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PERSPECTIVE PAPER

Genome editing of soybean inoculant using CRISPR/Cas9 system: enhancing agricultural sustainability

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Abstract

In modern agriculture, inoculants, consisting of various bacteria and fungi, are essential for promoting crop growth and sustainability while reducing reliance on agrochemicals. Despite their benefits, there have been no recent significant advances in enhancing their efficacy. Notably, *Bradyrhizobium japonicum* E109 and *Bradyrhizobium japonicum* SEMIA 5079, the most globally utilized, were isolated decades ago, underscoring the need for genetic enhancement. Recently, efforts have focused on selecting spontaneous mutations in genes associated with denitrification and oxidative stress. While this technology has shown promise in reducing nitrous oxide emissions and enhancing root colonization, selecting mutants remains costly and challenging without clear phenotypic markers. The CRISPR/Cas9 system offers a potential solution, although validation is limited to model strains such as *Sinorhizobium meliloti* 1021. Using the CRISPR/Cas9 system and three sgRNAs, we have edited the genes 16S, *napA* and *glxA* from strains E109 and SEMIA5079, generating mutants with streptomycin tolerance, reduced nitrate reductase activity, and increased catalase activity, respectively. Finally, we discuss how the CRISPR/Cas9 system can contribute to bridging the gap between crop and inoculant improvement, and its possible role in producing a new generation of climate-smart inoculants.

Key message

We present the first genome editing of an inoculant using the CRISPR/Cas9 system.

Keywords Elite bacteria, Inoculant, Rhizobia · Soybean · Genome editing

In modern agriculture, a wide range of bacteria and fungi, collectively known as inoculants, are utilized to promote the growth, quality, and health of crops. Inoculants help reduce the use of agrochemicals, including fertilizers, fungicides, and insecticides, thus improving the sustainability of the agricultural sector. The most widely consumed inoculants globally are nitrogen-fixing rhizobia for soybean cultivation (Brambilla et al. 2022; Santos et al. 2019). Although these inoculants have significantly reduced the use of synthetic nitrogen fertilizers derived from petroleum, no new isolates with higher efficiency have been developed in the last years. In fact, the two most widely used inoculants in the world, *Bradyrhizobium japonicum* SEMIA 5079 and *Bradyrhizobium japonicum* E109, were isolated over four decades ago (Cassán et al. 2009; Santos et al. 2019). Thus, the need to enhance the genetics of these elite bacteria becomes clear.

In recent years, efforts have been made to achieve this goal by selecting spontaneous mutants in the *nap* and *glx* genes associated with denitrification and oxidative stress tolerance. The loss and gain of function in these genes result in inoculants with improved environmental and economic quality, respectively (Brambilla et al. 2020; Liebrenz et al. 2022). While this technology has facilitated the development of prototype inoculants with reduced nitrous oxide emissions and enhanced root colonization capacity, selecting these spontaneous mutants entails significant costs and is not feasible for genetic enhancements lacking clear phenotypic indicators, such as diminished nitrate reductase (Griess assay) and catalase (hydrogen peroxide assay) activities in

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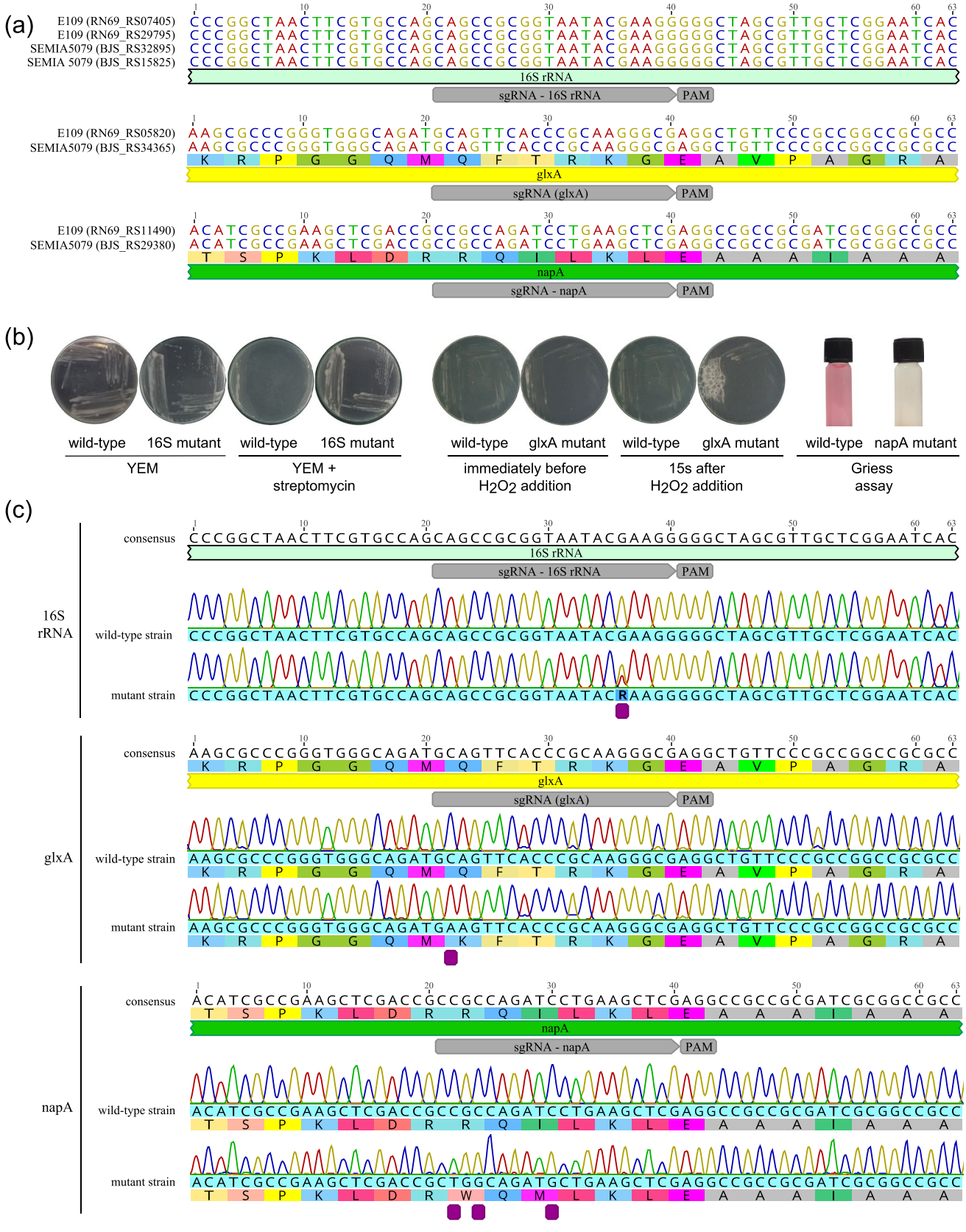
(a)
E109 (RN69_RS07405)
E109 (RN69_RS29795)
SEMIA5079 (BJS_RS32895)
SEMIA 5079 (BJS_RS15825)
16S rRNA
sgRNA - 16S rRNA
PAM
E109 (RN69_RS05820)
SEMIA5079 (BJS_RS34365)
glxA
sgRNA (glxA)
E109 (RN69_RS11490)
SEMIA5079 (BJS_RS29380)
napA
sgRNA - napA
(b)
wild-type
16S mutant
YEM
YEM + streptomycin
glxA mutant
immediately before H2O2 addition
15s after H2O2 addition
napA mutant
Griess assay
(c)
consensus
16S rRNA
wild-type strain
mutant strain
glxA
napA

◂**Fig. 1** Generation of mutant inoculants via the CRISPR/Cas9 system. (**a**) Nucleotide sequence of wild-type alleles of the genes 16S rRNA, *napA* and *glxA* from wild-type inoculants E109 and SEMIA5079. In accordance with their extreme phylogenetic affinity, the target sequences have 100% nucleotide identity. In this picture, we highlighted the guides (sgRNAs) and the sequence recognized by the Cas9 nuclease (PAM sequences). (**b**) The editing efficiency in each gene for each strain (E109 and SEMIA5079) was calculated by analyzing the phenotype of between 21 and 27 individual clones. Contrary to wild-type strains E109 and SEMIA5079, mutant strains containing edited alleles of the genes 16S rRNA, *napA*, and *glxA* exhibited growth on YEM agar supplemented with !00 µg/ml of streptomycin, a significant release of oxygen bubbles after exposure to H2O2, and no color change after the addition of the Griess reagent, respectively. These phenotypic assays were performed as previously described (Brambilla et al. 2020; Liebrenz et al. 2022). (**c**) The visualization of the Sanger sequencing analysis for studying the edition of bacterial genes 16S rRNA, *napA*, and *glxA*. By using this genotyping approach, it is possible to determine the genotype of an individual clone rapidly and at low cost. Wild-type strains were used as controls. The colors in a read denote the quality of an individual base, where light blue and blue indicate high and low quality, respectively. The presence of more than one peak in the edited base of the 16S rRNA gene (highlighted with a violet square) suggests that the evaluated clone (mutant strain) possesses both wild-type and mutant alleles (A or G nucleotides, respectively) for that multi-copy gene. In the mutant strains *glxA* and *napA*, there are one and two non-synonymous substitutions (highlighted with violet squares) that produce one or two amino acid changes, respectively

the *napA* and *glxA* mutant strains. The CRISPR/Cas9 system presents a potential solution to this challenge. However, its validation has been limited to model strains, such as the nitrogen-fixing bacterium *Sinorhizobium meliloti* 1021. In this article, we examine the feasibility of editing the genomes of the E109 and SEMIA5079 inoculants as an initial step towards developing a new generation of environmentally intelligent inoculants.

Three editing vectors were constructed containing the CRISPR/Cas9 system, where Cas9 is modified to induce single nucleotide substitutions in the genes 16S rRNA, *napA* and *glxA*, associated with protein biosynthesis, denitrification, and catalase activity (Additional File 1). These plasmids contain the classic genes *kmR* and *sacB* for the selection of recombinant strains in kanamycin and plasmid curing respectively (Kovach et al. 1995; Schäfer et al. 1994). The sgRNAs were directed to the region where streptomycin binds to 16S rRNA, and to regions of the structural domains of the NapA and GlxA proteins to suppress and activate expression of nitrate reductase and catalase activities, respectively (Fig. 1a). In all three cases, a significant proportion of the clones obtained via conjugation, originating from strains E109 and SEMIA5079, exhibited the anticipated phenotypes of streptomycin tolerance (18%), reduced nitrate reductase activity (36%), and increased catalase activity (30%), respectively (Fig. 1b). Consistent with these phenotypes, the mutant strains displayed the expected modifications at the target sites (Fig. 1c). Therefore, it can be concluded that the E109 and SEMIA5079 strains can be rapidly and efficiently edited using the CRISPR/Cas9 system.

Inoculants have historically been synonymous with sustainable agricultural production and can play a key role in the fight against global climate change. To achieve this, it is necessary to include new genetic improvement technologies for these inoculants and to understand them as part of the plant germplasm. This is particularly evident for the legume-rhizobia interaction, where without the nitrogen fixation carried out by rhizobia, the legume drastically reduces its productivity and nutritional quality. It is also highlighted that the CRISPR/Cas9 system can contribute to closing the historical improvement gap between crops and inoculants, and to planning new simultaneous improvements between plants and their host microorganisms. Considering climate change as a global concern demanding the reduction of greenhouse gas emissions and enhancement of productivity across vast geographical regions, the swift dissemination of CRISPR/Cas9 technologies in inoculants is imperative to mitigate its effects.

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Data availability The data used in this study are available from the corresponding author on reasonable request.

Declarations

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

References

Brambilla S, Soto G, Odorizzi A, Arolfo V, McCormick W, Primo E, Giordano W, Jozefkowicz C, Ayub N (2020) Spontaneous mutations in the nitrate reductase gene napc drive the emergence of eco-friendly low-N(2)O-emitting alfalfa rhizobia in regions with different climates. Microb Ecol 79:1044–1053

Brambilla S, Stritzler M, Soto G, Ayub N (2022) A synthesis of functional contributions of rhizobacteria to growth promotion in diverse crops. Rhizosphere 24:100611

Cassán F, Perrig D, Sgroy V, Masciarelli O, Penna C, Luna V (2009) Azospirillum brasilense Az39 and Bradyrhizobium japonicum E109, inoculated singly or in combination, promote seed germination and early seedling growth in corn (Zea mays L.) and soybean (Glycine max L.). Eur J Soil Biol 45:28–35

Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM 2nd, Peterson KM (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166:175–176

Liebrenz K, Gómez C, Brambilla S, Frare R, Stritzler M, Maguire V, Ruiz O, Soldini D, Pascuan C, Soto G, Ayub N (2022) Whole-Genome Resequencing of Spontaneous Oxidative Stress-Resistant Mutants Reveals an Antioxidant System of Bradyrhizobium japonicum Involved in Soybean Colonization. Microb Ecol 84:1133–1140

Santos MS, Nogueira MA, Hungria M (2019) Microbial inoculants: reviewing the past, discussing the present and previewing an outstanding future for the use of beneficial bacteria in agriculture. AMB Express 9:205

Schäfer A, Tauch A, Jäger W, Kalinowski J, Thierbach G, Pühler A (1994) Small mobilizable multi-purpose cloning vectors derived from the Escherichia coli plasmids pK18 and pK19: selection of defined deletions in the chromosome of Corynebacterium glutamicum. Gene 145:69–73