Research Article

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Overexpression of *EiKCS* confers paraquat-resistance in rice (*Oryza sativa* L.) by promoting the polyamine pathway

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Abstract

BACKGROUND: Paraquat is used widely as one of the bipyridine herbicides, which generates reactive oxygen species to cause cell death. With a growing number of paraquat-resistant weeds, the mechanism of paraquat-resistance in plants remains unclear. This research verified the functions of a previously confirmed putative paraquat-resistant gene, *EiKCS*, from paraquat-resistant goosegrass by genetic engineering in a single overexpressing line in rice.

RESULTS: Overexpression of *EiKCS* improved paraquat resistance in transgenic rice (KCSox). Pre-applied (12 h) exogenous spermidine (1.5 mmol L^{-1}), alleviated the injury of paraquat in rice. Paraquat induced injury in KCSox was 19.57%, which was lower than 32.22% injury it induced in wild-type (WT) rice. The paraquat-resistant mechanism was through the increased activity of antioxidant enzymes and the overproduction of endogenous polyamines. The spermine content in KCSox was more than 30 μg mL^{-1}, while that in WT rice was less than 5 μg mL^{-1}. Quantitative proteomics showed that β-ketoacyl-coenzyme A (CoA) synthase (51.81 folds) encoded by the transgenic *EiKCS* gene promoted the synthesis of the proteins involved with the polyamine pathway. The synthesized putrescine was promoted by the arginine decarboxylase (ADC) pathway. The spermidine synthase I (1.10-fold) and three eceriferum cofactors (CERs) were responsive to the paraquat stress. We validated putrescine ($C_{18}H_{20}N_2O_2$) spermidine ($C_{28}H_{31}N_3O_3$), and spermine ($C_{38}H_{42}N_4O_4$) in this study.

CONCLUSION: *EiKCS* encoding β-ketoacyl-CoA synthase from goosegrass has been shown as an ideal candidate gene for engineering genetically modified organism (GMO) crops, as its overexpression does not only bring paraquat-resistance, but also have potential benefits without decreasing yield and rice grain quality.

Supporting information may be found in the online version of this article.

Keywords: paraquat resistance; polyamines; goosegrass (*Eleusine indica* L.); GMO crops; rice (*Oryza sativa* L.)

1 INTRODUCTION

Paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride) is a photosystem I (PSI) inhibitor used in agriculture and gardening worldwide, because it rapidly kills weeds and green plants.[1] In green plants, paraquat interrupts normal electron flow through PSI by excepting electrons instead of ferredoxin. This turns paraquat^{2-} into a free radical, which, in turn, disrupts cell membranes.[2] This action of paraquat results in the production of toxic reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), hypochlorous acid (HClO), and free radicals O^{2-} which facilitate the toxic action of paraquat.[3] In addition to its control efficacy of weeds, paraquat is also one of the most widely used herbicides in bipyridine herbicides for its timely inactivation upon reaching the soil.[4]

It has been reported that over 30 species of weeds have developed resistance to paraquat due to the repeated applications of commercial paraquat for decades worldwide (http://www.weedscience.org). A dominant (or semi-dominant) single gene was identified to underlie the resistance to paraquat, which was also identified to be involved in vacuolar sequestration.[5–7] Goosegrass (*Eleusine indica* L.) is one of the most serious invasive weeds in crop fields in many countries due to its paraquat-resistance.[8–10] Polyamines have been identified to be associated with the

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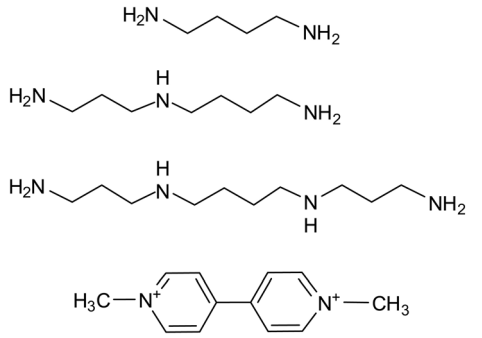

Figure 1. The structure of polyamines and paraquat: (from top to bottom) putrescine, spermidine, spermine and paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride).

resistance and detoxification mechanism in the paraquat-resistant biotypes of goosegrass.[11–13]

The structures of polyamines are similar to that of paraquat (Fig. 1).[14–17] Both of them are following similar characteristics of two or more nitrogen atoms surrounded by maximum positivity of charge, a non-polar group between charges, a minimum of steric hindrance, and an optimum distance between the changed nitrogen centers.[18,19] Polyamines can share a common uptake system with paraquat and are more successful endogenous substrates than paraquat due to their structural similarity.[20,21] An inhibitor of polyamines is a better inhibiting paraquat uptake than the endogenous polyamine uptake.[22] Polyamines are essential for the survival and growth of all living organisms. Examples are putrescine (Put), spermidine (Spd), and spermine (Spm), which are major polycationic compounds in plants. Polyamines are involved in cell death, seed germination, and root formation and involved in the ROS stress pathways induced by paraquat.[23–25] The relationship between polyamines and ROS showed exogenous polyamines lowered O^{2-} levels and remarkably reprogrammed oxidative status by enhancing observed changes in enzymatic activities in salt-treated plants.[25,26] The polyamine biosynthetic pathway in the plants has been elucidated. It involves the conversion of arginine into ornithine or *N*-carbamoylputrescine, which then converts to putrescine. Putrescine successively converts to spermidine and then spermidine to spermine.[27–29] Despite this progress, it remains unknown how paraquat regulates the polyamine pathway and cross-talk with other stress pathways. This study aimed to clone a putative paraquat-resistant gene, *β-ketoacyl-CoA synthase 11-like* (*EiKCS*), from a paraquat-resistant goosegrass and overexpress *EiKCS* in rice (*Oryza sativa* L.); in others to develop a paraquat resistant rice.

2 MATERIALS AND METHODS

2.1 Goosegrass planting and paraquat treatments

A susceptible (S) biotype of goosegrass was collected from the campus of South China Agricultural University (SCAU) (113°36′E, 23°16′N). The resistant (R) biotype of goosegrass was collected from the Teaching and Research Farm (113°409′E, 22°809′N) in the Panyu district of Guangzhou, China. The susceptible and resistance phenotypes of the two biotypes were confirmed by inhibiting more than and less than 50% growth of plants, respectively. as compared to the untreated controls, and the R biotype showed 59.48-fold higher level of resistance than that of the S biotype.[30] In this study, goosegrass plants of S and R biotypes were transplanted into pots at the 3-leaf stage, with each pot containing five seedlings. The plants were sprayed by a 3WP-2000 spray tower (Nanjing Research Institute for Agricultural Mechanization, Ministry of Agriculture, Nanjing, China) at the 6–9 leaf stage at the recommended rate of 0.6 kg active ingredient (a.i.) ha^{-1} paraquat (Syngenta Corporation, Shanghai, China) in our previous studies.[11,13] After treatment of paraquat at 30, 60, and 90 min, separately collected R and S goosegrass were named R1, R2, R3, S1, S2, and S3. And then, they were frozen in liquid nitrogen and stored in tin foil at −80 °C.

2.2 Construction of KCSox rice

The *EiKCS*-overexpression construct was prepared in a modified pCUbi1390 vector with an ubiquitin promoter from *Zea mays*.[31] And the sequence of the pCUbi1390 vector was confirmed by using a primer 5′-ATGTATAATTGCGGGACTCT-3′ (Nos-R). The total RNA was extracted from R goosegrass leaves under paraquats and reverse transcribed to obtain complementary DNA (cDNA) (Supporting Information, Table S1). Then the cDNA served as a template for amplifying the *EiKCS* gene (1551 bp) by the primer 5′-AATCAGGTCCATCAAGTGTCA-3′ (PqE-F)/PqE-R (5′-GGCG GTGTTATCTTCCCT-3′) (Supporting Information, Fig. S1). Polymerase chain reaction (PCR) (T100™ PCR, Bio-Rad, Santa Rosa, California, USA) (50 μL) was of cDNA (2 μL), primers (2 μL each), dNTP (1 μL), Pfu polymerase (0.4 μL, Sangon Biotech) and Pfu buffer (5 μL) with the following profile: 95 °C 3 min, 35 cycles for 94 °C 30 s, 58 °C 30 s and 72 °C 120 s, followed 72 °C 6 min. After gel extraction, 20 μL purified *KCS* gene (1563 bp) was recombined into the pCUbi1390 vector and digested with BamHI and SpeI. It was placed in a water bath at 37 °C for 3 h with BamHI/SpeI (1 μL each, QuickCut™, Takara, Shiga, Japan), Buffer (10 μL), pCUbi1390 vector (1.5 μg). After gel extraction again, two modified bands of *EiKCS* gene (50 ng) and pCUbi1390 vector (100 ng) were mixed with T4 DNA ligase (1 μL), 10× buffer (2 μL) in total 20 μL at 22 °C for 3 h.

The overexpression pCUbi1390::*EiKCS* vector was transformed into *Escherichia coli* DH5a, which was screened on an LB plate with gentamicin (50 mg mL^{-1}) and kanamycin (50 mg mL^{-1}) solutions. Positive colonies were detected by PCR with primers 5′-TTTAGCCCTGCCTTCATACG-3′ (pCUbi1390-F) and 5′-TTGCGGGAC TCTAATCATAA-3′ (pCUbi1390-R). Plasmid was extracted from *E. coli* cells using the HighPure Maxi Plasmid Kit (Tiangen Biotech, Beijing, China). The pCUbi1309::*EiKCS* overexpression construct were then introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation (1800 V/5 ms). Positive *Agrobacterium tumefaciens* clones were screened on an LB plate with hygromycin (50 mg mL^{-1}) and rifampicin (20 mg mL^{-1}). Calli were induced from mature rice seed embryos of the variety Zhonghua11 (ZH11) (*O. sativa* ssp. *Japonica* L.), which is a commonly-used variety for the verification of transgenic functions in rice.[32–33] *Agrobacterium tumefaciens*-mediated rice transformation was performed as described.[34,35]

2.3 Screening of KCSox rice

The TPS (tris physiological saline) method reduced the workload of extracting DNA from all KCSox rice leaves samples for screening transgenic plants (Figs S2 and S3). PCR amplification was used to detect the presence (1898 bp) of *EiKCS* in KCSox (T_1 and T_2) plants by primers pCUbi1390-F/R, along with the control detection of the hygromycin gene *(hptII)* with 5′-ACGGTGTCGTCCATCACAGTTTGC C-3′ (Hyg-F) and 5′-TTCCGGAAGTGCTTGACATTGGGGA-3′ (Hyg-R). In the T_3 KCSox plants, the presence of *EiKCS* (1468 bp) was further confirmed by another primer pair 5′-ATTGCTAACTTGCCAGTGT

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TTCTC-3′ (PqE-1-F) and 5′-ACGACCAGGATGCCGATGT-3′ (PqE-1-R). PCR was followed the profile of the TIANcombi DNA Lyse&-Det PCR Kit (Tiangen Biotech).

The wild-type (WT) (ZH11) rice and another rice cultivar Ruanhuayou1179 (RHY1179) were used as controls. T_2 KCSox (60 seeds) and WT (RHY1179) (60 grains) were soaked in hygromycin solutions (0, 25, 50, 75, 100, 200, 400, 800, and 1600 mg L^{-1}) for 2 days in a 35 °C incubator. Then 15 seeds of the KCSox plant as oval-shaped rice on the left, and WT (RHY1179) as long-shaped rice on the right, were placed on a filter paper in a culture dish. The germination in water of each rice material was repeated three times (15 grains × 3 repeats = 45 grains for each treatment). The remaining 15 seeds of the KCSox and WT (RHY1179) were planted in soil in a glasshouse at 34 °C/28 °C, 12 h:12 h (day/-night). T_2 KCSox (100 seeds) and WT (ZH11) (100 seeds) rice were soaked in 25 mg L^{-1} hygromycin solution for 2 days. Germination was calculated on the seventh day. After WT (RHY1179) seedlings sprayed with the gradient of hygromycin solutions, the weight of aboveground fresh biomass and photographs were taken on the seventh day.

2.4 Growth assay on paraquat-treatment and exogenous spermidine of KCSox

For evaluating the paraquat-sensitivity of the rice plants, the WT (RHY1179) seedlings were planted in soil in plastic bowls (r = 5 cm) in a glasshouse at 34 °C/28 °C, 12 h:12 h (day/night) (each bowls contained ten plants). At the 4–6 leaf stage, the WT rice seedlings (21 bowls) in seven groups were sprayed with 0, 10, 30, 90, 270, 810, and 2430 mg L^{-1} paraquat solutions. And WT rice seedlings (24 bowls) in eight groups were sprayed with 0, 90, 180, 270, 360, 450, 630, and 810 mg L^{-1} paraquat solutions. Determination of fresh weight was done as mentioned earlier.

For paraquat response, KCSox (T_3) and WT (ZH11) were planted in the bowls (r = 5 cm) in the glasshouse (each bowl contained one plant). At the 6–9 leaf stage, KCSox plants (12 bowls) and ZH11 (12 bowls) with similar growth state were selected. There were nine bowls each for KCSox, and ZH11 plants. The bowls were sprayed with 270 mg L^{-1} paraquat solutions. However, the remaining bowls were used as control and sprayed with double-distilled water (ddH_2O). Their paraquat-resistant phenotypes were photographed at 0, 12, 24, 36, 48, 60, and 160 h (Fig. S4).

To confirm the effect of polyamines on paraquat resistance, the WT (RHY1179) seedlings at 4–6 leaf stage (18 bowls) in six groups were sprayed with 0, 0.5, 1.0, 1.5, 3.0, and 8.0 mmol L^{-1} spermidine solutions (SKU-Pack Size 85 580-5G, Sigma-Aldrich, St Louis, MO, USA). Determination of fresh weight was done as mentioned earlier.

For evaluating the optimum time for prior-treatment with spermidine alleviating paraquat damage, RHY1179 plants were planted in plastic buckets (r = 10 cm) in the glasshouse (each bucket contained 15 plants). At the 6–9 leaf stage, the plants (18 buckets) were divided into six groups for six treatments (Table S2): (i) sprayed with ddH2O as control; (ii) sprayed with 270 mg L^{-1} paraquat 24 h after spraying with 1.5 mmol L^{-1} spermidine (Para24h + spd); (iii) sprayed with 270 mg L^{-1} paraquat 12 h after spraying with 1.5 mmol L^{-1} spermidine (Para12h + spd); (iv) sprayed with 270 mg L^{-1} paraquat immediately after spraying with 1.5 mmol L^{-1} spermidine (Para0h + spd); (v) sprayed with 270 mg L^{-1} paraquat 12 h before spraying with 1.5 mmol L^{-1} spermidine (Spd12h + para); (vi) sprayed with 270 mg L^{-1} paraquat 24 h before spraying with 1.5 mmol L^{-1} spermidine (Spd24h + para). Determination of fresh weight was done as mentioned earlier.

2.5 Endogenous polyamines analysis in the KCSox plants

For investigating the mechanism of paraquat resistance, the KCSox (T_3) and ZH11 plants grown in plastic bowls (r = 5 cm) in a glasshouse at 34 °C/28 °C, 12 h:12 h (day/night) (each bowl contained one plant) were used in the measurement of endogenous polyamines and physiological indices. At the 6–9 leaf stage, the KCSox (42 bowls) and ZH11 (36 bowls) plants were treated with spermidine and paraquat, and paraquat only, for 0, 30, 60, 90, and 180 min.

As a control, seedlings of RHY1179 at the 6–9 leaf stage were treated with pre-spermidine and paraquat (Spd + para), paraquat (Para), and ddH_20 (Ck) for 0, 30, 60, 90, and 180 min. The aboveground parts of the plants were collected as one sample, and immediately frozen in liquid nitrogen and stored in tin foil at −80 °C.

Extraction of free polyamines from the plants and benzoylation of the extracted polyamines was performed according to previous methods with modifications.[28,29] Rice leaves (0.2 g) were pulverized into powder with a mortar and pestle in liquid nitrogen. The powder was collected into a 10-mL centrifuge tube, to which 1.5 mL of 5% (*v/v*) cold perchloric acid (PCA) was added and kept on ice for 1 h. The samples were centrifuged at 10000 × *g* for 30 min at 4 °C. To 1.5 mL of the supernatant collected, 1 mL of sodium hydroxide (NaOH, 2 mol L^{-1}) was added and then shaken. Then, 10 mL of benzoyl chloride was added, mixed, and incubated at room temperature for 20 min. Thereafter, 2 mL of saturated sodium chloride was added to the samples. Then, 2 mL of ether was added and mixed, and the samples were centrifuged at 3000 × *g* at 4 °C for 10 min. A volume (1 mL) of the supernatant was taken and filtered with a syringe (aperture 0.2 mm). The filtrate was then dispensed into a 2-mL sample bottle. The organic solvent phase was evaporated with hot air, and the residue was resuspended in 1 mL of methanol.

The benzylated polyamines were analyzed by high-performance liquid chromatography-mass spectrometry (HPLC-MS, Uplc1290-6470A QQQ, eclipse plus, 2.1 mm × 50 mm, 1.8 um, Agilent, Santa Clara, CA, USA). The mobile phase was 0.2% formic acid aqueous solution and acetonitrile. External standards were used to determine the number of derivatized products of the three polyamine benzoyl chloride products. Standards of putrescine, spermidine, and spermine (SKU-Pack, Sigma-Aldrich) were used to prepare 1.5 mL (1 mmol L^{-1}) spermidine, spermine, and putrescine solution, respectively, then, prepared as 2 mL of ether phase. A volume (0.5 mL) of each ether phase of polyamines was taken and dispensed in a 2-mL sample bottle. To these, 1 mL of methanol was added after the ether solution had volatilized. Based on the linearity and optimum concentrations of the samples, the three derivatized polyamines, putrescine ($C_{18}H_{20}N_2O_2$, 297 > 105), spermidine ($C_{28}H_{31}N_3O_3$, 458 > 162), and spermine ($C_{38}H_{42}N_4O_4$, 619 > 497), were quantified using standard curves (Fig. S5). The limit of polyamines detection was 5 ng mL^{-1}, and the limit of quantification was 1 ng mL^{-1}.

2.6 Physiological indices in KCSox

The scavenging capacity for intracellular ROS and the extent of lipid peroxidation in rice were analyzed in the same materials used for polyamine detections. Rice leaves (0.1 g) were pulverized into powder with a mortar and pestle in liquid nitrogen. All parameters were measured with their corresponding detection kits following the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The total protein content was detected using the Coomassie brilliant blue protein

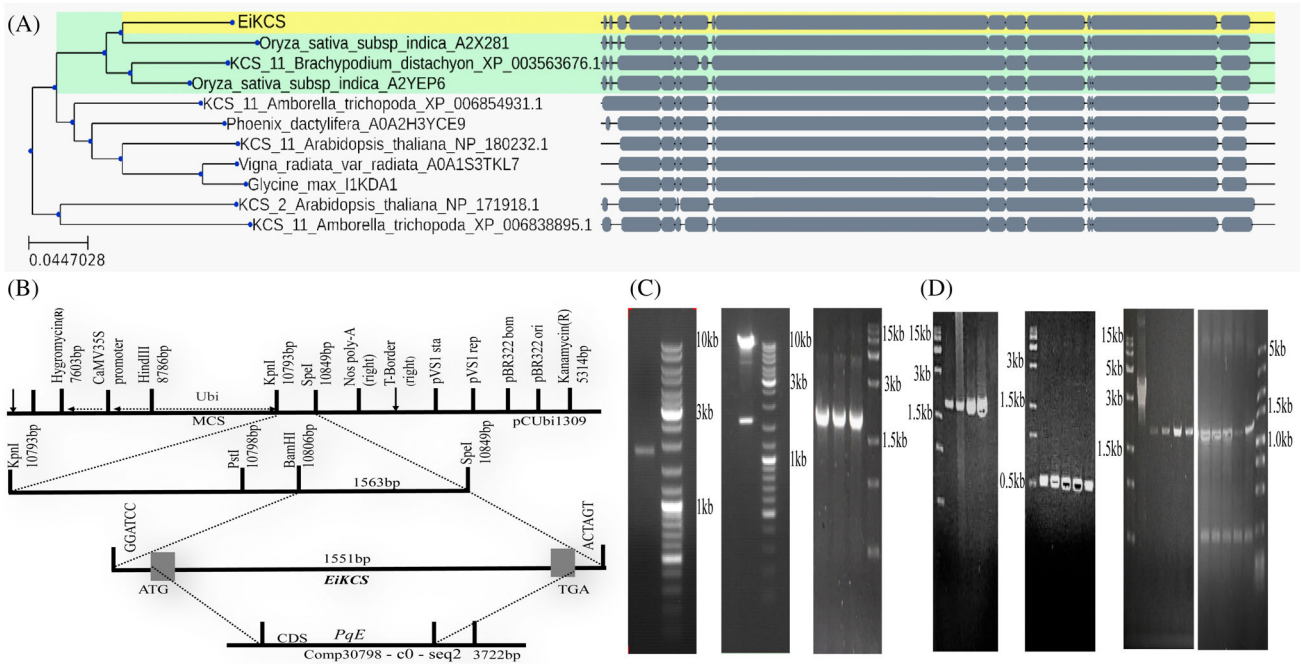

Figure 2. Construction and identification of KCSox rice. (A) An phylogenetic tree of EiKCS and its homologous proteins. The proteins of the genes for transcriptome analysis are colored yellow, and those with the closest relationship are colored green. (B) Genetic map of the pCUbi1309::*EiKCS* vector. (C) PCR amplification of *EiKCS* for construction of the vector. CDS sequence of *EiKCS* was amplified from goosegrass, the vector, and *Agrobacterium tumefaciens* colonies. (D) PCR amplification of T_0 to T_3 plants of KCSox. Hygromycin-resistace marker gene (289 bp) and a sequence (3413 bp) of pCUbi1309::*PqST3* vector were served as control.

determination kit (A045). This was used for the calculation of superoxide dismutase (SOD, A001-1), peroxidase (POD, A084-3), catalase (CAT, A007-1) activities, and malondialdehyde (MDA, A003-1) content in U mg prot^{-1}.[36–37]

To explore the role of EiKCS in the very-long-chain fatty acids (VLCFAs) biosynthesis, the analysis of cuticular wax, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were conducted in KCSox (T_4) followed the methods in a previous report.[38]

To assess the photosynthetic performance of rice after exposure to paraquat, we measured the maximum yield of photosystem II (Fv/Fm) using the previous method.[7] Using the smearing method (Fig. S6 and Table S3), leaves of ZH11 at 4–5 leaf stage were treated with 10 mg L^{-1} paraquat solution (para1) only, 1 mmol L^{-1} spermidine solution immediately after the application of 10 mg L^{-1} paraquat solution (spd + para1), and ddH_2O (Ck1). The flag leaves were randomly detected at 0, 30, 60, 90, and 180 min. Three lines of KCSox and ZH11 at the 4–5 leaf stage were treated with 10 mg L^{-1} paraquat. And the flag leaves were randomly detected at 0, 1, 2, 3, and 4 days. Fv/Fm determination was conducted by a chlorophyll fluorometer (Mini PAM, Waltz, Effeltrich, Germany).

2.7 The EiKCS protein and proteomics analysis of KCSox rice

The protein solution samples were hydrolyzed into peptide segments by trypsin (V9012, Promega (Beijing) Biotech, Beijing, China). The ketoacyl-coenzyme A (CoA) synthase protein encoded by the transgenic *EiKCS* had four unique peptides, which were determined using the Skyline software (v.3.6) for peptide synthesis. After the synthesis, the reductive alkylation experiment was carried out for PRM (parallel reaction monitoring) analysis.

Leaves of three KCSox plants (T_3), and leaves of three KCSox (Z1, Z2, and Z3) plants (T_2), and three plants of ZH11 (W1, W2, and W3) at 6–9 leaf stage (from three individual plants each) were treated with paraquat for 2 days. First, whole protein (2605.7 μg) was extracted from all the KCSox plants were combined as one mixed sample for Label-free proteomics analysis (Fig. S7(a) and Table S4). Second, 50 μg of total protein was separately extracted from Z1, Z2, Z3, W1, W2, and W3 leaves for PRM analysis (Fig. S7(b) and Table S5). Protein extraction was conducted by BCA protein assay kits (Shanghai Beyotime Biotech, Shanghai, China). The process of Label-free assay was performed based on previously reported methods.[39] The process of PRM assay followed as previously described.[40] And two processes were supported by Jingjie PTM BioLabs (China).

2.8 Data analysis

Significant differences between treatments were analyzed by analysis of variance (ANOVA) in Duncan's test. And these were carried out using Excel 2016, SPSS 17.0 (SPSS Inc., Chicago, IL, USA), and DPS 7.05 (Zhejiang, China). Rice Swissprot database (48 915 sequences) and InterPro database were used in the protein identification. WoLF PSORT was used to predict subcellular localization in eukaryotic sequences (https://omictools.com/wolf-psort-tool). Analysis of the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways was carried out according to the KEGG website (http://www.kegg.jp/).

3 RESULTS

3.1 Construction of the KCSox rice and confirmation of overexpression

A significantly increased transcript level of the *EiKCS* gene, which was previously named the *PqE* gene, was observed in a paraquat-resistant goosegrass under paraquat stress, as compared to the expression in susceptible goosegrass.[13] Phylogenetic analysis

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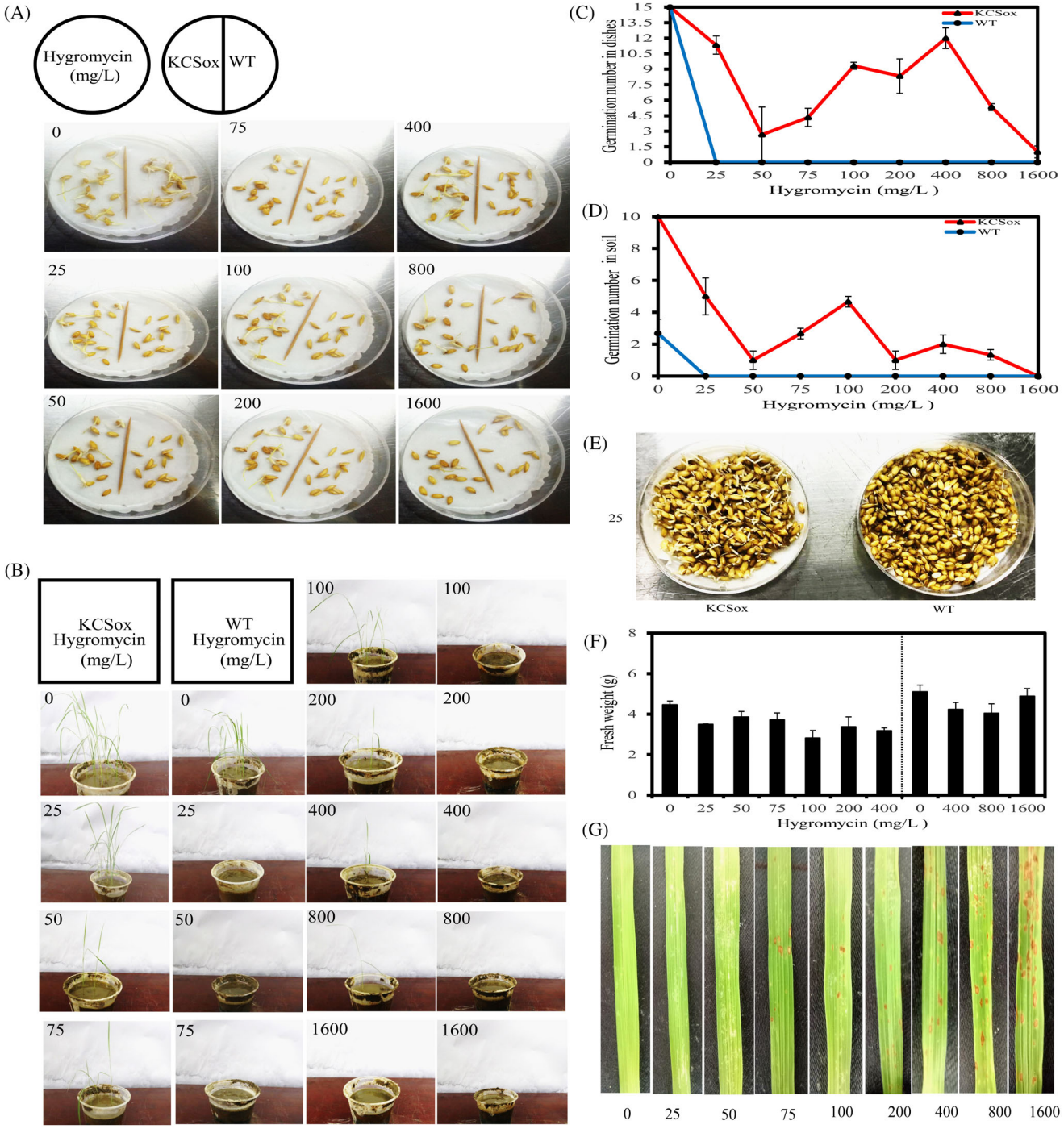

Figure 3. Screening KCSox rice progenies (T_2) by hygromycin assay. (A,B) Phenotypes of KCSox and WT seeds after hygromycin treatments. (C,D) Germination of KCSox and WT seeds after hygromycin treatments (three replicates). (E) Phenotypes of KCSox and WT seeds after hygromycin treatments. (F) The weight of aboveground fresh biomass of WT after hygromycin treatments (three replicates). (G) Phenotypes of WT leaves after hygromycin treatments.

showed that *EiKCS* was closest to the *KCS* gene (EC 2.3.1.199) in rice (*O_sativa*_subsp_indica_A2X281) (Fig. 2(A)). Thus, the CDS sequence of *EiKCS* (1551 bp) was amplified from the resistant goosegrass (Fig. 2(B)) and overexpressed (driven by the maize ubiquitin promoter) in the transgenic plants (KCSox) of the *japonica* rice (Fig. 2 (C)). The transgenic *EiKCS* gene and the T-DNA (carrying the *HPT* marker gene) in each generation of KCSox plants were confirmed by PCR (Fig. 2(D)) and by hygromycin-resistance assay (Fig. 3(A–D)).

The germination of KCSox seeds in the culture media after soaking in hygromycin solution (25 mg L^{-1}) for 2 days could effectively eliminate the non-transgenic seeds (Fig. 3(E)). This is also applicable in a screening of genetically modified organism (GMO) progenies with the *Hygromycin* marker gene. In addition, exogenous hygromycin was not applicable on rice leaves for screening because it inhibited the nutritional growth of rice and caused damages on leaves (Fig. 3(F,G)).

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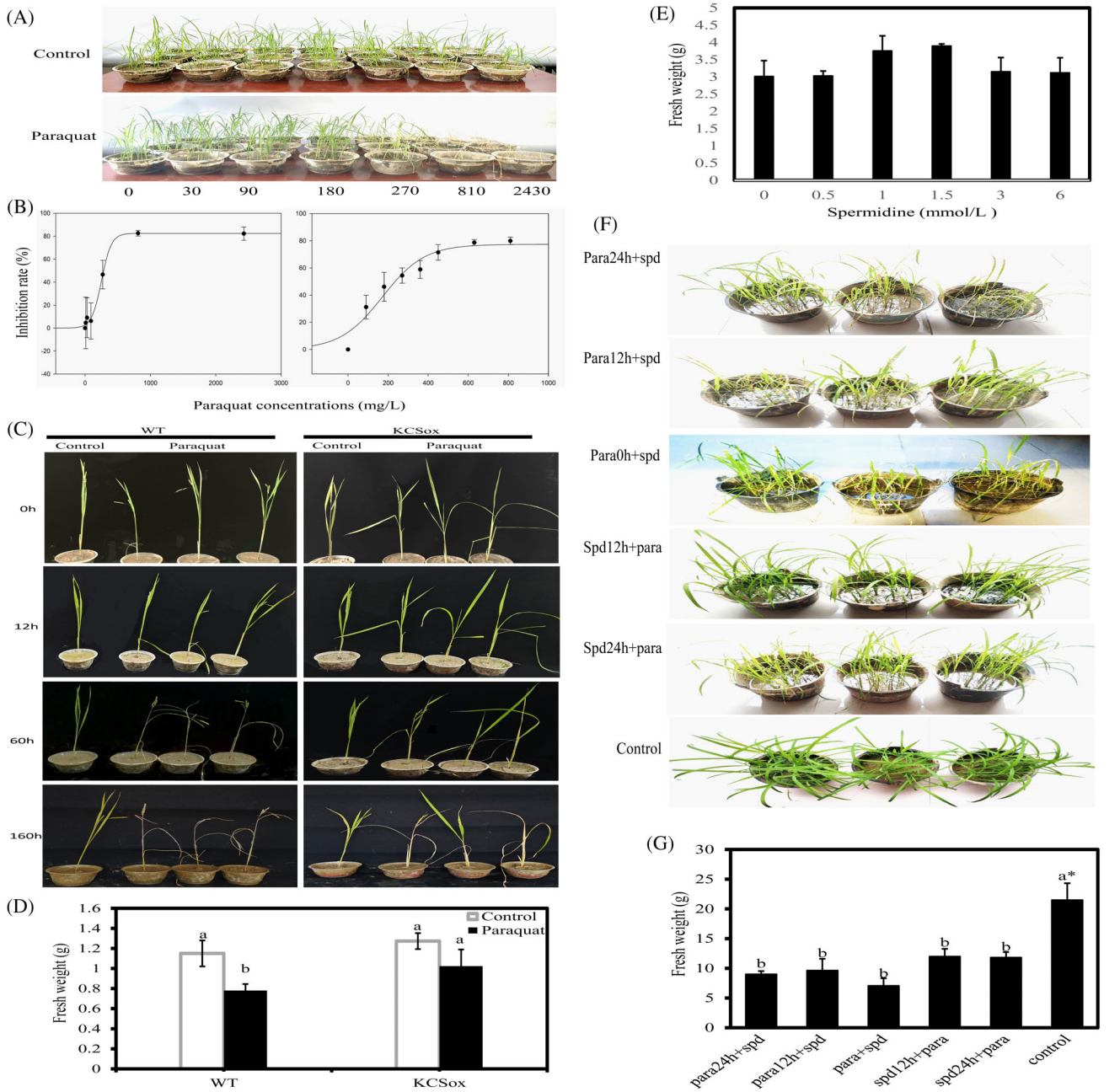

Figure 4. Paraquat-resistance of KCSox rice (T_3). (A) The phenotypes of WT after paraquat treatments. (B) Inhibition rates of WT after paraquat treatments (three replicates). (C) The phenotypes of KCSox and WT after paraquat treatments. (D) Fresh weights of KCSox and WT after paraquat treatments. Each had nine replicates under the paraquat treatment and three replicates of the mock (ddH_2O). (E) Fresh weights of WT after spermidine treatments (three replicates). (F) The phenotypes of WT after paraquat with spermidine treatments. (G) Fresh weight of WT after applying paraquat and spermidine (three replicates). Different letters indicate significant differences at $P < 0.05$. Asterisk (*) indicates significant differences at $P < 0.01$.

3.2 KCSox or pre-applied exogenous spermidine confers resistance to paraquat

Paraquat caused visible withering of WT leaves (Fig. 4(A)). And the discriminatory dose of paraquat to WT rice reached 54.42% at 270 mg L^{-1}, which were assessed by two gradients of paraquat solution (Fig. 4(B)). The KCSox plants showed resistance to paraquat, while the growth of WT rice was significantly inhibited after spraying paraquat at this concentration (270 mg L^{-1}) (Fig. 4(C)). The fresh weight of aboveground biomass of WT rice was significantly decreased by 32.22% compared to the mock (with ddH_2O) ($P < 0.05$), while that of the KCSox plants was decreased by 19.57% compared to the mock (Fig. 4(D)). Therefore, the results showed that the *EiKCS* gene could confer paraquat resistance in rice under the same background. Meanwhile, the exogenous spermidine also enhanced the resistance to paraquat in rice. Exogenous spermidine application promoted a maximum growth of WT leaves at 1.5 mmol L^{-1} and alleviated the damage of paraquat to the best at pre-applied 12 h (Fig. 4(E,F)). This was when the fresh weight decreased the least (Fig. 4(G)). Therefore, both KCSox or pre-applied exogenous spermidine conferred resistance to paraquat. To further study the mechanism of paraquat-resistance in KCSox relating to polyamines, these optimal treatment conditions were

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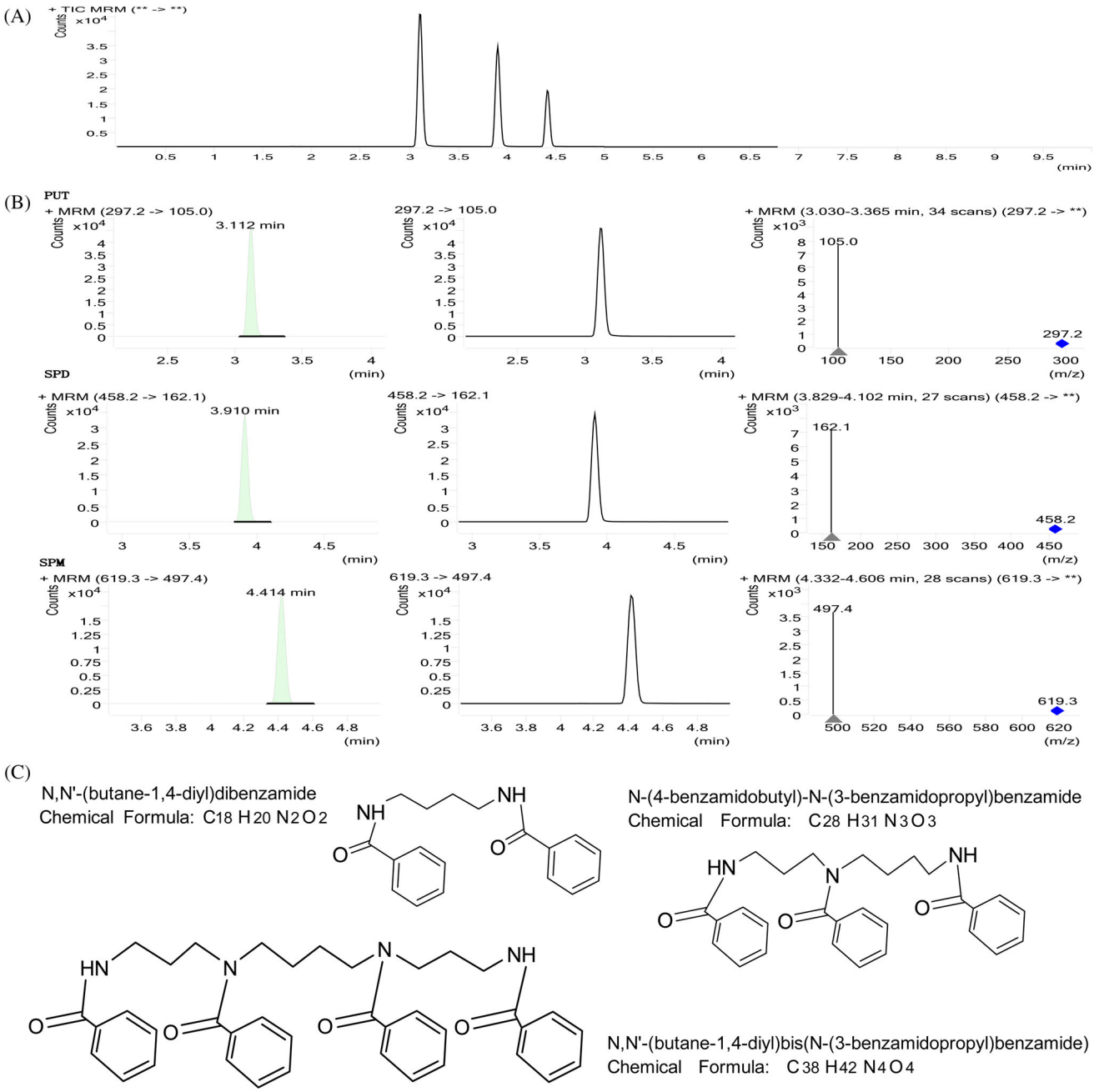

Figure 5. Confirmation of three polyamines extracted from rice plants. (A) The retention time of the standard of the polyamines by HPLC. (B) The retention time and the exact mass of the polyamines from rice leaves by HPLC-MS/MS. (C) Molecular structure of the polyamines identified in rice after derivatization.

used to determine endogenous polyamines, ROS, MDA, Fv/Fm, and proteins associated with the polyamine pathway.

3.3 Overexpression of *EiKCS* increases endogenous polyamines in KCSox

Total free polyamines extracted from KCSox and WT rice leaves were similarly derivatized and analyzed by HPLC. After benzoylation, the retention times of the peaks of the polyamine standards and the polyamines from the rice samples were consistent at *c.* 3.112 min (Put), 3.910 min (Spd), and 4.414 min (Spm) (Fig. 5(A)). We further carried out a quantitative analysis of the rice polyamines by HPLC-MS/MS to confirm this. The results clearly showed that the exact mass of the polyamine derivatization products was 297.2 (Put), 458.2 (Spd), and 619.3 (Spm) for the standards and rice samples (Fig. 5(B)). To further validate this method, all the possible molecular formulas of polyamines benzoylation derivatizations were analyzed. There are nine possible molecular structures for polyamines derivatizations. Because amidation of derivatizations is a classical reaction, in which each N site of polyamines may bind to benzene ring when acyl chloride reacts with amide completely. The HPLC-MS/MS results unequivocally showed that the molecular structures of the polyamine derivatization products were $C_{18}H_{20}N_2O_2$ (Put), $C_{28}H_{31}N_3O_3$ (Spd), and $C_{38}H_{42}N_4O_4$ (Spm) in rice samples (Fig. 5(C)). This confirmed that benzoylation reaction occurred at all N-sites, and the quantitative analysis method employed was correct.

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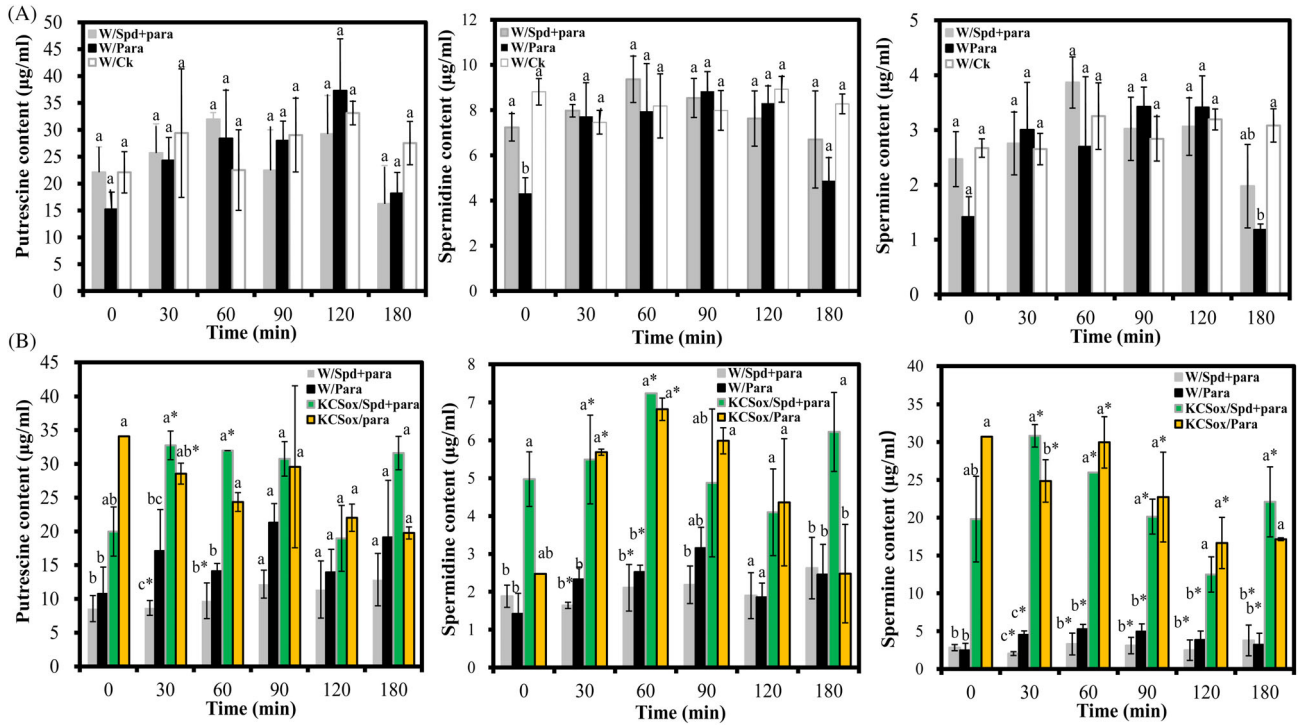

Figure 6. Quantitative analysis of endogenous polyamines in KCSox and WT rice. (A) Endogenous content of polyamines in WT under treatments of pre-spermidine and paraquat (Spd + para), paraquat (Para), and ddH_2O (Ck). (B) Endogenous content of polyamines in KCSox (T_2) and WT under treatments of pre-spermidine and paraquat (Spd + para), paraquat (Para), and ddH_2O (Ck). KCSox under the pre-spermidine and paraquat treatment had four replicates and others had three replicates. Different letters indicate significant differences at $P < 0.05$. Asterisk (*) indicates significant differences at $P < 0.01$.

Next, the endogenous polyamines contents of KCSox and WT rice leaves were quantitatively measured under the following treatments: pre-spermidine and paraquat, paraquat, and ddH_2O (mock). The contents of all three endogenous polyamines significantly increased in the KCSox rice than in WT rice under the same treatments ($P < 0.1$) (Fig. 6(A,B)). This showed that the *EiKCS* transgene was related to polyamine biosynthesis. Furthermore, the levels of polyamines in KCSox increased at different times. Putrescine and spermidine significantly increased within 30–60 min after both pre-spermidine and paraquat, paraquat treatments. But spermine increased within 0–180 min after both pre-spermidine and paraquat, paraquat treatments ($P < 0.01$). Consistent with this, the spermine content remarkably increased to a high level of 30 μg mL^{-1} in KCSox under both paraquat only and paraquat with pre-spermidine treatments compared to its level of < 5 μg mL^{-1} in the WT. The prolongation of the overproduction of spermine in the KCSox rice might be due to the transformation of endogenous putrescine and spermidine to spermine in the plant polyamine pathway.

3.4 Overexpression of *EiKCS* enhanced antioxidant capacity in rice

Different physiological indices such as the antioxidant enzymes of the ROS system, MDA, and Fv/Fm were also measured for the same rice samples, except for the endogenous polyamine assay. The total protein content in KCSox under paraquat treatment significantly decreased compared to that of WT (Fig. 7(A,G)). And the lipid peroxidation product in KCSox leaves induced by paraquat was evaluated in terms of the MDA level. In KCSox, the MDA content instantly increased 351.62-fold and subsequently decreased after 90 min. However, no changes in MDA level were detected in WT rice (Fig. 7(B,H)). The MDA accumulation may be explained by the role of EiKCS in the VLCFAs biosynthesis. The content of C_{19} and C_{21} fatty acids ($C_{19}H_{40}O_2Si$ and $C_{21}H_{44}O_2Si$) in KCSox was increased by 58.94 and 55.31 ($P < 0.05$), used C_{24} as an internal standard (Fig. 8(A)). Those compositions were all reduced in KCSox after paraquat treatment but no difference was observed in the VLCEAs-forming compositions. The VLCFAs also act as the substrate for the synthesis of wax. In this study, KCSox rice significantly differed in wax phenotypes (Fig. 8(B–E)). The phenotypes in inverse difference showed that total wax content extracted from KCSox rice was less by SEM and TEM compared to WT rice (Fig. 9(A,B)). It indicates that the *EiKCS* transgene may not be related to synthetic wax or not need the CER cofactors to form a cuticular barrier. Thus, these implied that wax formation could not be a major contributor to the enhanced paraquat resistance in the KCSox rice.

Also, the chlorophyll fluorescence parameter (Fv/Fm) in KCSox was higher than that in WT under paraquat treatment. This indicated that EiKCS might promote the light-harvesting in photosynthesis (Fig. 7(C,I)). As the biomarker of the antioxidant defenses, the activities of SOD, POD, and CAT (per unit protein) in KCSox showed great increases throughout the trial period than in the WT (Fig. 7(D–F,J–L)). In particular, the activities of SOD and POD in KCSox increased significantly 12.52-fold and 7.39-fold higher (at 60 min) and 44.64-fold and 28.40-fold higher (at 90 min) respectively (Fig. 7(J, K)). Thus, overexpression of the *EiKCS* gene enhanced the antioxidant capacity in KCSox. These results clearly demonstrated that the overexpression of *EiKCS* in rice combated the oxidative stress induced by paraquat. Exogenous spermidine could advance the increase of these physiological indices in KCSox to 30 min. Again, it suggested that *EiKCS* may function to polyamine biosynthesis.

3.5 Identification of the EiKCS protein and its cofactors CERs in rice

To further study the mechanism of paraquat-resistance in KCSox relating to polyamines, an overview of proteomic analysis was

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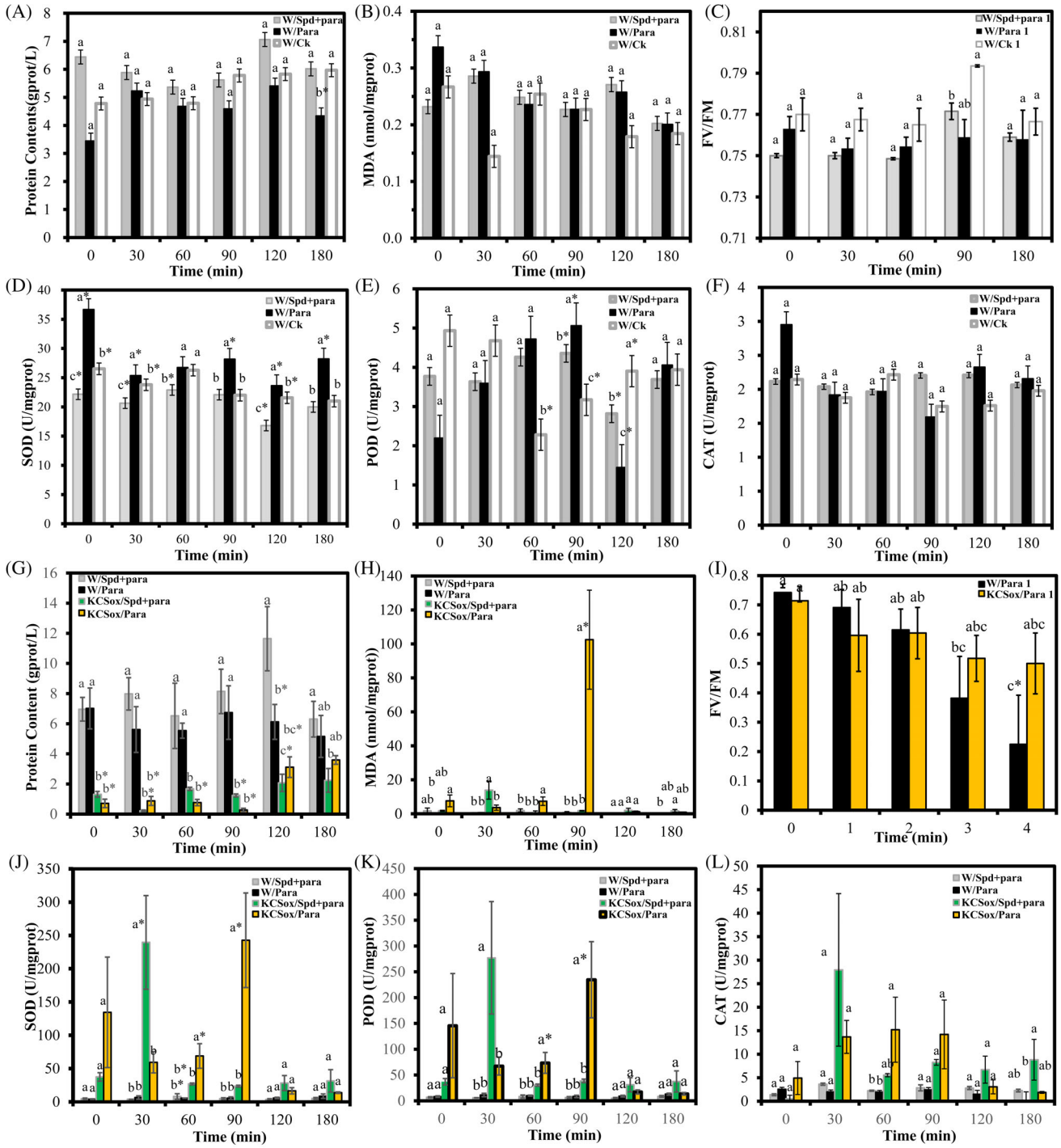

Figure 7. Different physiological indices of the KCSox and WT rice. (A,B,D–F). Total proteins and MDA contents, and the activities of SOD, POD, and CAT in the WT rice under treatments of pre-spermidine and paraquat (Spd + para), paraquat (Para), and ddH_2O (Ck) (three replicates). (G,H,J–L) The total protein and MDA contents, and the activities of SOD, POD, and CAT in both KCSox (T_2) and WT under treatments of pre-spermidine and paraquat (Spd + para), paraquat (Para), and paraquat (Ck) (three replicates). KCSox under the pre-spermidine and paraquat treatment had four replicates and others had three replicates. (C,I) Chlorophyll fluorescence (Fv/Fm) of KCSox and WT under treatments of pre-spermidine and paraquat (Spd + para1), paraquat (para1), and ddH_2O (Ck1) (three replicates). Different letters indicate significant differences at $P < 0.05$. Asterisk (*) indicates significant differences at $P < 0.01$.

performed on mixed samples of KCSox and WT respectively under the paraquat treatment (three replicates) using Label-free qualitative proteomics. The results showed that 4035 proteins were identified, which were predicted to be related to the chloroplast (43.82%), cytoplasm (25.53%), and nucleus (14.47%) (Fig. 10(A)), including the light-harvesting chlorophyll proteins (LHC) and subunits of the core complex of PSI (Fig. 10(B,C)). This might explain that paraquat affected the energy capture and electron transport-chain of the photosynthesis process in rice to the ROS response induced by paraquat stress, consistent with differences recorded for Fv/Fm. However, it could not be confirmed that if the identified KCS protein in KCSox corresponded to the

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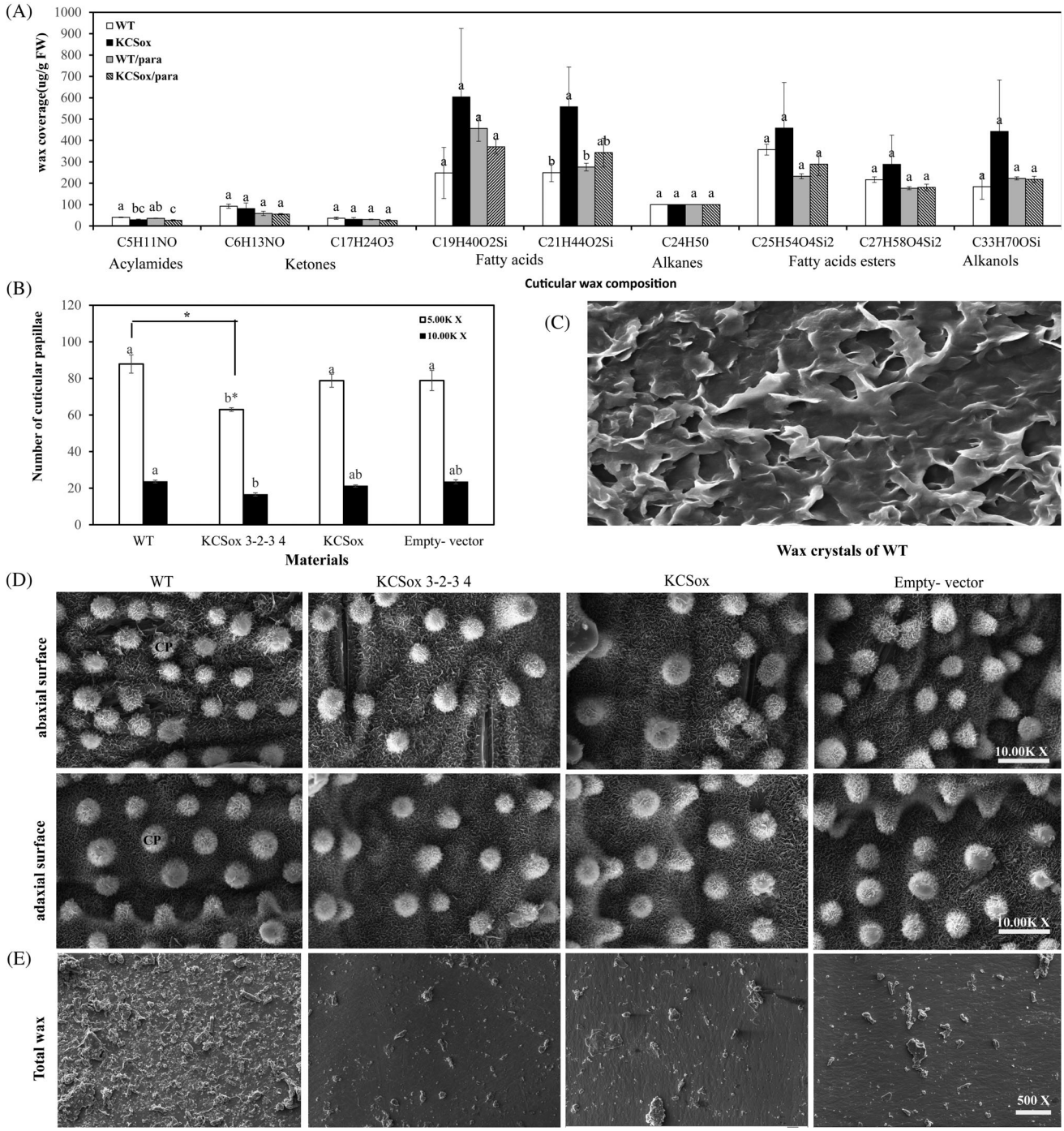

Figure 8. The analysis of cuticular waxes in rice leaves. (A) Compositional content of main wax components in T_3 generation KCSox and WT rice at 6–9 leaf stage sprayed with 270 mg L^{-1} paraquat solution (para) and ddH_2O as a control for 2 days. The $C_{24}H_{50}$ in alkanes is 20 μg tetracosane (C_{24}) as the internal standard. (B) Number of cuticular papillae of rice materials on each fixed view is counted. Each material had more than six replicates of views. Different letters indicated different significances in 5.00K× or 10.00K× using Tukey's honestly significant difference test ($P < 0.05$) among all treatments, and asterisk (*) indicates significant differences ($P < 0.01$). (C) Wax on 50.00K× of SEM. (D) Cuticular wax formed by WT rice and KCSox and empty vector (Control) visualized by SEM with 10.00K×, on the adaxial and the abaxial surface of the flag leaf. CP indicated cuticular papillae. (E) Total wax extracted from 0.2 g flag leaves of rice materials visualized by SEM with 500×.

overexpressed *EiKCS* transgene. Thus, non-isotopic labeled peptides for EiKCS were designed and synthesized based on the protein sequence encoded by *EiKCS* (Table 1). Two unique peptides, SGLGEETYLPAAVLR and CFGCVTQEEDGEGR, were confirmed to be useful for specific and quantitative detection of EiKCS protein content (Fig. 10(D)). Based on Blast analysis in the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/), there are two EiKCS homologues (Q2R3A1 and Q0J9M3) in rice. The Q0J9M3 protein was detected in the proteomics analysis of this study (Fig. 10(E)). Similarly, the three CERs cofactors of the EiKCS protein, Q9FTH0, Q9LGQ6, and Q9FTG9, were also identified in rice (Fig. 10(F–H)), based on the homologous CERs

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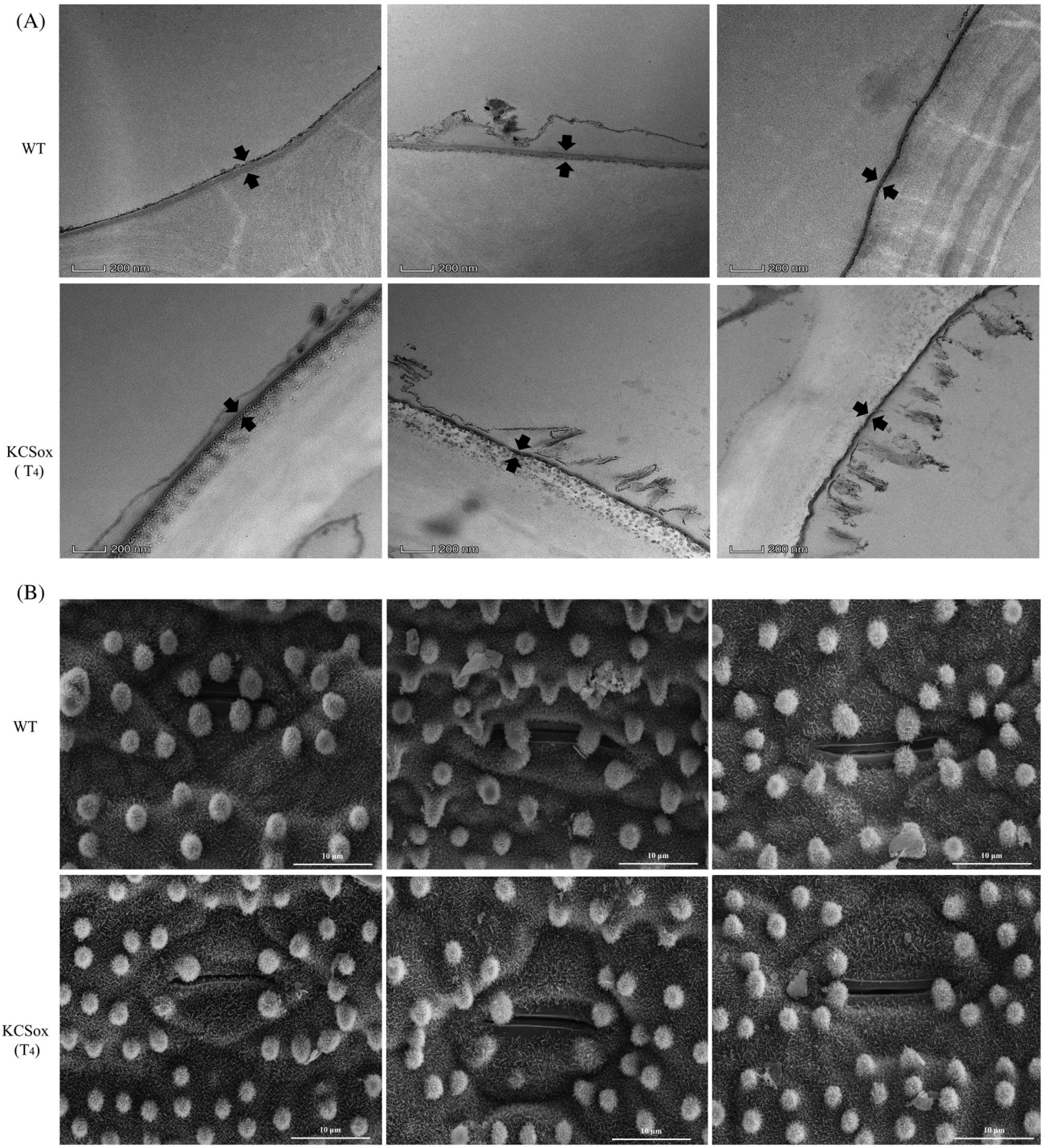

Figure 9. The phenotype of cuticular waxes in rice. (A) TEM analysis of leaf cuticle membranes in T_4 generation KCSox and WT rice. Black arrowheads indicate the zone of cuticle membranes. Bars = 200 nm. (B) SEM analysis of leaf cuticular stomata in T_4 generation KCSox and WT rice. Bars = 10 μm.

sequence (OsCER2, Accession numbers Os04g0611200) previously reported.[42]

3.6 Polyamine pathways were promoted in KCSox rice under paraquat stress

Quantitative proteomics analysis was performed to measure the expression levels of KCS proteins, CERs cofactors, and proteins associated with the polyamine pathway and other cross-talk pathways by PRM (Table 2). The results showed different expression levels of the 15 target proteins of KCSox and WT (three replicates) under paraquat treatment (Fig. 11(A)). The level of the KCS proteins was 51.81-fold higher in KCSox compared to WT. This suggested an overexpression of the *EiKCS* transgene at the protein level in KCSox under paraquat stress. There were also high increases in the levels of the CERs cofactors in KCSox, which were Q9FTG9 (1.41-fold), Q9LGQ6 (1.61-fold), and Q9FTH0 (1.98-fold). This positive correlation between the expression levels of the three CERs cofactors and EiKCS confirmed their function in

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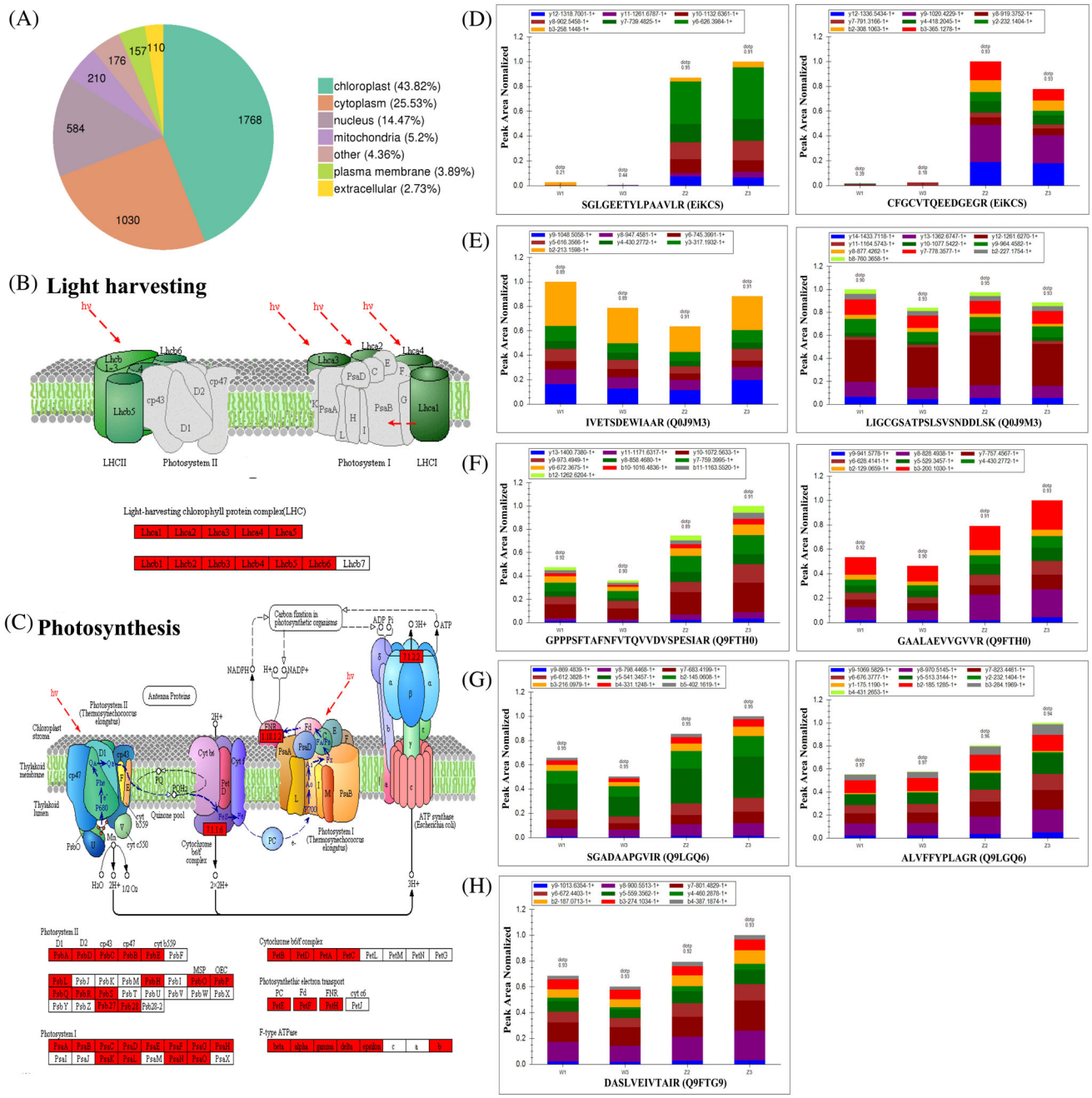

Figure 10. Identified proteins of rice under paraquat by Label-free proteomic. (A) Subcellular localization of identified proteins under paraquat treatment. (B,C) Schematic representation of light-harvesting and photosynthesis in photosystems I and II in rice under paraquat treatment. (D) The distribution of fragmentation peak area for the identification of the EiKCS protein based on two synthetic peptide segments. (E) The distribution of fragmention peak area for identification of the homologous rice proteins (Q0J9M3) of the EiKCS protein based on two unique peptide segments. (F–H) The distribution of fragmention peak area for the identification of the cofactor CERs protein (Q9FTH0, Q9LGQ6, and Q9FTG9) based on their unique peptide segments.

Table 1. Designed unique peptide segments of EiKCS for synthesis

Peptide list of protein synthesis of Ei-ketoacyl-coenzyme A synthase (EiKCS)	
1	LTGSFTDASLDFQR
2	SGLGEETYLPAAVLR
3	CFGCVTQEEDGEGR
4	VGVSLSR

assisting EiKCS in catalyzing VLCFAs into the wax. Further, the expression levels of the key proteins involved in regulating polyamines synthesis also increased, which were Q7X7N2 (1.12-fold), Q93XI4 (1.32-fold), and Q9SMB1 (1.10-fold). This directly promoted the formation of putrescine and its conversion to spermidine and spermine. The expression levels of the proteins Q69P84 (1.15-fold), Q9FRX7 (1.20-fold), B9F3B6 (1.24-fold), and O04226 (1.13-fold), which are mainly associated with the metabolism of putrescine and spermidine, were enhanced. In contrast, that of proteins such as Q6KAJ2 (0.93-fold), B9FK36 (0.84-fold),

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Table 2. Information of 15 targeted proteins with functional description

Protein accession	Gene name	Subcellular localization	Protein description	KEGG gene	KEGG KO number	KEGG pathway
Q7X7N2	*ARG1*	Cytoplasm	Arginase 1, mitochondrial	EC 3.5.3.1	K01476	osa00330 Arginine and proline
Q6ZG77	*LYSA*	Chloroplast	Ornithine decarboxylase	EC 4.1.1.20	K01586	osa00300 Lysine biosynthesis
Q93XI4	*CPA*	Cytoplasm	N-carbamoyl putrescine amidase	EC 3.5.1.53	K12251	osa00330 Arginine and proline metabolism
Q9SMB1	*SPDSYN1*	Cytoskeleton	Spermidine synthase 1	EC 2.5.1.16	K00797	osa00330 Arginine and proline metabolism
Q69P84	*OJ1344_B01.27–1*	Chloroplast	Aldehyde dehydrogenase family 7 member A1	EC 1.2.1.3	K14085	osa00071 Fatty acid degradation and osa00330
Q9FRX7	*Aldh2b*	Mitochondria	Aldehyde dehydrogenase (NAD^+)	EC 1.2.1.3	K00128	osa00071 Fatty acid degradation and osa00330
B9F3B6	*ALDH5F1*	Chloroplast	Succinate-semialdehyde dehydrogenase	EC 1.2.1.24	K17761	osa00250 Alanine, aspartate and glutamate metabolism
O04226	*P5CS1*	Chloroplast	Delta-1-pyrroline-5-carboxylate synthase 1	EC 1.2.1.41	K12657	osa00330 Arginine and proline metabolism
Q6KAJ2	*Os02g0797500*	Chloroplast	Aspartate aminotransferase	EC 2.6.1.1	K00811	osa00330 Arginine and proline metabolism
B9FK36	*ACC2*	Chloroplast	Acetyl-CoA carboxylase 1	EC 6.4.1.2	K11262	osa00061 Fatty acid biosynthesis
Q0J9M3	*Os04g0643300*	Chloroplast	3-Oxoacyl-[acyl-carrier-protein] synthase III	EC 2.3.1.180	K00648	osa00061 Fatty acid biosynthesis
EiKCS	*EiKCS*	Cytoplasm	3-Ketoacyl-CoA synthase 11	EC 2.3.1.199	K15397	ko00062 Fatty acid elongation
Q9FTG9	*Os01g0615300*	Cytoplasm	3′-*N*-debenzoyl-2′-deoxytaxol *N*-benzoyl transferase		Cofactors	
Q9LGQ6	*AT9*	Cytoplasm	Acyl transferase 9			
Q9FTH0	*Os01g0615200*	Endoplasmic reticulum	10-Deacetylbaccatin III-10-*O*-Acetyl transferase-like			

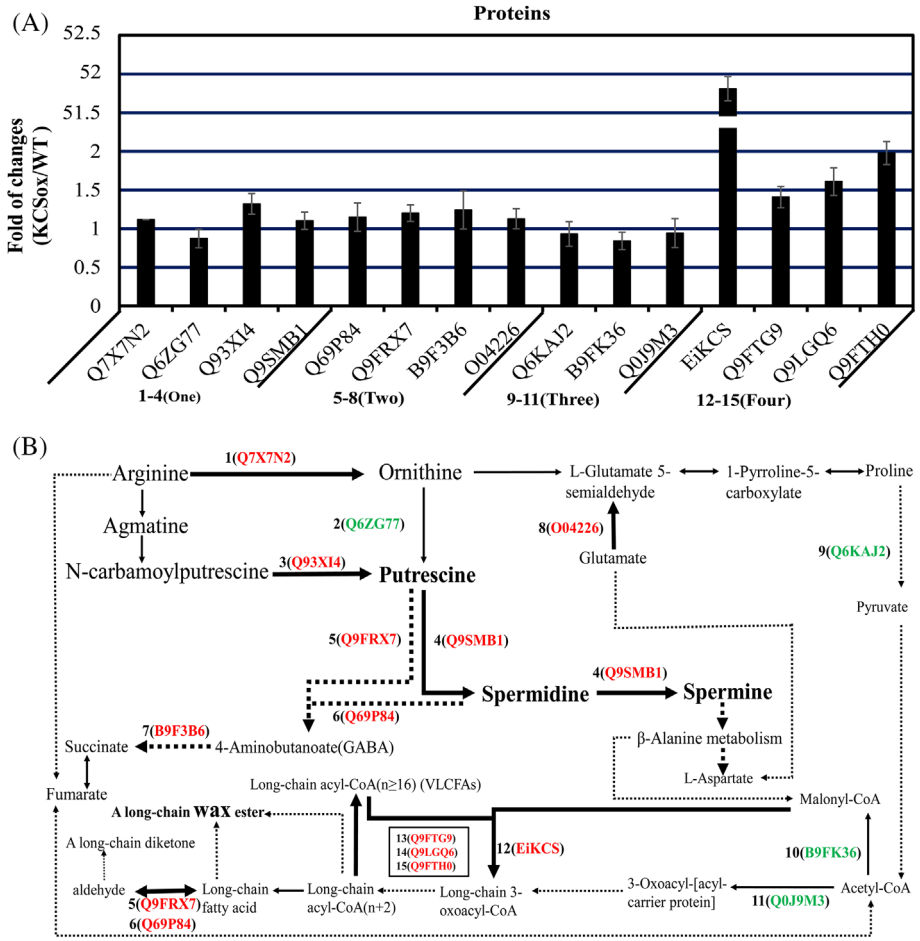

Figure 11. Expressions of proteins in KCSox related to polyamines pathways under paraquat. (A) The fold of changes in the levels of 15 selected target proteins is shown as two logarithmic values of the ratio of KCSox/WT in KCSox and WT by PRM (three replicates). Group 1 is of polyamine biosynthesis pathway, group 2 is of the polyamine metabolism pathway, group 3 is of TCA and fatty acid biosynthesis pathways, and group 4 is of fatty acid elongation. (B) The schematic representation of the 15 selected target proteins in the polyamine pathway and cross-talk with other pathways. Red-marked proteins with a bold indicator line indicate up-regulation, and green-marked proteins with a thin indicator line indicate down-regulation. The solid line represents direct regulation and the dotted line represents indirect regulation.

and Q0J9M3 (0.94-fold), which link the metabolism of the polyamines to EiKCS via fatty acid pathway and tricarboxylic acid (TCA) pathway, were decreased or not affected significantly.

The KEGG pathway database was used to map the pathways of the 15 proteins related to polyamines to illustrate their changes from their interactions with EiKCS (Fig. 11(B)). It showed that the overexpression of *EiKCS* promoted the synthesis of putrescine by the arginine decarboxylase (ADC) pathway rather than the ornithine decarboxylase (ODC) pathway in rice, for the reason that the expression level of Q6ZG77 (ODC) decreased 0.87-fold. Also, that Q9SMB1 (spermidine synthase I) promoted the unidirectional transformation from putrescine to spermidine. Then, spermidine was transferred to spermine, consistent with the overproduction of endogenous polyamines in the KCSox rice. The up-regulation of Q9FRX7 and Q69P84 separately enhanced the metabolism of putrescine and spermidine, explaining the initial increase and then decrease of spermidine content and the delayed accumulation of spermine. Moreover, the proteins Q9FRX7 and Q69P84 also play functional roles in the fatty acid degradation pathway by directly regulating the conversion of long-chain fatty acids to aldehydes. This may explain the transient increase in the MDA contents in rice under paraquat stress. In summary, overexpression of EiKCS protein promoted the polyamine pathway and inhibited its links to the fatty acid pathways to respond to the paraquat stress in the KCSox rice.

4 DISCUSSION

This study using these paraquat-resistant goosegrass accessions to identify a novel paraquat resistance gene (*EiKCS*) involved in non-target resistance mechanisms, such as endogenous polyamines and antioxidant activities. Similarly, a transporter gene ABCC8 was found from a weed species, endowing a non-target resistance of glyphosate in crops.[41] In glyphosate-resistant cropping systems, paraquat is used as an alternative herbicide to control resistant weeds.[42] But paraquat is only labeled preplant in rice for the sensitivity of rice to off-target paraquat movement, and pre-applied exogenous spermidine can aid rice recovery from paraquat, compared to nitrogen fertilizer programs following rice exposure to a sub-lethal concentration of paraquat.[43] The gene *EiKCS* confers paraquat-resistance in rice could deal with paraquat-injury for rice producers. Overexpression of *EiKCS* in rice increased antioxidant enzymes to scavenge ROS, consistent with loss of *OsPQT3*, *AtPQT3*, and *AtPDR11* from paraquat resistance

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mutants.[44] These results may explain that polyamines metabolized paraquat based on their chemistry homology and interacted with the ROS generated by paraquat.

This study provides the latest results from manipulating the polyamine biosynthetic pathway in cereals using genetic engineering since it was reported 20 years ago.[45] Molecular formulae of the dominant benzoylated polyamines in rice were further derived in this study based on other previous reports.[28] The overexpression of the *EiKCS* gene, which significantly increased endogenous polyamines in rice, can be used to improve paraquat resistance in crops and shows great potential in medical and industrial applications.

In human medicine, it is known that polyamines treat cancer by the designing of anticancer agents, and polyamine-regulated genes are worth being identified in links of carcinogenesis or apoptosis.[46] The spermine and spermidine in tumor cells can inhibit cellular apoptosis.[47] The polyamines are synthesized from arginine and proline metabolism, and the polyamine pathway is a potential target for cancer chemoprevention.[48,49] The gene of ODC in the polyamine pathway making polyamine synthesis associated with cancer polyamine function for the therapeutic intervention.[50,51] We propose that *EiKCS* in plants related to polyamine synthesis may be of medicinal value. For example, the ectopic expression of a *KCS* gene from *Cardamine graeca* in *Brassica* oilseeds produces more nervonic acid oils that are beneficial for human and animal health.[52]

In addition, this study demonstrated that the polyamine metabolic pathway was associated with the enhanced paraquat-resistance in the KCSox rice, providing new evidence for the interaction of the polyamine metabolic pathway with other metabolic routes.[27] The polyamine synthetic pathway in plants showed that putrescine was synthesized via ODC or ADC.[29] In *Arabidopsis thaliana*, the lack of an *ODC* gene in its genome causes the absence of ODC.[28] In this study, both ADC and OCD were found, but putrescine is mainly synthesized through the ADC pathway in the KCSox rice under paraquat stress.

Moreover, significant expression variations of 15 proteins involved with polyamine metabolism were detected under paraquat stress. And the folds of the expression changes were much higher than those detected by the same quantitative proteomic methods in a previous study.[53] The increased expression of the proteins well explained the accumulation of polyamines, especially the Q9SMB1. And the lack of spermine metabolism protein could infer that spermine accumulation in rice was the main response to paraquat stress. The transient increase of MDA content in the KCSox rice could infer that MDA forming was related to fatty acid degradation affected by the *KCS* gene. Also, we discovered an association between the EiKCS protein and three CER cofactors in rice, which expanded the understanding of the function of CERs in VLCEAs extension in other species.[54–57]

By now, 21 *KCS* genes have been identified in the *Arabidopsis* genome, but their substrates are still unknown. It is well recognized that the same kind of KCSs can catalyze the acyl extension of VLCEAs with different chain lengths, and different KCSs can catalyze substrate of the same structure.[58] *WSL1* encodes β-ketoacyl CoA synthetase (KCS) in rice, and the content of VLCEAs C20-C24 decreased significantly in leaf and sheath of the *wsl1* mutant.[59] Osgl1-1/WSL2 was found to be homologous to CER3 in *Arabidopsis* and GL1 in maize.[38] Another *KCS* gene, *WSL4*, was found in rice, whose defective mutation leads to loss of wax crystals, while overexpression leads to increased wax content.[60,61] Meanwhile, the gene encoding KCS in FAE1 mutation was identified in *Brassica napus* L., which caused accumulation of both C20 and C22 fatty acids.[62] A *HvKCS6* gene homologous to *AtCER6* (*AtKCS6/AtCUT1*) was identified in a barley mutant, which changed sensitivity responding to water limitation.[63] And 33 genes in barley from the *KCS* gene family were identified in an annotation map of the KEGG metabolic pathway, which had up-regulated or down-regulated trends under drought stress.[64,65]

Although the paraquat-tolerant KCSox rice showed similar effects on proteins involved in photosynthesis and light-harvesting processes with a mesosulfuron-methyl resistant *Alopecurus aequalis*,[40] no significant decline was observed in the yield and quality of the KCSox rice (Tables S6 and S7). The 1000-grain weight and grain filling rate were not affected by water limitation as a drought treatment in the field. All these traits give the *EiKCS* gene a niche as a candidate gene for engineering GMO crops. Its overexpression provides crops with enhanced paraquat resistance and other potential benefits without decreasing yield and quality.

5 CONCLUSIONS

We successfully cloned *EiKCS* gene from the paraquat-resistant goosegrass and transformed it into rice plants. Its overexpression and characterization demonstrated that *EiKCS* conferred paraquat-resistance in rice. This study provided useful insight for further functional studies of genes in GMO that show resistance to non-selective herbicides.

ACKNOWLEDGEMENTS

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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