Construction of SHP-GLOX Straw Lignin Degulation System and Its Application Effect in Rice

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Method Article

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Abstract

Backgroud:

Rice straw is the crucial biomass for raw materials in China. Rice straw usage is limited due to its high lignin content, and the reduction of its lignin content could have economic and environmental benefits. Soybean hull peroxidase (SHP), a class III plant peroxidase, is derived from multiple sources. It has several advantages, such as high resistance to heat, high stability under acidic and alkaline conditions, and broad substrates range. SHP is speculated to be useful for lignin degradation. Glyoxal oxidase (GLOX) is an extracellular oxidase that can oxidize glyoxal and methylglyoxal in the extracellular medium to generate H_2O_2.

Results

In the present study, the *SHP* gene and the *GLOX* gene in the pCAMBIA3301-glycine-rich protein (GRP)-SHP-GLOX, designated as the K167 vector, was optimized and introduced into rice embryos using *Agrobacterium*-mediated transformation. Positive transgenic rice embryos were examined using molecular, physiological, biochemical tests and fermentation tests. The outcomes suggested that SHP degraded lignin effectively.

Conclusions

This research has created a breeding material that does not basically affect the normal growth and yield of rice, but the rice stalks are more likely to be degraded in the later stage, and used to breed rice varieties whose stalks are easy to use for energy. Our results will improve the industrial and commercial applications of rice straw.

Backgroud

China produces rice (*Oryza. Sativa L. spp. japonica*) straw in large quantities, which is also a crucial source of biomass for raw materials. It primarily constitutes cellulose, hemicellulose, and lignin. Lignin has a complex structure that makes its degradation difficult and, in turn, limits the utility of rice straw. Lignin is a highly abundant and renewable resource across the globe, which holds great potential for the production of value-added chemicals[1–3]. China produces lignocellulosic resources in large quantities in the form of agricultural waste, industrial production residues, and residual wood. Crop straw accounts for a large proportion of lignocellulosic agricultural resources in agriculture-based countries; besides, it is widely used as the raw material for animal feed, fertilizer, alkali source, and for energy production[4].

The precise underlying mechanism for lignin biosynthesis remains unknown so far. However, as per previous studies, broadly, it involves three steps: shikimate metabolism, phenylpropane metabolism, and

lignin synthesis pathways. Lignin expression or content can be altered by regulating the expression of related enzymes, which can be attained via genetic engineering[5–7].

Lignin is formed from peroxidase catalyzed polymerization of lignin monomers. Peroxidase is an oxidoreductase enzyme that utilizes hydrogen peroxide (H_2O_2) to oxidize organic compounds. It plays a crucial role in a myriad of physiological and biochemical processes in plants, such as photosynthesis, respiration, disease resistance, and biotic-abiotic stress[8]. Peroxidases are ubiquitous in plants, animals, and microorganisms. Besides, peroxidases have found wide applications in multiple areas, such as biodegradation, sewage treatment, bio-catalysis and as a biosensor. Horseradish peroxidase is a well-known commercial enzyme. Soybean hull peroxidase (SHP), a class III peroxidase, is derived from the soybean seed coat, a by-product generated during the industrial oil production process. SHP has multiple substrates, resistant to high temperature[9, 10], and functions over a broad pH range, which makes it a valuable research tool[11].Glyoxal oxidase (GLOX) is an extracellular oxidase that can oxidize glyoxal and methylglyoxal in the extracellular medium to generate H_2O_2. As a source of physiological H_2O_2, GLOX is also an important class of enzymes on the degradation of lignin by enzymes.

Transgenic technology has been employed to enhance the degradation of straw, which has reduced the cost of feed production and environmental pollution[12]. SHP is used to treat phenol-containing wastewater released by the industrial pulping processes as SHP effectively degrades the lignin present in this wastewater[10]. It has crucial implications in bioremediation and other research investigations[13].In the current study, we constructed the K167 vector containing the *SHP* and *GLOX* gene and introduced it into the rice genome through the *Agrobacterium*-mediated transformation method to generate transgenic rice plants with enhanced lignin degradation.

Results

Vector construction and verification

In order to verify the successful construction of K167 vector. First, it was verified by electrophoresis (Fig. 1A) and double enzyme digestion (Fig. 1B) to check the release of the insert. Subsequently, K167 was sent to Shanghai Bioengineering Co., Ltd. for sequencing. The sequencing results combined with the results of enzyme digestion proved that K167 vectors had been successfully constructed, and these vectors were used for subsequent Agrobacterium transformation.

PCR-based screening of transgenic rice plants

PCR was employed to validate the transgene insertion using SHP-F and SHP-R primers. The amplified PCR product of 750 bp band size validated the *SHP* positive transgenic rice plants. The strip gels of the appropriate size were recovered and sent to Shanghai Bioengineering Co., Ltd. for sequencing. The

sequencing results proved that the gene fragments were correct, indicating that the genomes of these strains all carried foreign genes, and these 21 strains were identified as positive plants (Fig. 1D, E).

Southern blot analysis of transgenic rice plants

Southern blotting confirmed the site of transgene integration. To extract pure rice leaf DNA, repeated extractions using RNase A was performed to eliminate RNA contamination. Lane1 is the positive control, and this lane is where the target gene fragment is melted and combined with the probe, so this lane has only one band and the color is darker. Lane 9 is the negative control, and this lane has no band. K1, K2, K3, and K4 all show two bands in the lanes, so it can be determined that the number of copies of the target gene contained in the genomes of these four lines is double copy, which is a double copy line. K5, K6, and K7 all show a single band in the lanes, so it can be determined that the number of copies of the target gene contained in the genomes of these three lines is a single copy, which is a single copy line. Southern blot result showed that exogenous genes were integrated into the rice genome. (Fig. 2A).

Real-time Fluorescent Quantitative PCR（RT-qPCR）of transgenic rice plants

RT-qPCR validated the presence of the *SHP* gene in transgenic rice plants. *GAPDH* was used as an internal reference gene, and cDNA from the wild-type rice line was considered as a negative control. RT-qPCR detection was performed on the positive plants of transgenic rice, and the results proved that the exogenous gene has been transcribed and expressed normally in rice, and the expression level has certain differences in different periods. The *SHP* gene of transgenic rice was up-regulated during the flowering and maturity stages. The *GLOX* gene expression was normal during the flowering period and was significantly up-regulated during the maturity period (Fig. 2B, 2C).

Basta smear experiment

The *Bar* gene in the K167 vector serves as a screening marker, and it imparts resistance against Basta herbicide, also known as glufosinate. The *Bar* gene expression in transgenic rice was analyzed using Basta. The resistance of transgenic rice plants to Basta demonstrated the *Bar* gene expression (Fig. 2D). Conversely, negative control plants were found to be sensitive to Basta as they did not express the *Bar* gene.

Measurement of the agronomic traits

As shown in Figures 3.A, 3.B, 3.C and 3.D, the wild-type and transgenic rice both grow well and develop normally. There are no significant difference of the wild-type and K-series transgenic rice lines in grain morphology, panicle shape and whole plant morphology.

The agronomic data of the transgenic lines are shown in Table 2, and the agronomic data of the wild-type are shown in Table 3. In terms of plant height, the wild type is not significantly different from K8, K18, and K19 (*P> 0.05*), but is significantly different from other strains (*P <0.05*). In terms of ear length, wild-type and K1, K4, K7, K8, K9, K10, K12, K14, K15, K16, K18 were not significantly different (*P□0.05*), and were significantly different from other strains (*P□0.05*). In terms of the number of tillers, the wild type was not significantly different from K2, K3, K5, and K13 (*P□0.05*), but was significantly different from other strains (*P□0.05*). In terms of total grains per ear, wild type has no significant difference with K1, K4, K5, K7, K8, K16, K18, K20 (*P□0.05*), and significant difference from other strains *(P□0.05*). In terms of the number of grains per panicle, the wild type is not significantly different from K1, K4, K5, K7, K8, K11, K16, K20 (*P>0.05*), and significantly different from other strains (*P <0.05*). In terms of seed setting rate, there was no significant difference between wild-type and all transgenic rice (*P□0.05*). In terms of 1000-seed weight, wild type has significant differences with K2, K3, K6, K9, and K12 (*P <0.05*), but not significantly different from other strains (*P> 0.05*).

Measurement of peroxidase activity

Peroxidase activity was detected spectrophotometrically in transgenic plants, and these plants were identified through PCR-based screening using wild-type rice plants as control. Transgenic rice leaves demonstrated higher peroxidase activity than the control leaves, but activity levels varied notably among the transgenic rice leaves (Fig. 4A). According to SPSS analysis, the peroxidase activity of wild-type rice and transgenic rice lines are extremely different *(P <0.01*). It can be seen from Figure 4B that the peroxidase activity in the leaves of the transgenic rice at the mature stage is higher than that in the wild-type rice, and there are also certain differences between different transgenic lines. Compared with the wild type, the highest value of peroxidase activity of the transgenic rice line K4 increased by 65.38%, and the lowest value of K3 increased by 22.16% compared with the wild type. The results showed that the exogenous genes *SHP* and *GLOX* can be expressed normally and efficiently under the guidance of the promoter, the peroxidase activity of the transgenic rice plants was significantly increased.

Determination of heterocellulose content

Heterocellulose content is the sum of cellulose and hemicellulose content. However, when cellulose and hemicellulose content are measured separately, a partial loss in yield is witnessed, which results in an inaccurate measurement of lignin components. This inaccuracy can be eliminated by measuring the heterocellulose content. In this study, the holocellulose content of rice in the flowering stage is shown in Fig 4B. The holocellulose content of wild-type rice is 61.12% ± 0.89%, the highest holocellulose content in transgenic rice lines is K4, which is 62.85% ± 0.28%, and the lowest holocellulose content is K2, which is 60.48% ± 0.74%. Data analysis using SPSS 26.0 showed that the content of cellulose in wild-type rice lines was not significantly different from that of transgenic rice lines (*P>0.05*), indicating that the introduction of exogenous genes *SHP* and *GLOX* did not affect the synthesis of cellulose in plants during

blooming. The total cellulose content of rice at the mature stage is shown in Fig 4C. The holocellulose content of wild-type rice is 63.35% ± 0.78%, the highest holocellulose content of transgenic rice lines is K6, which is 66.04% ± 0.87%, and the lowest holocellulose content is K15, which is 63.30% ± 0.52%. Data analysis using SPSS 26.0 showed that the content of cellulose in wild-type rice lines was not significantly different from that of transgenic rice lines (*P 0.05*), indicating that the expression of exogenous genes *SHP* and *GLOX* did not affect the synthesis of cellulose in plants at the mature stage.

Determination of lignin content

The lignin content of rice in the flowering stage is shown in Fig 4D. According to measurement, the lignin content of wild-type rice lines is 33.63% ± 0.76%, the highest lignin content of transgenic rice lines is K8, which is 35.90% ± 0.70%, and the lowest lignin content of transgenic rice lines is K10, which is 32.47. % ± 0.81%. Using SPSS 26.0 for data analysis, the lignin content of wild-type rice lines was not significantly different from that of transgenic rice lines (*P> 0.05*), and the lignin content of some transgenic rice lines was slightly higher than that of wild-type rice. Studies have shown that the exogenous gene *SHP* can promote the synthesis of lignin in the early stage of plant development, and can effectively degrade lignin under the action of H_2O_2 in the later stage. Therefore, it is speculated that the expression of the exogenous gene *SHP* does not affect the lignin synthesis in the early stage of the plant, and may also promote the lignin synthesis.

The lignin content of rice in the mature stage is shown in Fig 4E. According to the measurement, the lignin content of wild-type rice lines is 35.17% ± 0.84%. the highest lignin content of transgenic rice lines is K9, which is 30.45% ± 0.66%, and the lowest lignin content of transgenic rice lines is K5, which is 24.58. % ± 0.27%. Using SPSS 26.0 for data analysis, the lignin content of wild-type rice lines was significantly different from that of transgenic rice lines (*P <0.01*). It can be seen from Fig 5.E that the lignin content of transgenic rice at the maturity stage is lower than that of wild-type rice, and there are also certain differences between different transgenic lines. Compared with the wild type, the lignin content of the transgenic rice line, the highest value K9, was reduced by 13.42%, and the lowest value, K5, was reduced by 30.11% compared with the wild type. The results show that *GLOX* can provide H_2O_2 for *SHP*, and *SHP* can effectively degrade lignin under the action of H_2O_2. Therefore, it is speculated that the expression and interaction of exogenous genes *SHP* and *GLOX* can effectively reduce the lignin content.

Results of ethanol fermentation experiments of transgenic plants

Analysis of the Determination of Reducing Sugar Content

The reducing sugar concentration determined after cellulase hydrolysis is shown in Fig. 5A. The experimental data results represent three groups of duplicates. After the no pretreatment group and the treatment with ddH_2O group, the contents of reducing sugars obtained from cellulase-degraded transgenic rice and the control (WT) were essentially same. However, after 50% sulfuric acid pretreatment,

both the transgenic rice and the control showed an increase in the content of hydrolyzed sugars (23.2 g/L); this was significantly higher ($p < 0.05$) than that in the untreated group (5.8 g/L).

Determination of Ethanol Content

Ethanol fermentation experiments were performed on ddH_2O treated transgenic rice, 50% sulfuric acid-pretreated rice, and non-pretreated rice. Compared with the same fermentation conditions, the three pre-fermentation treatments resulted in changes in the amount of ethanol produced. Under an initial fermentation pH of 5.0 and temperature of 30°C, and an inoculation amount of 10%, fermentation products were obtained at 24 h, 48 h, 72 h, and 96 h in order to determine the ethanol concentration. As shown in Fig 5B, the ethanol content determined at each period of pretreatment with 50% sulfuric acid was much higher than the corresponding value of the untreated group and ddH_2O pretreatment. The final ethanol concentration of transgenic rice (9.1 g/L) was significantly higher than that of non-transgenic rice (2.6 g/L), but slightly lower than the concentration of the acid pretreatment group (9.9 g/L).

Discussion

The expression vector plays a crucial role in transgene expression[19]. In this study, we constructed a K167 backbone vector from pCAMBIA3301, which contained herbicide and kanamycin-resistant gene. The K167 vector contained a β-glucuronidase gene in the T-DNA region of the SHP-GLOX gene, harboring the GRP peptide. The gene cassette in the K167 vector is under the control of the constitutive CaMV 35S promoter that is active throughout the plant development[20]. Thus, in this study, we used CaMV 35S to enhance the *SHP* and *GLOX* gene expression in rice plants.

Rice is a vital food crop worldwide. The main objective of rice plant's agronomic research is genetic engineering mediated improvement of the overall characteristics and resistance to biotic and abiotic stress through genetic engineering[21]. *Agrobacterium*-mediated transgenic technology is widely used in research and development of rice plants [22]. It is influenced by a myriad of factors, such as embryos pretreatment, concentration and time of infection, temperature and time of co-culture, and concentration of screening agents[23][24][25].

In this study, the rice was transfected with the *SHP* and *GLOX* gene using Agrobacterium-mediated transformation. Big young rice embryos with the highest growth were selected for this transformation using phosphinothricin as the screening agent. In the screening stage, callus appeared brown in color, and at the end of the screening stage, resistant calluses appeared yellowish in color with a fluffy texture and strong regenerative capacity[7][26]. A total of 380 seeds were transformed in the experiment. After a series of transformations, a total of 21 transformation events were obtained, and the transformation efficiency was 5.53%.

Generally, a high DNA and RNA concentration with low RNA and protein contaminants is a prerequisite for southern hybridization. Herein, the phenol-chloroform extraction method was used to remove impurities,

such as polysaccharides and proteins; besides, RNase A was used to reduce RNA contaminants from DNA precipitate after dissolution[27]. In conventional southern blotting, radioisotopes are used to label probes that have potential safety hazards and require proper handling under specialized laboratory conditions. The currently used high-symplectic labeling method is safe and efficient for probe labeling. Southern hybridization can be used to detect foreign genes in a genome and evaluate species homology[28]. The commonly used RNA extraction reagents include TRIzol, sodium dodecyl sulfate (SDS), and CTAB. As shown in a previous comparative analysis of several RNA extraction methods, the SDS extraction method yielded the highest RNA purity and integrity[29].

In this study, we did not observe any significant phenotypic differences between transgenic and wild-type rice plants. There was a significant difference ($P < 0.01$) and the control peroxidase activity in transgenic rice, the maximum increase compared to control up to 65.38%. It is speculated that the exogenous genes SHP and GLOX can be expressed normally and efficiently under the guidance of the promoter, and the peroxidase activity of the transgenic rice plants is significantly improved. There was no significant difference in the content of cellulose in the transgenic rice lines at the flowering and maturity stage compared with the wild type ($P > 0.05$). It is speculated that the introduction of exogenous genes SHP and GLOX has no significant effect on the synthesis of plant cellulose and hemicellulose. The lignin content of transgenic rice lines during blooming stage was not significantly different from that of wild type ($P > 0.05$). The lignin content of rice at the mature stage was determined, and it was found that the transgenic rice lines were significantly different from the wild type ($P < 0.01$), and the lignin content of the transgenic rice lines were lower than those of the control group. It is inferred that the exogenous gene GLOX is at full maturity. At the beginning of the period, it expressed and provided H_2O_2 for SHP, which may have participated in the process of lignin depolymerization and reduced the lignin content of the plant. The commonly used methods to determine lignin content are acetic acid, acid washing, and Klason methods[30]. To determine the lignin content, these three methods were compared by determining the saccharification efficiency of various *Miscanthus* cultivars. Klason method was found to be the most effective and suitable method for high-throughput analyses[18]. It suggested that *SHP* expression promoted lignin synthesis as well as lignin degradation in rice plants, which with GLOX expression.

Lignin has a complex structure, and it degrades slowly under natural conditions. Conventional physical and chemical methods used for lignin degradation have a low efficiency of lignin removal, culminating in environmental pollution[31]. Current strategies for lignin removal have been primarily focused on the synthesis of lignin monomers, and the genes associated with this process is being studied in most plants; however, other approaches such as lignin polymerization have not been extensively investigated[32]. Peroxidase and laccase promote the polymerization of lignin monomers[33][34] as well as lignin degradation under certain conditions [35][36]. Restricted genetic diversity can supply only a limited number of elite genes for modern plant cultivation and transgenesis[37].Thus, peroxidases are expected to enhance lignin degradation in transgenic crops for the effective utilization of biomass resources[38].

Conclusion

This research has created a breeding material that does not basically affect the normal growth and yield of rice, but the rice stalks are more likely to be degraded in the later stage, and used to breed rice varieties whose stalks are easy to use for energy. Our results will improve the industrial and commercial applications of rice straw.

Methods

K167 plasmid optimization

Escherichia coli DH5α cells were procured from TransGen Biotech (Beijing, China). The pCXUN plasmid and plant expression vector pCAMBIA3301-glycine-rich protein (GRP) -SHP -GLOX(K167) used in this study was taken from our laboratory stocks. GRP protein guides the localization of the SHP-GLOX fusion protein to the plant cell wall in peptide-producing rice[14]. K167 contains the constitutively expressed active cauliflower mosaic virus (CaMV) 35S promoter, which is widely used to generate transgenic plant varieties.

Total RNA is extracted from soybean hulls, and then reverse transcription of RNA is performed by reverse transcription polymerase chain reaction technology to obtain first-strand cDNA. Using the synthesized cDNA as a template, the SHP gene was amplified by PCR. In order to express the SHP gene in rice, a recombinant vector was constructed on the basis of the pCAMBIA3301 vector. First connect the SHP with the pMD-20T vector, and then connect the vector strains pNMCS-ZSAG12p-GRP-GLOX-NOS, pMD 20-T-GRP0.9, pMD 20-SHP and pCAMBIA3301 constructed and stored in the laboratory. After screening and identification, a new recombinant plasmid named GRP-SHP-GLOX expression vector (K167) was obtained for the production of transgenic rice (Figure 1C). (The specific construction process is in Annex 1)

Genetic transformation of rice

Competent *Agrobacterium* cells were prepared from *Agrobacterium tumefaciens* EHA105 colonies cultured on YEB medium. The cells were transformed with using the electroproration method, and the transformants were identified using PCR. The transformed *A. tumefaciens* cells were preserved using a 20% glycerol solution and stored at −80°C. The *Oryza. Sativa L. spp. japonica* rice lines were transformed with the K167 vector using *Agrobacterium* strain EHA105[15].

Identification of transgenic rice by PCR

Genomic DNA was extracted from the leaves of transgenic rice plants using the cetyltrimethylammonium bromide (CTAB) method. The extracted DNA was subjected to PCR-based screening of primer SHP and Bar.

Southern blot analysis

Preparation of digoxigenin (DIG)-dUTP-labeled probe

The *Bar* gene in the rice genome was amplified using PCR. The amplified PCR product was visualized using AGE and recovered through the gel extraction procedure, which was later denatured in boiling water for 10 min. The denatured PCR product was transferred to an ice bath for 2-3 min, and 4 µl of DIG-High Prime was added to it. This mixture was incubated overnight at 37°C, and the reaction was terminated by heating it at 65°C for 10 min. The labeled probe was stored at 4°C until further use.

Southern blotting

The rice genome was digested overnight at 37°C with a restriction enzyme, HindIII (New England Biolabs), in a reaction containing 30 µl of genomic DNA extracted from the rice leaves, 5 µl of HindIII, 10 µl of buffer, and 55 µl of ddH_2O. The digested product was resolved on 1% agarose gel for 24 h at 25 V. The agarose gel was later transferred to a nylon membrane, washed with 2X saline–sodium citrate, blotted dry, and placed in a transparent Ziploc bag. The DNA was fixed on the membrane by ultraviolet irradiation for 5 min, and the membrane was stored at 4°C until use.

In a bag, the nylon membrane was treated with 10 ml of preheated (37°C water bath) hybridization solution. Pre-hybridization was performed at 37°C with shaking at 80 rpm for 30 min. 5 µl *Bar* probe was denatured for 5 min, cooled on an ice bath, and centrifuged at 12000 rpm for 30 s. It was followed by the addition of 500 µl hybridization solution and the mixture was incubated overnight at 37°C with shaking at 80 rpm. The film was washed and developed as per the standard protocol[16]. Briefly, the membrane was transferred to a new bag, washed gently with light shaking at 65°C, blocked for 30 min at room temperature (24℃), and incubated with the antibody solution (1:10,000 dilution) for 30 min at room temperature. After washing, the membrane was equilibrated in the assay solution for 2–5 min followed by incubation with the test solution for 5 min. This membrane was developed by exposing it to X-ray film, observed and scanned the membrane hybridized with a digoxigenin-labeled probe for overnight and was detected using the digoxigenin luminescence detection procedure with the Roche DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science, Penzberg, Upper Bavaria, Germany), as per the manufacturer's instructions.

Real-time Fluorescent Quantitative PCR（RT-qPCR）

RNA was extracted from fresh rice leaves using a plant RNA extraction kit (Kangwei Century Biotech Co., Taizhou City, China), as per the manufacturer's instructions. The diluted cDNA was used as a PCR template. G*lyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) was used as a reference gene. The sequences of primer are in Table 1.

Basta smear

1:5000 dilution of 10% Basta solution (Bio Basic, Markham, ON, Canada) was prepared using deionized water. Transgenic rice plant leaves were treated with this diluted Basta solution, and changes at the site of application were evaluated after 8-10 days.

Biochemical analysis of transgenic rice plants

The agronomic traits

Randomly select 20 well-growing mature rice and control rice, and measure their plant height, panicle length, effective tiller number, total grain number, real grain number, seed setting rate per plant and 1000-seed weight.

Peroxidase activity assay

Crude enzyme extract was prepared from leaves of transgenic rice, and the peroxidase activity in this extract was evaluated spectrophotometrically (Shanghai Jingke Industry Co., Ltd). A unit of enzyme activity was calculated as an increase of 0.01 light absorption units per minute (i.e., change in absorbance at 470 nm/raw weight).

Determination of hemicellulose content

The hemicellulose content of transgenic rice which in the flowering stage and the maturity stage was determined using the $NaClO_2$ method, as described previously (GB2677.10-1995)[17].

Determination of lignin content

The lignin content in transgenic plants which in the flowering stage and the maturity stage was determined using the Klason method[18].

Assessment of ethanol fermentation of transgenic rice

Stems of the same parts of the transgenic and control plants at the mature stage were cut into sections of about 3 cm and divided into three groups. The first group was immersed in ddH_2O in a 45°C water bath for 1 h. The second group was treated with 50% sulfuric acid and hydrolyzed at 120°C for 1.5 h. The third group was no treatment. After hydrolysis, the solution was neutralized to pH 5.0 with sodium hydroxide. The stem sections of the two groups were placed in an oven for drying (100℃), ground into a powder, and then sieved through a 40-mesh sieve. Then, acetic acid-sodium acetate cellulase solution (pH 4.8) was added and the solution was immersed in a water bath at 37°C for 24 h. The DNS (dinitrosalicylic acid) method was used to determine reducing sugars. Reducing sugars were used as a carbon source after the rice was hydrolyzed; the fermentation medium was added at a solid-liquid ratio of 1:10, and sterilized at 121°C for 20 min. Preserved Saccharomyces cerevisiae was isolated from the slant culture

medium and cultured in a seed medium at 28°C with shaking at 200 rpm for 16 h, and inoculated at a 10% inoculation amount. Under the fermentation pH 5.0 and the temperature 30°C, the products of fermentation for 24 h, 48 h, 72 h, and 96 h were obtained for detection of ethanol content.

Declarations

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Authors' Contributions

QW performed physiological and biochemical analyses, designed and coordinated the study, analyzed the data, JZ constructed and optimized the vectors and drafted the manuscript. YL performed PCR, RT-qPCR. RW performed Southern blot analyses, drafted the manuscript and participated in experimental design and data analysis. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated from this study are included in this published article and supporting materials.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors have read and agree to publish the paper.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1-3 are available in the Supplementary Files section.

Figures

Figure 1

Vector construction and PCR-based identification. (A). Electrophoretic identification of K167 plasmid. M:Star Marker(DL 2000 Plus); 1 - 3：Recombinant plasmid to be teste. (B). Double-enzyme digestion verification of recombinant plasmid. M：1 kb Plus；2 - 3：*NcoI/PmlI* enzyme digestion；4 - 5：*PstI/StuI* enzyme digestion. (C). Schematic diagram of the recombinant plasmid GRP-SHP-GLOX (K167). (D). PCR identification of transgenic rice. M：Star Marker Plus；D2000 Plus；;1 - 19：K1 - K19. (E). PCR identification of transgenic rice. M：Star Marker；D 2000；；1 - 2：K20 - K21；3：WT；4：Plasmid K167.

Figure 2

Southern blot, RT-qPCR and Basta smearing identification of rice. (A). Southern blot detection of rice. M： Marker；1：Positive control；2 - 8：Transgenic rice K1 - K7；9：Negative control. (B). The expression levels of SHP and GLOX genes in rice at flowering stage. (C). The expression levels of SHP and GLOX genes in rice at the maturity stage. (D). Basta smear experiment on rice leaves. A, C：Transgenic rice before and after smear；B, D：Wide-type rice before and after smear

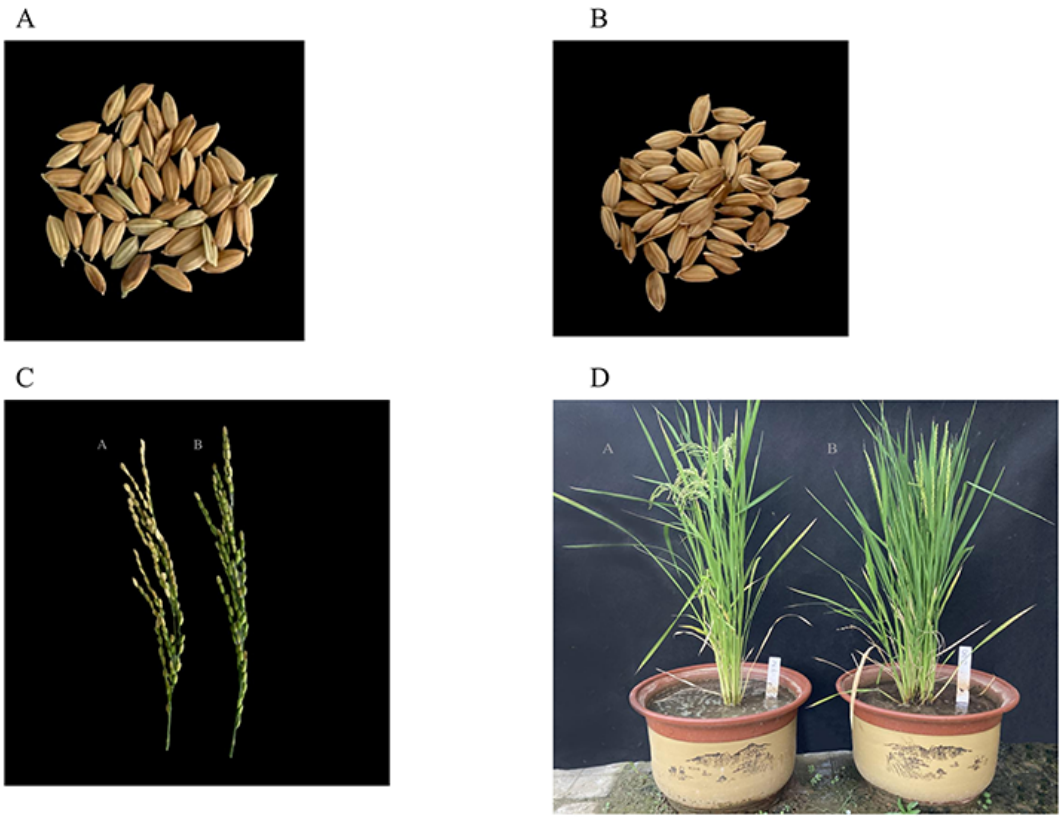

Figure 3

Phenotypes of transgenic rice and wild-type rice. (A). Phenotypes of grains of transgenic rice. (B). Phenotypes of grains of wild-type rice. (C). Comparison of the panicle types between wild-type rice and transgenic rice. A: Wide-type rice; B: transgenic rice. (D). Comparison of the shapes of wild-type rice and transgenic rice. A：Wide-type rice of the control group；B：transgenic rice.

Figure 4

Physiological and biochemical measurements. (A). Detection of peroxidase activity in transgenic maize plants. (B). Holocellulose content in transgenic rice and wild-type rice at blooming stage. (C). Holocellulose content in transgenic rice and wild-type rice at the mature stage. (D). Lignin content of transgenic rice and wild-type rice at blooming stage. (E). Lignin content in transgenic rice and wild-type rice at the mature stage.

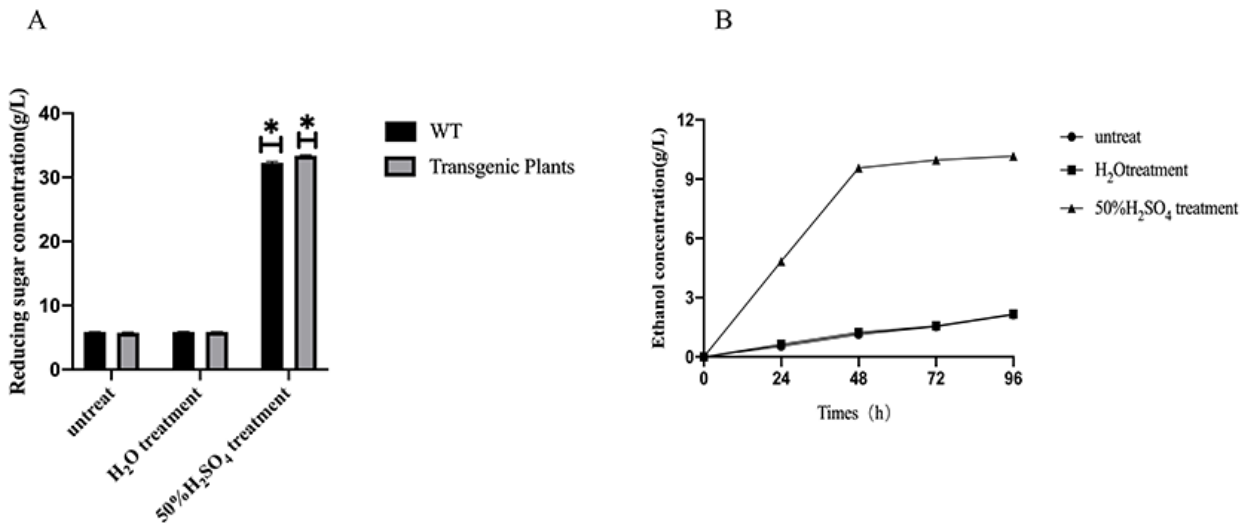

Figure 5

Ethanol fermentation experiments of transgenic plants. (A). Hydrolytic reducing sugar content. (B). Ethanol concentration during fermentation

Supplementary Files

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- Table.xlsx