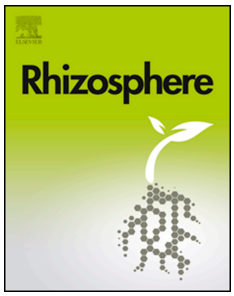

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Oligopeptide uptake improves oxidative stress resistance and nodulation competitiveness in soybean rhizobia

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Key words: *Bradyrhizobium*; soybean; aspartate kinase; oxidative stress; colonization starvation.

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Abstract

We here explored the mechanisms associated with oxidative stress resistance and root colonization by soybean rhizobia. Genomic and physiological analyses supported the fact that the mutation of a highly conserved valine at position 263 to alanine in an aspartate kinase-like protein associated with oligopeptide uptake (LysC) enhances the oxidative stress resistance of the commercial inoculant *Bradyrhizobium japonicum* E109. Integrated genetic and physiological studies suggest that LysC is part of a conserved substrate transport system in *Bradyrhizobium*, and that the LysC-V263A mutation in strain E109 significantly increased its oligopeptide uptake, survival during periods of starvation and nodulation competitiveness. Finally, the potential utilization of the CRISPR system to promptly propagate this gain-of-function mutation in soybean production worldwide is discussed.

Inoculants play a pivotal role in enhancing both environmental and economic sustainability in agricultural practices. They contribute to improved nutrient uptake, reduced fertilizer usage, and minimized soil degradation. These biotechnological products promote soil health, increase crop productivity, and lead to cost savings for farmers, making them indispensable tools for sustainable agriculture and key contributors to mitigating climate change (Brambilla et al., 2022; Cao et al., 2023). Soybean cultivation has been the largest consumer of inoculants, particularly nitrogen-fixing symbiotic rhizobia belonging to *Bradyrhizobium japonicum* and *Bradyrhizobium diazoefficiens* related species. Among the prominent commercial inoculants are strains such as *Bradyrhizobium japonicum* SEMIA 5079, *Bradyrhizobium japonicum* E109, and *Bradyrhizobium diazoefficiens* SEMIA 5080, which are closely related to the model rhizobium *Bradyrhizobium diazoefficiens* USDA110 (Santos et al., 2019).

A recent study involving genome resequencing of spontaneous oxidative stress-resistant mutants derived from the commercial strain *B. japonicum* E109, in combination with molecular and physiological investigations, led to the identification of an antioxidant cluster named BjAC (*Bradyrhizobium japonicum* antioxidant cluster). This cluster contains a transcriptional regulator (GlxA) that controls the expression of a catalase (CatA) and a phosphohydrolase (YfbR) involved in the hydrolysis of hydrogen peroxide and oxidized nucleotides, respectively (Liebrenz et al., 2022). Evolutionary analysis and empirical complementation experiments further supported the hypothesis that the emergence of BjAC in the *B. japonicum* lineage occurred after its divergence from the *B. diazoefficiens* lineage (Liebrenz et al., 2022). Additionally, genetic engineering of this cluster significantly enhanced the nodulation competitiveness of strain E109 (Liebrenz et al., 2022). However, the application of this strategy to improve plant colonization is highly restricted due to the absence of BjAC in different legume rhizobia, including soybean inoculants belonging to the *B. diazoefficiens* species. In light of this limitation, we undertook an empirical exploration aiming to identify novel mutations within conserved genes that could enhance both oxidative stress resistance and nodulation competitiveness.

We here selected and studied a spontaneous hydrogen peroxide (H_2O_2) - resistant mutant (AK) derived from the wild-type strain E109 (Fig. 1a). This mutant displayed intermediate resistance to H_2O_2 compared to strain E109 and the previously described glxA mutant under rich medium conditions (Fig. 1a). In line with this phenotype, the AK strain showed no increase in the expression of the *catA* gene (Fig. 1b), which encodes catalase, when compared to its wild-type counterpart E109, and it also exhibited no evident modifications in catalase activity (Fig. 1c). Under these conditions, strain AK also exhibited higher resistance to osmotic and low-temperature stresses than strain E109 (Additional File 1). These findings indicate that the resistance of strain AK to oxidative stress could be a result of a greater overall resistance to abiotic stress.

Genomic analysis revealed that the mutant strain AK is completely isogenic with their wild-type parental strain E109, except for a single nucleotide substitution in the locus RN69_RS01030, which encodes for LysC, an aspartate kinase-like protein (Fig. 1d). Specifically, we showed the emergence of

a nucleotide change within the *lysC* gene in strain AK (T788C), which changes the valine 263 site to alanine (Fig. 1d). We showed that this site is highly conserved in different nitrogen-fixing strains from *Bradyrhizobium* genus (Fig. 1e), including model strain USDA110 and inoculants *B. diazoefficiens* SEMIA 5080, *B. arachidis* CCBAU 051107 and *B. elkanii* SEMIA 587 (Additional File 1). Comparison of the nucleotide sequence of the region around the *lysC* gene of strain E109 with other genomes of *Bradyrhizobium* species, supported that this region is a preserved feature of the genus *Bradyrhizobium* (Fig. 1f). Therefore, the *lysC* gene represents an excellent target for enhancing these N-fixing symbionts. To explore the potential use of this strategy to other strains within the same genus, we selected and analyzed four spontaneously generated H_2O_2-tolerant mutants derived from the USDA110 strain. Then, we amplified the *lysC* gene from these strains and analyzed its nucleotide sequence. Remarkably, we identified the LysC-T788C-V263A modification in three independent mutants (Additional File 1). These results not only highlight the potential of this improvement strategy but also suggest the presence of other potential modifications capable of conferring the same oxidative stress-resistant phenotype.

Evolutionary analysis and functional domain prediction of different isoforms of the aspartate kinase protein in *Bradyrhizobium* were consistent with synteny analysis and suggest that the *lysC* gene (blr0216) of the USDA110 strain is the orthologous gene to the *lysC* gene (RN69_RS01030) of the E109 strain (Fig. 1f) (Additional File 1). This *lysC* gene from the *Bradyrhizobium* species was located immediately downstream of the *ptsP* and *prfA* genes (Fig. 1f), which are critical components of the bacterial phosphotransferase system (PTS) involved in the translocation and phosphorylation of numerous substrates (Deutscher et al., 2006). RNase protection analysis of *lysC* (blr0216) and *ptsP* (blr0217) expression in recombinant strains derived from USDA110 supported that these adjacent genes are expressed as separate transcriptional units (King and O'Brian, 2001). *In vitro* and *in vivo* assays have showed the physical interaction between the LysC and PtsP proteins, their essential role in oligopeptide transport, and the regulatory function of LysC in this transport through its phosphorylation activity (King and O'Brian, 2001). Strain AK exhibited a lower duplication time and higher resistance to hydrogen peroxide than strain E109 in minimal medium supplemented with Pro-Gly-Gly (Fig. 1g), suggesting that LysC-V263A mutation could enhance peptide uptake and, probably indirectly, increase resistance to oxidative stress (Fig. 1h).

A successful inoculant needs to compete with a vast diversity of indigenous microbes and efficiently colonize plant organs, including legume nodules (de Souza et al., 2020). To evaluate competition for nodulation, we inoculated soybean with 1:1 mixtures containing the wild-type parental strain E109 together with spontaneous oxidative stress (AK) and streptomycin (StrR)-resistant mutants, and identified the bacterial strains occupying each nodule by their antibiotic resistances and catalase phenotype. In these assays, we found that most of the nodules were colonized by the mutant strain AK (>90%), which contrasts with the 1:1 ratio (approximately 50%) in the control strain StrR (Fig. 1i). These findings are in line with the understanding of the LysC-V263A mutation as a putative trait for soybean yield. Moreover, this result is consistent with the previous results showing the relevance of the

oxidative stress response in soybean colonization by different nitrogen-fixing symbionts from different genera, including *Rhizobium leguminosarum* 3841 (Hu et al., 2021) *Sinorhizobium fredii* HH103 (Crespo-Rivas et al., 2019) and *B. japonicum* E109 (Liebrenz et al., 2022).

Ensuring the successful delivery of a substantial number of viable bacterial cells to the plant is a prerequisite for achieving a satisfactory colonization rate. This, in turn, amplifies the desired effects in the field. The viability of inoculants may encounter challenges at various stages, both prior to and during application. Initially, a product must exhibit an adequately prolonged shelf life, reflecting its stability across the entire production process, packaging, storage, and transportation conditions (Berninger et al., 2018). In this line, we studied the survival capacity of strains E109 and AK under conditions of low nutrient availability. Strain AK demonstrated a considerable survival advantage over strain E109 following two months of exposure to these adverse conditions (Fig. 1j). These results support the hypothesis that the LysC-V263A mutation enhances survival during periods of starvation.

Genetic engineering methods for improving inoculants have typically involved modifying bacteria by either enhancing or suppressing specific genes. This often requires the introduction of foreign DNA to trigger trait expression, behaving like a dominant allele. Then, transferring knowledge from model species (e.g. *E. coli*) to inoculants has been relatively straightforward, even when dealing with bacteria of differing lifestyles. However, widespread reluctance exists among countries to permit the release of genetically modified bacteria into agricultural systems. This limits the application of the valuable traits commonly achieved via genetic engineering. One alternative involves the identification of dominant or partially dominant mutations induced by gain-of-function alterations. These mutations can be created through single nucleotide substitutions using the CRISPR system. As legal frameworks for the use of gene-edited organisms evolve, covering bacteria, plants, and animals, without the presence of foreign DNA, in an increasing number of countries (Lewi and Vicién, 2020; Mallapaty, 2022), it becomes plausible to anticipate the rapid and widespread adoption of gain-of-function mutations (e.g. LysC-V263A) in crop production in the near future.

Declaration of competing interest. The authors declare no conflict of interest.

Data availability. Data will be made available on request.

Figure 1. The V263A mutation in LysC improves oxidative stress resistance and nodulation competitiveness in soybean inoculant E109. (a) The disk inhibition assay in YEM agar confirms the tolerance to H_2O_2 (30%) of a spontaneous mutant (AK) derived from the wild-type strain E109. (b) Real-time RT-PCR studies of *catA* expression in *B. japonicum* E109 and its *lysC* mutant derivative, strain AK. (c) Contrary to E109 and AK colonies, glxA colonies produced a large amount of oxygen bubbles after exposure to H_2O_2. (d) As compared to the genome of their parental strain E109, the genome of the oxidative-resistant strain AK displays a mutation only within the *lysC* (yellow) gene, which code for an

aspartate kinase-like protein. The mutation is highlighted in gray and its impact on nucleotide and amino acid sequences are described on the left. (e) The consensus sequence of the LysC protein in the genus *Bradyrhizobium* reveals that valine at position 263 and the majority of nearby amino acids are highly conserved in this group of microsymbionts. (f) The gene *lysC* from strains belonging to *Bradyrhizobium* genus (e.g. strain E109) is situated within a conserved region of the *Bradyrhizobium* genome, and specifically, immediately downstream of two components of the bacterial phosphotransferase system (PTS) related to the translocation and phosphorylation of numerous substrates (the *ptsP* and *prfA* genes). Putative transcriptional units are highlighted in light blue (www.microbesonline.org). (g) Duplication time of the wild-type strain E109 and the mutant strain AK were evaluated in exponential growing cells in RMM medium, with or without the addition of 50 µg/ml prolyl-glycyl-glycine (PGG). (h) The disk inhibition assay using H_2O_2 (30%) on RMM agar, with or without the supplementation of 50 µg/ml PGG. (i) **S**train E109 and its derived spontaneous mutants StrR and AK were inoculated at a 1:1 ratio, using 10^3 CFU/plant. Nodule occupancy was determined using selective media containing streptomycin or H_2O_2. (j) **S**trains E109 and AK were growth one week in inoculated at a 1:1 ratio, using 10^3 CFU/plant. Nodule occupancy was determined using selective media containing streptomycin or H_2O_2. (j) To assess cell survival under low-nutrient conditions, YEM cultures of strains E109 and AK were transferred to a physiological solution (0.9% NaCl) and incubated for two months. Survival was expressed as a percentage of the number of colony forming units at time zero, that was taken as 100%. Physiological and molecular studies were performed as previously described (Liebrenz et al., 2022). Values are mean ± SEM (n=4-20). Asterisks indicate a statistically significant difference (Student's t test: *$p<0.05$; **$p<0.01$; ***$p<0.001$).

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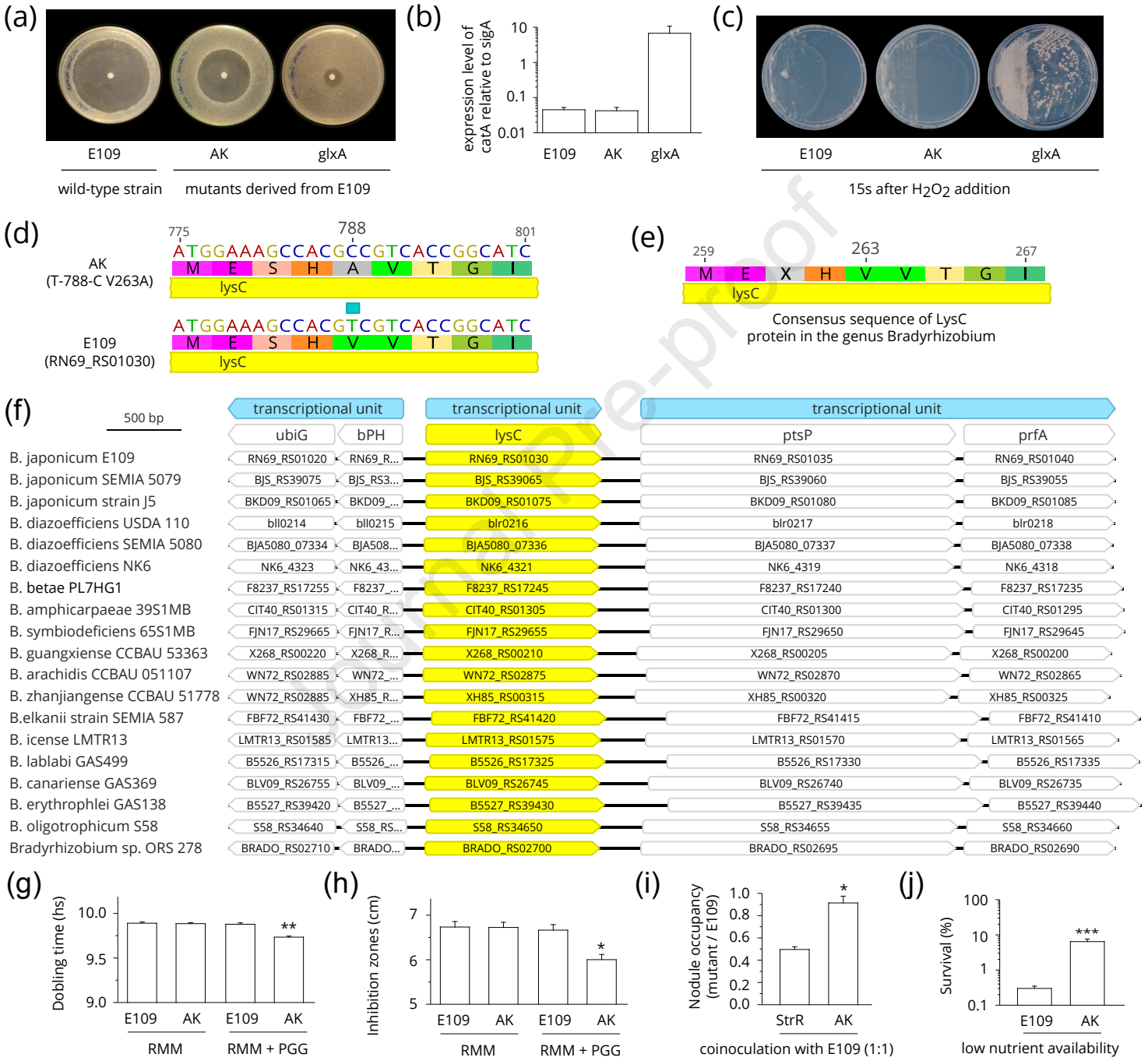
Figure 1



Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: