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Original article

Improved stress tolerance and productivity in transgenic rice plants constitutively expressing the *Oryza sativa* glutathione synthetase *OsGS* under paddy field conditions

Seong-Im Park[a,c,1], Young-Saeng Kim[b,1], Jin-Ju Kim[a,c], Ji-Eun Mok[a], Yul-Ho Kim[d], Hyang-Mi Park[e], Il-Sup Kim[c,*], Ho-Sung Yoon[a,c,**]

[a] *Department of Biology, College of Natural Sciences, Kyungpook National University, Daegu 702-701, Republic of Korea*
[b] *Research Institute of Ulleung-do & Dok-do, Kyungpook National University, Daegu 41566, Republic of Korea*
[c] *School of Life Sciences, BK21 Plus KNU Creative BioResearch Group, Kyungpook National University, Daegu 41566, Republic of Korea*
[d] *Highland Agriculture Research Institute, National Institute of Crop Science, Rural Development Administration, Pyeongchang 25342, Republic of Korea*
[e] *National Institute of Crop Science, Rural Development Administration, Wanju 54955, Republic of Korea*

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ABSTRACT

Reactive oxygen species, which increase under various environmental stresses, have deleterious effects on plants. An important antioxidant, glutathione, is used to detoxify reactive oxygen species in plant cells and is mainly produced by two enzymes: gamma-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GS). To evaluate the functional roles of the glutathione synthetase gene (*OsGS*) in rice, we generated four independent transgenic rice plants (TG1–TG4) that overexpressed *OsGS* under the control of the constitutively expressed *OsCc1* promoter. When grown under natural paddy field conditions, the TG rice plants exhibited greater growth development, higher chlorophyll content, and higher GSH/GSSH ratios than control wild-type (WT) rice plants. Subsequently, the TG rice plants enhanced redox homeostasis by preventing hydroperoxide-mediated membrane damage, which improved their adaptation to environmental stresses. As a result, TG rice plants improved rice grain yield and total biomass following increases in panicle number and number of spikelets per panicle, despite differences in climate during the cultivation periods of 2014 and 2015. Overall, our results indicate that *OsGS* overexpression improved redox homeostasis by enhancing the glutathione pool, which resulted in greater tolerance to environmental stresses in the paddy fields.

1. Introduction

Rice, which is the most commonly consumed food for a large part of the human population, has been subjected to climate changes as a result of global warming and industrialization. These changes, which are related to drought, flooding, salinity, extreme temperatures (both low and high), ultraviolet radiation, ozone, contamination by heavy metals, and nutrient deprivation, represent environmental stresses that cause unfavorable conditions for crops and greatly diminish the yields of agricultural systems (Mittler, 2002). Plants exposed to these environmental stresses exhibit increased levels of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), and hydroxyl radicals (Wang et al., 2007), which function as signaling sensors of growth and defense pathways but are toxic when produced in excess (Mittler et al., 2004). More specifically, high ROS levels damage biomolecules such as nucleic acids, proteins, and lipids, and cell organelles, including chloroplasts (Dat et al., 2003). For example, lipid peroxidation, a process by which ROS remove electrons from lipids in cell membranes, affects membrane permeability and causes ion leakage (Jambunathan, 2010).

To repair ROS damage, plants use complex antioxidant systems. There are two types of antioxidant constituents: antioxidant enzymes such as superoxide dismutase, catalase, peroxiredoxin, ascorbate peroxidase, dehydroascorbate reductase, and glutathione reductase; and non-enzymatic constituents, such as lipid-soluble tocopherols and water-soluble reductants, which include ascorbic acid (AsA) and

Abbreviations: DAB, 3,3-diaminobenzidine; ECS, Glutamylcysteine synthetase; GS, Glutathione synthetase; GSH, Reduced glutathione; GSSG, Glutathione disulfide; H_2O_2, Hydrogen peroxide; MDA, Malondialdehyde; MV, Methyl viologen; NBT, Nitro blue tetrazolium; Os, *Oryza sativa* L; O_2^-, Superoxide anion; ROS, Reactive oxygen species

* Corresponding author.
** Corresponding author at: Department of Biology, College of Natural Sciences, Kyungpook National University, Daegu 702-701, Republic of Korea.
E-mail addresses: 92kis@hanmail.net (I.-S. Kim), hsy@knu.ac.kr (H.-S. Yoon).
[1] These authors contributed equally to this work.

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glutathione (Shah et al., 2001). In particular, antioxidant systems are closely associated with the AsA-GSH cycle in plants, and in almost all living organisms, GSH is an abundant and ubiquitous thiol compound involved in this cycle and in a number of biochemical reactions. GSH is a tripeptide (γ-Glu–Cys–Gly) that occurs interchangeably in two distinct redox forms. It is highly soluble in water, owing to the thiol group, and can be either an electron acceptor or a donor in physiological reactions. Furthermore, it can participate in regeneration of another potential antioxidant, AsA, via the AsA-GSH cycle. GSH recycles AsA from its oxidized form to its reduced form using the enzyme DHAR; acts as a precursor of phytochelatins, which combine with oxidants such as menadione and methyl viologen and heavy metals such as cadmium; and regulates redox homeostasis in cells (Sharma et al., 2012). In addition, xenobiotics and other organic radicals are neutralized by GSH through a cascade of detoxification mechanisms involving glutathione peroxidases (GPx), glutathione-S-transferases (GST), and glutathione reductase (GR) (Rahman et al., 2006). GSH synthesis requires two enzymatic steps involving ATP. GCL, also known as γ-glutamylcysteine synthetase, mediates the first reaction between glutamate and cysteine to form a dipeptide, γ-glutamyl-cysteine (γGluCys), which in turn reacts with the glycine catalyzed by glutathione synthetase (GS) to produce GSH (Aoyama et al., 2008). Accordingly, GS is considered the main enzyme of GSH synthesis. Therefore, we postulated that the *GS* gene has an important role in the improvement of environmental adaptation and rice yield in paddy fields.

Recently, we demonstrated that overexpression of *Oryza sativa* γ-glutamylcysteine synthetase could increase acquired tolerance to ROS-induced oxidative stress in transgenic (TG) rice plants (Choe et al., 2013), which suggests that GSH plays a critical role in protecting plant cells from abiotic stresses. To expand this evidence for GSH synthesis, we generated TG rice plants harboring the *O. sativa* L. *japonica* 'Ilmi' chloroplast *GS* gene (*OsGS*) from LOC_Os12g16200, examined how the transgene was expressed under paddy field conditions, and then functionally characterized the agronomic traits and adaptation of the transgenic rice plants against environmental stresses. In this study, our results indicate that, in response to the environmental stresses of natural paddy fields, TG rice plants with a constitutively expressed cytochrome c (*OsCc1*) promoter exhibit greater acquired adaptation and better early growth development, phenotypes, and rice yield, including grain yield and biomass. These improvements are achieved via enhanced GSH biosynthesis, which results in improved redox homeostasis and an augmented GSH pool, which helps the plant to adapt to various environmental stresses under natural paddy fields despite climate changes during the cultivation period.

2. Materials and methods

2.1. Plasmid construction and transformation into rice

The full-length *OsGS* cDNA (GenBank accession no.: AY453405) was synthesized with total RNA isolated from leaves of rice seedlings (*O. sativa* L. *japonica* 'Ilmi') using reverse transcription-polymerase chain reaction (RT-PCR) with the following primer set: forward 5′-GGG**GAATTC**TACTGCCAATGTC-3′ and reverse 5′-AAATTAT**GAATTC**TCTG-3′ (the restriction enzyme site for each primer is bolded). The cDNA was then cloned between the rice *OsCc1* promoter and the nopaline synthase gene (*nos*) terminator of the pMJ101 vector using *Eco*RI restriction endonucleases. The resulting plasmids were introduced into *Agrobacterium tumefaciens* LBA4404, which was used to transform rice calli from the scutellum of mature 'Ilmi' seeds, in accordance with a previously described *Agrobacterium tumefaciens*-mediated transformation method (Hiei et al., 1994).

2.2. Plant materials and growth conditions

Oryza sativa L. was used as a host for *OsGS* overexpression. The T_3 (2014) and T_4 (2015) generations of four independent homozygous TG rice plants and a single wild-type (WT) rice plant were used to examine genotypes and phenotypes. Accordingly, the TG and WT seeds were disinfected with 0.05% fungicide (Sportac: prochloraz 25%) for 24 h and soaked in distilled water for 4 days in a growth chamber in the dark at 28 °C. Thereafter, the seeds were transplanted into soil pots (8 cm in diameter and 12 cm in height; 100 plants/pot) and then grown in a greenhouse (16 h light: 8 h dark) at 28–30 °C for 4 weeks. Next, the seedlings were transplanted into a natural paddy field located on the campus of Kyungpook National University in Gunwi, South Korea, and cultivated from June to October.

2.3. Genomic DNA isolation and PCR

For molecular analysis, genomic DNA was manually extracted from leaf samples. Briefly, approximately 0.1 g of leaf samples were placed into 2-mL microfuge tubes, immediately soaked in liquid nitrogen, and ground. The ground leaves were added to 300 μL DNA extraction buffer, which contained 100 mM Tris-HCl (pH 9.0-9.5), 1 M KCl, and 10 mM EDTA (pH 8.0), vortexed for 10 min, and then incubated at 70 °C for 30 min. After incubation, the samples were vortexed for 30 s, added to 300 μL distilled water, vortexed for 5 min, and then centrifuged at 12,000 rpm for 5 min at 4 °C (Bae et al., 2013). The supernatant (~400 μL) was carefully transferred into a new tube and used as template for PCR. The PCR reaction was performed using a PCR premix (Bioneer, Daejeon, Korea) and the OsCc1-F1 (5′-CTACCAGAGCACGTGGACC-3′) and OsGS-R1 (5′-CCTAGTAAGACCCAACCTAACATCC-3′) primer set, with the following conditions: an initial denaturing step for 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 56 °C, and 50 s at 72 °C, and a final extension of 5 min at 72 °C. The amplified DNA fragments (10 μL reaction mixture) were visualized, along with 5.0 μL of 1-Kb plus DNA Ladder Marker (Elpis-Biotech, Daejeon, Korea), on a 1.0% (w/v) agarose gel (Lonza, Rockland, ME, USA) that contained 10 μL 100 mL^{-1} EcoDye DNA staining solution (SolGent, Daejeon, Korea).

2.4. RNA extraction and RT-PCR

Total RNA was isolated from the leaves of TG and WT rice plants at 4 weeks after transplantation, using TRIzol reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. The reverse transcription reactions were conducted with 2 μg of total RNA, using the SuperScript III First-Strand Synthesis System (Invitrogen). To monitor *OsGS* expression, the OsGS-F2 (5′-GCGGAATGGAGAGCAAGACTCTTGAT-3′) and OsGS-R2 (5′-AGCCCTGCAAAGCATTTCCG-3′) primer set was used. The rice *tubulin* (*Tub*) gene with the Tub-F (5′-GAGTACCCTGACCGCATGAT-3′) and Tub-R (5′-GTGGTCAGCTTGAGAGTCCT-3′) primer set was used as a positive housekeeping control under the same PCR conditions. The semi-quantitative RT-PCR reactions consisted of 26 cycles of 5 min at 94 °C, 30 s at 55 °C, and 50 s at 72 °C, and then a final extension for 5 min at 72 °C. For quantitative real-time PCR analysis, 2 μL of the diluted (1:10) first-stand cDNA templates and SYBR Green PCR master mix (Applied Biosystems, Foster, CA, USA) were used in a total volume of 20 μL. The reaction consisted of an initial denaturation step for 10 min at 95 °C, then 40 cycles of 15 s at 95 °C and 10 min at 60 °C. Data were collected and analyzed using StepOne Software v2.1 (Applied Biosystems). Normalization and relative quantification were conducted using the $2^{-\Delta\Delta Ct}$ method (Ferreira et al., 2006).

2.5. Protein extraction and western blot analysis

For western blot analysis, rice leaves (0.1 g) were harvested, frozen in liquid nitrogen, and ground to a fine powder. The protein was then extracted as described by Shultz et al. (2005), with slight modifications. The protein concentration was determined using Protein Assay Dye