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Multiple ways to evade the bacteriostatic action of glyphosate in rhizobia include the mutation of the conserved serine 90 of the nitrogenase subunit NifH to alanine

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Abstract

The genome resequencing of spontaneous glyphosate-resistant mutants derived from the soybean inoculant E109 allowed identifying genes most likely associated with the uptake (*gltL* and *cya*) and metabolism (*zigA* and *betA*) of glyphosate, as well as with nitrogen fixation (*nifH*). Mutations in these genes reduce the lag phase and improve nodulation under glyphosate stress. In addition to providing glyphosate resistance, the amino acid exchange Ser90Ala in NifH increased the citrate synthase activity, growth rate and plant growth-promoting efficiency of E109 in the absence of glyphosate stress, suggesting roles for this site during both the free-living and symbiotic growth stages.

Introduction

Glyphosate (N-phosphonomethyl glycine) is the most used herbicide in the world. Since the first glyphosate-resistant crop, a transgenic soybean variety, was introduced to the US market in 1996, the use of this herbicide has increased drastically. During the last decades, the adoption rates of glyphosate-resistant germplasms, including a vast diversity of major legumes (e.g. soybean and alfalfa) and non-legume crops (e.g. cotton, maize and rice), have been extremely high in both developed (e.g. US) and developing (e.g. Argentina) countries. The cultivation of these transgenic crops in association with glyphosate has provided the most effective and inexpensive weed management technology in history. However, although glyphosate is toxicologically safe for humans and animals (both wildlife and domesticated) and its environmental impact is lower than that of the multiple herbicides and tillage that it replaces, the extended use of glyphosate generates the occurrence of glyphosate-resistant weeds, alters the mineral nutrition, affects the animal microbiota, and increases the susceptibility to plant pathogens [1].

Glyphosate inhibits the enzyme 5-enolpyruvyl-3-shikimate phosphate synthase (EPSPS) involved in *de novo* synthesis of aromatic amino acids in plants, bacteria and other organisms. A key feature of this systemic herbicide that explains its commercial success is the particular ability to translocate rapidly to metabolic sinks, killing meristematic tissues away from the application site and controlling a broad-spectrum of weeds, including perennial plants. Current commercial glyphosate-resistant crops, including transgenic soybean cultivars, express an insensitive EPSPS gene and not a glyphosate degradation gene, and thus, their natural endophytes and commercial inoculants are indirectly exposed to glyphosate. A clear example of the negative impact of glyphosate on plant-growth promoting bacteria is the reduction of nitrogen fixation and yield in transgenic soybean production via the growth inhibition of

rhizobia [2]. This long-term situation evidences the need to improve the current inoculants for the maximization of symbiotic nitrogen fixation in glyphosate-resistant crops. Unfortunately, the potential benefits of engineered microbes in this agronomic topic is limited by the ecological and health risks of the massive release of genetically modified microbes in agroecosystems [3]. Thus, the production of spontaneous glyphosate-resistant mutants could be an attractive alternative to rapidly improve the inoculants available in the market.

Several glyphosate resistance mechanisms, including target alteration and control of the uptake, export and degradation of glyphosate, have been reported in different microbes [4]. However, the emergence of glyphosate resistance in rhizobia has not been studied at the genetic level. In this context, we here selected and studied spontaneous glyphosate-resistant mutants derived from the soybean inoculant *Bradyrhizobium japonicum* E109 [5].

Materials and methods

Spontaneous glyphosate-resistant mutants derived from the wild-type strain E109 were selected in RMM medium [6] supplemented with 7 mM glyphosate. Resistance to glyphosate of each independent spontaneous mutant clone was confirmed by plating isolated colonies on this selective medium (Fig. S1). The mutation sites associated with the stress-resistant phenotype were identified as previously described [5]. The nucleotide sequences of the mutant strains and wild type strain E109 were deposited in the EMBL Nucleotide Sequence Database, accession numbers GR1 (SAMN24505280), GR2 (SAMN24518821), GR3 (SAMN24519010), GR4 (SAMN24527104), GR5 (SAMN24529885) and E109 (SAMN24528291). For complementation analysis, strain E109 was transformed with the plasmid pBBR1-MCS3 containing mutant alleles, as previously described [5]. For physiological studies under aerobic conditions, bacterial cultures were grown in 125 mL Erlenmeyer flasks containing 25 mL of

RMM medium supplemented with 50 mg/L yeast extract (RMM2 medium), incubated at 28°C with shaking (250 rpm). Cells from stationary-growth cultures were used to inoculate fresh RMM2 medium supplemented with 0 or 0.7 mM glyphosate at an initial optical density (OD 580 nm) of 0.05. Growth was monitored by measuring OD, and citrate synthase (CS) activity and doubling time evaluated in exponentially growing cells as described previously [7].

The symbiotic efficiency was analyzed by growing rhizobia-inoculated soybean plants in hydroponics and irrigated with the minimal medium INTA13 without nitrogen, as previously described [5]. To analyze the nodulation abilities of mutants under herbicide stress, young seedlings of transgenic glyphosate-tolerant soybean were exposed to an application of 90 mg/L glyphosate at the stage of cotyledons. A week after herbicide treatment, the presence of nodules was determined by visual evaluation. The plant growth-promoting efficiency of mutants in wild-type glyphosate-sensitive soybean without glyphosate stress was analyzed two months after inoculation, as previously described [5]. Total nitrogen content in plant matter was established by the Kjeldahl method. The glyphosate-sensitive and glyphosate-tolerant soybean seeds used were the commercial varieties Alim 5.09 and Andrea 63.1, respectively. The effect of bacterial treatments on N_2O soil emissions was analyzed as previously described [8] with slight modifications. Microcosms were prepared by placing approximately 150 g of agronomic soil containing 2 µM of nitrate [9] into 500 mL sterilized bottles exposed to 150 µL of bacterial culture grown for seven days in YEM medium and washed twice in physiological solution. The microcosms were incubated at 25°C for 24 h, and the N_2O fluxes were measured by gas chromatography [10].

Results and Discussion

Genomic analysis revealed that the spontaneous glyphosate-resistant mutant strains GR1-GR5 are isogenic to their wild-type parental strain E109, with the exception of single nucleotide substitutions on the genes *gltL* (Fig. 1a), *cya* (Fig. 1b), *zigA* (Fig. 1c), *betA* (Fig. 1d), and *nifH* (Fig. 1e). These genes code for a glutamate transporter, an adenylate cyclase, a Zn-binding metallochaperone, a choline dehydrogenase and an essential structural subunit of nitrogenase, respectively (Fig. S2). Of these five well-known proteins, the only protein that has been found to be directly associated with the emergence of glyphosate-resistant bacteria is the glutamate transporter [11]. The transformation of strain E109 with the mutant alleles of *zigA*, *betA* and *nifH* but not of *gltL* and *cya* increased its natural tolerance to glyphosate (Fig. S3), suggesting that these are probably gain and loss-of-function mutations, respectively.

Previous studies have shown the essential roles of the glutamate transporter and the adenylate cyclase in the uptake of glyphosate in *Bacillus subtilis* [11] and of other phosphonate antibiotics (fosfomycin and fosmidomycin) in *Escherichia coli* [12], respectively. Here, we showed the emergence of a nonsense mutation within the *gltL* gene in strain GR1 (Fig. 1a) and of a consensus sequence binding site for the transcriptional repressor factor HipB [13] within the promoter of the *cya* gene in strain GR2 (Fig. 1b). Consequently, these mutant strains are probably defective in glyphosate uptake.

Recent reports have described the important functions of the Zn-binding metallochaperone ZigA and the choline dehydrogenase BetA in histidine degradation and glycine-betaine biosynthesis in *Acinetobacter baumannii* [14] and alfalfa rhizobia [15], respectively. In addition, glyphosate exposure induces the depletion of intracellular zinc bioavailability and the expression of choline dehydrogenase [16, 17]. In this study, we showed the occurrence of non-synonymous nucleotide substitutions within the *zigA* gene in strain GR3 (Fig. 1c) and within the *betA* gene in strain GR4 (Fig. 1d), which improved the ability of E109 to grow under glyphosate stress. Considering the previous works describing the functions of

ZigA and BetA in amino acid metabolism and our results, we propose that the *zigA* and *betA* mutations probably modified the glyphosate metabolism.

Glyphosate acts as a bacteriostatic antibiotic against both Gram-negative and -positive bacteria [18, 19]. In accordance with their higher resistance to glyphosate under solid media, strains GR1-GR5 showed a shorter initial lag (Fig. 1f) and earlier nodulation (Fig. 1g) than strain E109 under glyphosate stress, supporting the ability of these mutants to evade the bacteriostatic effects of glyphosate in pure culture and *in planta*. These findings provide the first genetic evidence that the alteration of the soybean-rhizobia symbiosis under glyphosate stress is directly related to the antibiotic action on soybean rhizobia. In addition, significant amounts of *nifH* transcript and NifH protein has been observed in soybean rhizobia under a wide-range of free-living conditions including aerobic cultures [20-23], suggesting that the role of NifH may not be restricted to the nitrogen fixation process. To our knowledge, however, this is the first report describing a phenotype produced by *nifH* mutation outside of the nitrogen fixation process.

Recently, phosphoproteome analysis of the bacterium *Zymomonas mobilis* ZM4 has shown that the nitrogenase subunit NifH is phosphorylated at serine 90 under nitrogen-fixing conditions [24]. Currently, there are no reports describing the possible role of this post-translational modification. We have shown that this site is highly conserved in different nitrogen-fixing strains from diverse phyla, including commercial inoculants E109 and *Azospirillum brasilense* Az39 (Fig. 2a). In addition, we showed the emergence of a nucleotide change within the *nifH* gene in strain GR5, which changes the serine 90 site to alanine (Fig. 1e). Based on this result, we propose future studies exploring the probable phosphorylation of this residue in NifH from E109 and other legume microsymbionts.

Considering that the growth inhibition of microbes by bacteriostatic antibiotics has been associated with reduced metabolism and suppressed cellular respiration [18, 25, 26], we studied

the activity of CS, which plays crucial roles in central carbon and energy metabolism [7], and the bacterial growth rate. In contrast with other glyphosate-resistant mutant strains (GR1-GR4), strains GR5 showed increased CS activity (Fig. 2b) and lower duplication time (Fig. 2c) than strain E109 in exponentially growing cells without glyphosate stress. Similar to commercial alfalfa inoculants [27], several soybean inoculants including strain E109 have conserved nitrate, nitrite, and nitric oxide reductases related to the production of the greenhouse gas nitrous oxide (N_2O) from nitrate, but lost the N_2O reductase associated with the degradation of N_2O to gas nitrogen [28]. Consequently, strain E109 and other important legume inoculants are high N_2O-emitting rhizobia [8, 28]. As expected, the complementation of strain E109 with the pYC7 cosmid containing the N_2O reductase cluster, suppressed the high N_2O-emitting phenotype of strain E109 (Fig. 2d). In addition, strain GR5 showed significantly decreased N_2O emissions compared to strain E109 in microcosm assays (Fig. 2d). Therefore, our results suggest a possible link between glyphosate resistance and the metabolic shifts induced by the S90A mutation.

Interestingly, large differences in plant productivity (Fig. 3a) and nodule biomass (Fig. 3b) were observed between strain E109 and strain GR5 in soybean inoculation assays without glyphosate stress, suggesting that the putative phosphorylation site of NifH from strain E109 can also play functions in the symbiotic nitrogen-fixing process. Soybean plants inoculated with GR5 had increases between 21% (data not shown) and 8% (Fig. 3c) in nitrogen content of leaves with respect to plants inoculated with the parental strain E109 in chamber and field conditions, respectively. These results are in line with the positive effects of a high CS activity on the bacterial growth rate of *Rhizobium tropici* and nodulation of common bean [29]. A possible mechanism to explain the benefits of S90A mutation and high CS activity on symbiotic nitrogen fixation could be the respiratory protection of nitrogenase and the increase of the reducing equivalents as well as of ATP needed for the nitrogen fixation process [30]. In biotechnological

terms, the non-genetically modified mutant strain GR5 can be applied to increase the production, environmental safety and nutritional quality of both conventional and glyphosate-tolerant soybean varieties.

Figure Legends

Figure 1. Mutations responsible for the glyphosate resistance phenotype in spontaneous mutant strains derived from the commercial soybean inoculant E109 and the ability of these mutants to bypass the bacteriostatic effects of glyphosate both in pure culture and *in planta*. As compared to the genome of their parental strain E109, the genomes of the glyphosate-resistant strains (a) GR1, (b) GR2, (c) GR3, (d) GR4 and (e) GR5 display mutations only within the *gltL* (yellow), *cya* (green), *zigA* (light blue), *betA* (red) and *nifH* (orange) genes, which code for a glutamate transporter, an adenylate cyclase, a Zn-binding metallochaperone, a choline dehydrogenase and a subunit of nitrogenase, respectively. Mutations are highlighted in gray and their impact on nucleotide and amino acid sequences are described on the right. (f) The duration of the lag phase of E109 (a slow-growing bacterium) and its glyphosate-resistant derived mutants GR1-GR5 was evaluated in RMM2 medium with or without glyphosate stress. Values represent mean $\pm$ SD (n=3). (g) The ability of strains E109 and GR1-GR5 to nodulate glyphosate-tolerant soybean was analyzed in minimal medium INTA13 without nitrogen. Rhizobia-treated plants were either exposed or not to an application of glyphosate. The inoculation experiment was performed twice using eight replicates for each treatment, with the same results. Roots with (+) or without (-) nodules are highlighted.

Figure 2. Effects of the S90A mutation on the citrate synthase activity and growth rate of strain E109. (a) Alignment of amino acids of partial NifH proteins from strains belonging to

different phyla showing that the serine 90 phosphorylation site recently identified in *Zymomonas mobilis* ZM4 is a conserved site of this nitrogenase subunit. *Bradyrhizobium japonicum* E109 (WP_011084578), *Zymomonas mobilis* ZM4 (WP_011241556), *Azospirillum brasilense* Az39 (AIB12323), *Clostridium bornimense* (WP_044035927), *Nostoc* sp. PCC 6720 (CAA83510). (b) Citrate synthase activity and (c) duplication time of the wild-type strain E109 and the mutant strains GR1-GR5 were evaluated in exponential growing cells without glyphosate stress. Strain GR5 contains the S90A mutation. Values represent mean ± SD (n = 4). Significant differences between inoculants were analyzed with ANOVA followed by Dunnett's multiple comparisons test (n.s.: not significant, ****$p < 0.0001$, ***$p < 0.001$). (d) Average N2O fluxes from the soil microcosm inoculated with soybean rhizobia. Values represent mean + SEM (n = 20). *$p < 0.05$; **$p < 0.01$, t-test.

Figure 3. Effects of the S90A mutation on the plant growth-promoting efficiency of the inoculant. The productivity (a) and nodule biomass (b) of glyphosate-sensitive soybean plants inoculated with the wild-type strain E109 and the mutant strains GR1-GR5 were analyzed in the absence of nitrogen in the substrate and without glyphosate stress. Strain GR5 contains the S90A mutation. All values are means + SEM (n = 24). Significant differences between inoculants were analyzed with ANOVA followed by Dunnett's multiple comparisons test (n.s.: not significant, ****$p < 0.0001$). (c) Leaf nitrogen content in 3-month-old soybean plants treated with different bacterial inoculants under field conditions. Values represent mean + SEM (n = 9). *$p < 0.05$; t-test.

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Conflict of interest. The authors declare that they have no conflict of interest.

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Figure 1

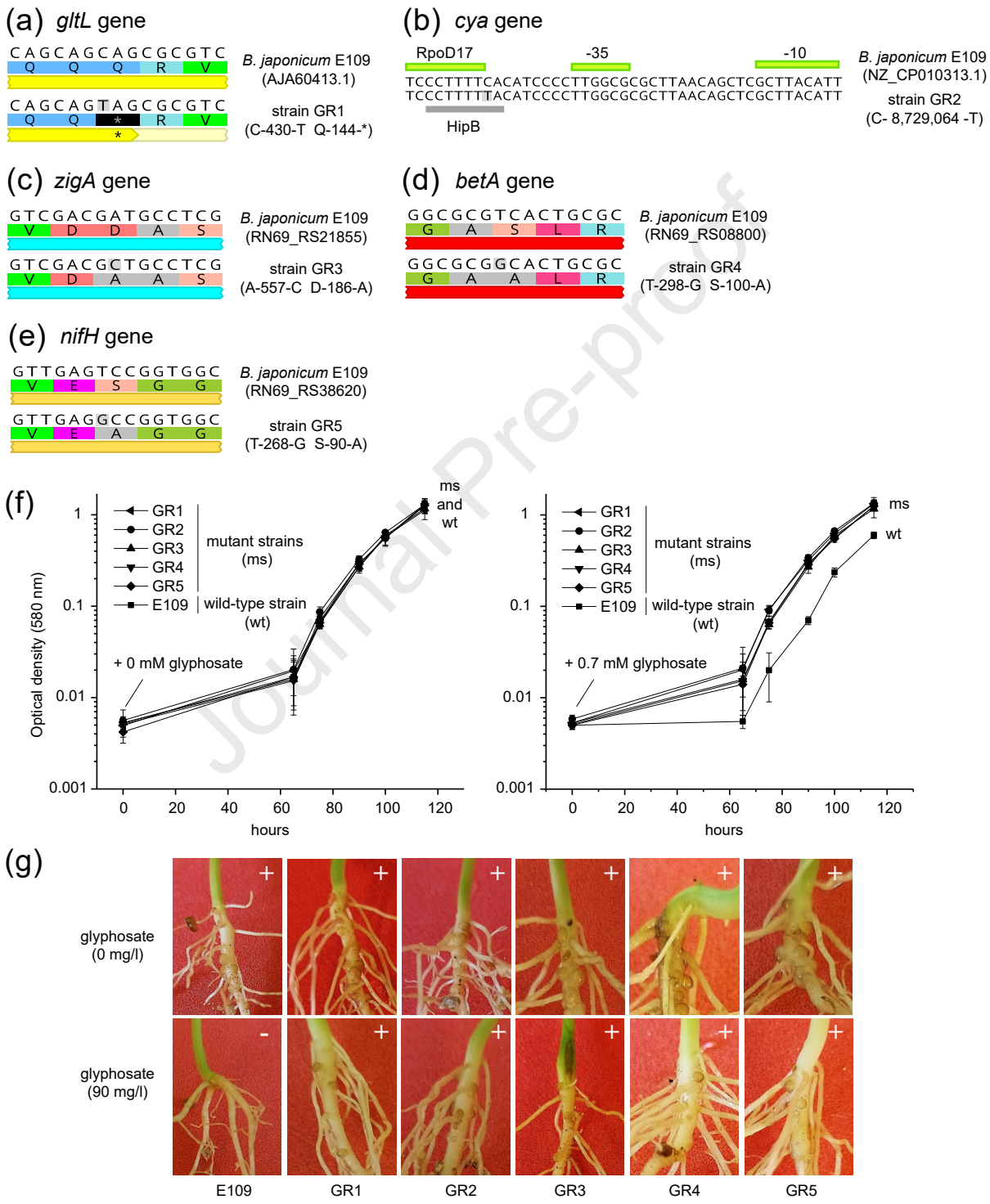

Figure 2

(a)

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                                  *     ****************** ** *  **
B. japonicum E109                 EDVMKVGYQDIRCVESGGPEPGVGCAGRGVITSINFLEEN
Z. mobilis ZM4                    EDVLKLGYKDIKCVESGGPEPGVGCAGRGVITSINFLEEN
A. brasilense Az39                EDVLKIGYKGIKCVESGGPEPGVGCAGRGVITSINFLEEN
C. bornimense                     DSIMKIGYGGTKCVESGGPEPGVGCAGRGIITSIGMLERL
Nostoc sp. PCC 6720               HEVMLTGFRGVRCVESGGPEPGVGCAGRGIITAINFLEEN
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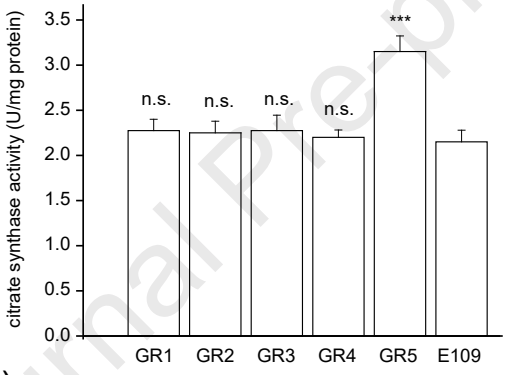
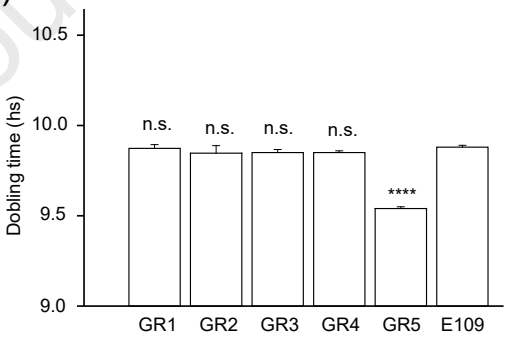
(b)



(c)



(d)

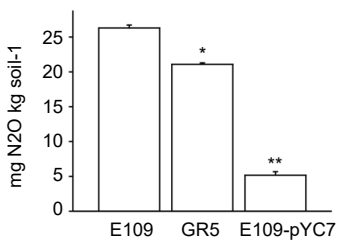


Figure 3