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Genetic engineering of RuBisCO by multiplex CRISPR editing small subunits in rice

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Summary

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is required for photosynthetic carbon assimilation, as it catalyses the conversion of inorganic carbon into organic carbon. Despite its importance, RuBisCO is inefficient; it has a low catalytic rate and poor substrate specificity. Improving the catalytic performance of RuBisCO is one of the key routes for enhancing plant photosynthesis. As the basic subunit of RuBisCO, RbcS affects the catalytic properties and plays a key role in stabilizing the structure of holoenzyme. Yet, the understanding of functions of RbcS in crops is still largely unknown. Toward this end, we employed CRISPR-Cas9 technology to randomly edit five *rbcS* genes in rice (*OsrbcS1–5*), generating a series of knockout mutants. The mutations of predominant *rbcS* genes in rice photosynthetic tissues, *OsrbcS2–5*, conferred inhibited growth, delayed heading and reduced yield in the field conditions, accompanying with lower RuBisCO contents and activities and significantly reduced photosynthetic efficiency. The retarded phenotypes were severer caused by multiple mutations. In addition, we revealed that these mutants had fewer chloroplasts and starch grains and a lower sugar content in the shoot base, resulting in fewer rice tillers. Further structural analysis of the mutated RuBisCO enzyme in one *rbcs2,3,5* mutant line uncovered no significant differences from the wild-type protein, indicating that the mutations of *rbcS* did not compromise the protein assembly or the structure. Our findings generated a mutant pool with genetic diversities, which offers a valuable resource and novel insights into unravelling the mechanisms of RuBisCO in rice. The multiplex genetic engineering approach of this study provides an effective and feasible strategy for RuBisCO modification in crops, further facilitate the photosynthesis improvement and sustainable crop production.

Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is the key enzyme responsible for catalysing the first step of the Calvin-Benson-Bassham (CBB) cycle in photosynthesis, which converts inorganic CO_2 into organic carbohydrates, and it is the major rate-limiting factor that determines carbon assimilation efficiency (Bar-On and Milo, 2019; Sharkey, 2023). RuBisCO plays crucial roles in photosynthesis, biomass accumulation and global carbon cycling; however, it exhibits a low catalytic rate (3–20 CO_2/s) and poor substrate specificity, due to the relatively ineffective discrimination between CO_2 and O_2 (Liu, 2022). Consequently, genetically engineering more catalytically efficient RuBisCO enzymes has become a major pursuit in the field of photosynthesis research (Bailey-Serres *et al.*, 2019; Lin *et al.*, 2014; Orr and Parry, 2020; Taylor *et al.*, 2022).

Among the distinct evolutionary lineages of RuBisCO proteins found in nature, RuBisCO is classified into six forms: I, I′, II, III, II/III and IV, with form I being the most abundant and found in both eukaryotes and bacteria (Banda *et al.*, 2020; Hayer-Hartl and Hartl, 2020; Schneider *et al.*, 1992; Schulz *et al.*, 2022). In plants, RuBisCO is a hexadecameric complex consisting of eight RuBisCO large subunit (RbcL) proteins and eight RuBisCO small subunit (RbcS) proteins. The RbcL—encoded by the chloroplastic gene *rbcL*—contains the active site of RuBisCO, while the RbcS—encoded by the nuclear gene *rbcS*, typically consists of a gene family with multiple members—stabilizes the holoenzyme structure (Bracher *et al.*, 2017). Ancestral sequence reconstruction was used to trace the evolutionary history of form I RuBisCO, revealing that the small subunit was acquired long before increases in atmospheric O_2 concentrations, and thereafter rapidly became the critical determinant of RuBisCO catalytic activity and specificity (Schulz *et al.*, 2022). In addition, *Escherichia coli*-based characterization of predicted ancestral forms of RuBisCO within the *Solanaceae* family demonstrated that even minor variations in the RbcS sequence could alter catalytic efficiency (Lin *et al.*, 2022). The assembly of different small subunits can also modify RuBisCO carboxylation efficiency, subsequently impacting plant photosynthetic activity (Brand and Tissier, 2022; Laterre *et al.*, 2017; Lin *et al.*, 2020; Martin-Avila

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