SIL-20A, column oven CTO-20A, UV detector SPD-20A and controller CRMC-10A. A column Phenomenex Luna 5 μm (PP2 150 × 2 mm, 100A particle size) was used and chromatography was performed with a flow of 200 μL/min using acetonitrile:H2O (1:10 formic acid) as mobile phase in a gradient of 0 to 5 min 60% of acetonitrile, from 5 to 30 min 60% to 100% of acetonitrile. The column oven was kept at 40°C, UV detector was recorded at 400 and 600 nm. The mass spectrophotometer was operating in electrospray positive mode, with a nebulization and drying gas at 4 Bar and 8 L/min, respectively. Capillary voltage was set to 5000 V and drying gas temperature in 200°C. Column effluent and quadrupole energy were set to 20 eV and 10 eV, respectively. The molecular formula of pheophytin a is C55H74N4O5 and the base peak was detected at [M+H]+ = 872.5731.

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and re-suspended in 0.1 mM desferroxamine. DNA concentration was measured spectrophotometrically at 260 nm.

Enzymatic hydrolysis of DNA from A. tequilana. See Supplementary Note 3.

HPLC-MS/MS analysis of DNA from A. tequilana. HPLC-MS/MS analysis was performed using an Agilent HPLC (1200 series, Agilent Waldbronn, Germany) coupled to a linear ion trap mass spectrometer (4000 QTRAP mass spectrometer, Applied Biosystems, Foster City, CA, USA) with electrospray ionization source. The column oven and auto sampler temperatures were set at 25 °C and 4 °C, respectively. For the separation, a reversed phase column was used ([C18(2)]: HST Luna, Phenomenex, 100 mm × 2.0 mm, 2.5 μm particle size). Flow rate was set at 0.2 mL/min. Gradient elution was carried out with 0.1% formic acid (A) and acetonitrile:0.1% formic acid (B). The separation was conducted with 0% to 40% B during the first 15 minutes, 40% B for 10 minutes, 40% to 0% B for 1 minute and 0% B for 35 minutes. MS/MS spectrometry analysis was performed in positive ion mode and using SRM mode for each 2'-deoxynucleoside that corresponded to the loss of 2'-deoxyribose moiety: 2'-deoxyguanosine (m/z 273–157), 2'-deoxycytidine (m/z 231–115), 2'-deoxyadenosine (m/z 257–141), 2' deoxythymidine (m/z 245–129), 5-methyl-2'-deoxycytidine (m/z 245–129) and N'-methyl-2'-deoxyadenosine (m/z 271–155) (Fig. 3 and Supplementary Fig. 39 to 41) (17). Mass spectrometry analyses were performed with the following parameters: collision excitation potential, 10 V; collision activated dissociation gas flow, medium; pause time, 5 s; dwell time, 300 ms; curtain gas, 12 psi; ion source, 5500 V; temperature, 650 °C; gas 1 and gas 2, 40 psi; declustering potential, 31 V and entrance potential, 10 V.

Analysis of 2'-dC- and 2'-dA-extracted from DNA of plants treated for two months with live or heat-killed 15N-labeled 2'-labeled bacteria was performed as described above.

HPLC-MS analysis of DNA from bacteria. See Supplementary Note 4.

Experiment 2

Plant mass accumulation study. To evaluate whether soil absorption from dead bacteria could account for some 15N movement into plant tissues, we developed an experiment in which we treated plants (six replicates per treatment) over a two-month period with live or heat-killed 15N-labeled B. tequilensis (heated to 121 °C, 10 min), and with 50% MN solution, non-endophyte E. coli (not labeled) and water as controls. Bacteria were applied as described in Experiment 1. To assess increase of 15N in leaves of Agave plants treated with 15N-labeled bacteria, deoxynucleosides were extracted from leaves and 2'-deoxycytidine and 2'-deoxyadenosine were measured. Measurements of plant mass with water-washed roots were made before and after two months of treatments. Biomass increase is the change in whole plant wet weight as a result of the treatments.

Statistical analyses. Statistical tests were performed with Origin (version 8.0) and GraphPad Prism (version 5.0) programs. Significant differences were determined by t-test and one-way ANOVA (applying Dunnett’s post-test) with the level of significance set at p < 0.05 for 15N-Trp quantification, detection of nuclease (2'-dC- and 2'-dA) and biomass increase, respectively.

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19. The authors acknowledge the research funding institutions FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo; No. 2012/21663-1, 2011/10048-5 and 2009/51850-9), CNPq (Conselho Nacional para o Desenvolvimento Científico e Tecnológico), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), PRONEX/FINEP (Agência Nacional de Pós-Graduação e Pesquisa), PROMEX-IPME (Programa de Apoio ao Núcleos de Excelência), PRUP (Pro-Rede) of the Federal University of São Paulo, Instituto do Milênio-Redoxoma (No. 42011/2006-5), INCT Redoxoma (FAPESP/CNPq/CAPES; No. 573530/2008-4), NAP Redoxoma (PRUP; No. 11.2011.1.9352.1.8), CEPID Redoxoma (PRUP; No. 2013/07937-8), John Simon Guggenheim Memorial Foundation (P.D.M. Fellowship), the John E. and Christina C. Craighead Foundation, USDA-NIFA Multistate Project W3147, the New Jersey Agricultural Experiment Station, National Council of Science and Technology of Mexico and the Program of Estancias Sabaticas y Posdoctorales para la Consolidacion de Grupos de Investigación (CONACYT 212875) and Project 207400 of Bilateral Cooperation Mexico-Brazil funded by CONACYT and CNPq (Brazil, No. 490440/2013-4). We also acknowledge Cristobal Fonseca-Sepulveda for conducting nitrogen fixation tests on B. tequilensis, and Joan W. Bennett for reviewing an early draft of this manuscript.

Author contributions

M.J.B.-G., P.D.M. & J.E.W. developed the concept of the experiments and analyses. M.J.B.-G., F.M.P., K.R.P. and M.S.T. implemented experiments. F.M.P., K.R.P., M.H.G.M.,
L.F.Y. & M.J.K. conducted biochemical analyses. M.S.T. isolated and identified bacteria. All authors contributed to data interpretation and writing of the manuscript.

Additional information
Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Beltran-Garcia, M.J. et al. Nitrogen acquisition in Agave tequilana from degradation of endophytic bacteria. Sci. Rep. 4, 6938; DOI:10.1038/srep06938 (2014).

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