Enhanced expression of the rice gene OsCTF using epigenetic regulation for low cadmium planting in Rice

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ABSTRACT:

Rice is a major food crop and one of the most cadmium absorbing and bulk cereal crops. OsCTF, a gene involved in the efflux of heavy metal Cd in rice, which is closely related to the amount of Cd accumulation in rice. In this study, CRISPR-dCas9 technology was used to regulation of OsCTF 3 '- UTR demethylation in rice to improve the amount of OsCTF gene expression, which was detected by direct detection, using a Chop-PCR method to detect the methylation level in rice, and real-time quantitative PCR to detect the level of OsCTF gene expression; at the same time,cadmium stress was detected, DNA methylation was analyzed by sulfite sequencing and McrBC-PCR, and OsCTF gene expression was detected by differential expression gene analysis. OsCTF expression and cadmium accumulation in rice after demethylation of the OsCTF 3 '- UTR were measured by both direct and cadmium stress assays. Then, OsCTF 3 '-UTR demethylation level, OsCTF gene expression and cadmium accumulation in offspring were measured by self-cross method of transgenic rice. Finally, a new rice line with low cadmium accumulation was formed.

KEYWORDS: Rice, Epigenetic, OsCTF 3 '- UTR, Demethylation, CRISPR-dCas9 ,OsCTF expression, Low cadmium accumulation, New rice lines

1. Introduction

In recent years, with the industrial development of our country, more and more pollutants have been introduced with the discharge of industrial wastewater waste into agricultural fields, soil pollution problems are increasing. Cadmium (CD), as a heavy metal contaminated substance in soil pollution, will cause serious hazards to human health after being absorbed by agricultural crops and consumed by people. The World Health Organization has classified cadmium as a class I carcinogen and specifies a daily intake for adults of no more than 70 μg. Rice (Oryza sativa L.) is one of the important food crops worldwide, and almost half of the world's population eats rice, playing a pivotal role in safeguarding food security in China, but rice is also one of the top cereal crops with the strongest contribution to Cd uptake and accumulation, therefore, it is important to study the regulatory mechanism of rice on Cd uptake, accumulation, and translocation, and further explore the relationship between DNA demethylation, gene expression, and Cd stress tolerance responses in rice, It is an undesired goal to achieve as low CD cultivation of rice as possible.

2. Literature Review

2.1 *Response mechanisms of rice to Cd stress*

The operational mechanisms of rice for heavy metal Cd^{2+} ions can be divided into uptake, transport, accumulation, and efflux, which involves several gene families. The Nramp family as an integral regulatory gene in the response mechanism of rice, and its related studies have been gradually enriched. For example, the Nramp family was found to participate in the root transport of cadmium ions in rice, and they were OsNramp1 and OsNramp 5 [1]. In recent years,many researchers have explored low cadmium accumulation in rice by gene editing two genes of the Nramp family involved in transport of cadmium ions in rice. Li et al [2] used CRISPR/Cas9 technology to knock out OsNramp5 gene in Huazan rice and Dronaceae rice 6385, and the result showed that OsNramp 5 mutant reduced cadmium content in the grain and had no effect on plant yield. However,the direct knockout of OsNramp 5 also drastically reduced manganese ions uptake by rice, resulting in poor plant growth in response to manganese deficiency or relatively low manganese supply levels in rice [3-4]. Chang et al [5] obtained OsNramp5 transgenic plants under the W4 background under the control of OsActin1. Rice will absorb large amounts of Cd ions and accumulate Cd ions abundantly in the root tips and lateral root primordia, which greatly reduces Cd ion accumulation in the grain, but also causing a dramatic decrease in the yield of rice. This method is very important for the purification of contaminated soil, but is unfavorable for rice harvest. Zhao et al [6] used CRISPR / Cas9 technology to knock out OsNramp1 in a nipponbear rice cultivars, and found that knocking out this gene resulted in decreased Cd

and Mn absorption in rice and significantly lower levels of Cd and Mn ions in the aerial parts and grain. However,compared with directly knocking out OsNramp5, knocking out of OsNramp1 had less effect on rice growth and yield,while knocking out of OsNramp1 and OsNramp5 at the same time in rice caused a sudden shortage of Mn ions and failed to grow normally and eventually died. Other genes involved in cadmium ion transport in rice are OsZIP1, OsZIP5, OsZIP7, OsZIP9 in the ZIP family [7-8] ,among which OsZIP1 is a metal efflux transporter that limits the excessive accumulation of cadmium, zinc, and copper in rice, and when rice is subjected to cadmium stress, demethylation occurring within 246 bp of the exon of this gene results in increased expression of OsZIP1 that assists in the efflux of heavy metal ions in rice and acts as a detoxification [9]. A similar gene is OsCTF. When rice is subjected to cadmium stress, CG demethylation leads to upregulation of OsCTF expression in order to limit Cd transportation and accumulation in cells [10]. Cadmium induced DNA demethylation mediates OsCTF regulation by Shengjun Feng's study on the mechanism of cadmium resistance in rice further confirmed that epigenetic could regulate OsCTF expression, and this epigenetic regulation was specifically involved in the detoxification of the heavy metal cadmium in response to cadmium stress. It is not directly related to the essential elements Cu, Zn, Fe, and Mn [11]. Although targeted demethylation of the OsCTF gene has been confirmed as an important regulatory mechanism in the detoxification of cadmium ions in rice, no corresponding planting lines of rice with low cadmium have been formed.

2.2 *DNA methylation / demethylation*

DNA methylation is one of the important genetic markers whose methylation activity is specific to the target region and can be inherited in mitosis [12]. In plants, DNA methylation is an important epigenetic modification that plays an important role in regulating gene expression, mediating plant responses to stress, and controlling plant growth and development. DNA methylation is the process by which the methyl group of S-adenosyl-L-methionine (SAM) is transferred to DNA cytosine under the catalysis of DNA methyltransferase (DNMT) to form 5-methylcytosine (5 - mC) [13]. At the same time, DNA methylation is a reversible epigenetic modification. Demethylation of plant genomes can activate genes in a silenced state, which is important in maintaining transgene activity in transgenic organisms and improving growth subsequently, accepting environmental changes, or preventing diseases [14]. Jin (2023) et al [15] found that ROS1 mediated DNA demethylation regulates the ABA pathway related gene AtACO3 in response to various abiotic stresses,which is essential for many important physiological processes, such as fruit ripening, biotic and abiotic stress responses, and gene imprinting.

2.3 *Research progress of CRISPR-dCas9 targeted demethylation*

The CRISPR / Cas9 system consists of a single stranded gRNA and an endonuclease active Cas9 protein that specifically recognizes the target gene sequence and cuts double stranded DNA at the target site. The non-homologous end-link repair mechanism of the cell reconnects the genome at the fracture site and introduces insertion or deletion mutations. It is also possible to provide an exogenous double stranded donor DNA fragment to integrate into the genome at the breakpoint by homologous recombination,so as to achieve the purpose of DNA modification [16-17], and the CRISPR / Cas9 system plays an important role in the precise improvement of rice yield traits, quality traits, and improving resistance for the purpose of rice. Meanwhile, gRNAs can mediate dCas9 protein binding to DNA, and use the ability of dCas9 to bind to DNA sequences to fuse dcas9 with other proteins, allowing it to function as a transcription factor and promote or inhibit gene expression [18].

CRISPR-dCas9 plays an essential role in the regulation of DNA demethylation by epigenetic mechanisms. TET1 is a α-ketoglutaric acid and Fe^{2+} dependent dioxygenases, which oxidizes 5mC and 5hmc in DNA to 5caC, the modified 5caC is specifically recognized and resected by thymine DNA glycosylase (TDG), and then converts this site to cytosine by the base excision repair pathway, constituting an active DNA demethylation pathway [19]. (Wang 2022) [20] The CRISPR / dcas9-TET1cd system is used to efficiently target a reduced methylation level of the self published epitranscriptomic allele-NMR19-4 and successfully induced PPH gene expression changes, thereby causing changes in Arabidopsis phenotypes that could be stably inherited in the progeny. Recently, it was shown that there is a gene OsCTF in rice that can control Cd accumulation, and demethylation of the 3 '- UTR can increase OsCTF gene expression [21].

With the increasing attention to food safety and the continuous improvement of breeding technology, there is more and more research on rice cultivation with low cadmium by using transgenic and gene editing technologies. But the existing research mainly focuses on Rice Nramp family, ZIP family, ABC Family, etc. However, most genes regulate the absorption of multiple ionic elements simultaneously and it is difficult to realize the specific regulation effect on Cd ions. At present, there are still few related studies on the OsCTF genes that specifically regulate cadmium ions, so it is an important issue to explore the roles and genetic mechanisms of the OsCTF genes to form stably inherited low cadmium cultivated rice lines.

3. Materials and method

3.1 *Construction of a rice plant with OsCTF 3 '-*

UTR demethylation

CRISPR/dCas9-TET1 expression vector was constructed by CRISPR/ DCas9-targeted editing of OsCTF gene 3 '-UTR, and transgenic rice plants with OsCTF gene 3' -UTR end demethylation were constructed by Agrobacterium transformation method. Chop-PCR method and transcriptome analysis were used. The methylation level and gene expression of OsCTF gene in transgenic rice plants were examined, and rice plants with OsCTF 3 '-UTR demethylation and OsCTF gene high expression were selected as T0 generation for follow-up experiments.

3.1.1 *CRISPR / dCas9-TET1 vector construction*

According to the principle of gRNA design, the gRNA was designed for the OsCTF 3 '- UTR site and performed by choosing the appropriate PAM sequence before designing primers, and then adaptors matching the vectors were added at both ends of the primers. The designed gRNA was ligated separately with the At7SL-2 promoter, and then the gRNA and dCas9 were expressed together with TET1 to create a construct with At7SL-2 driving gRNA expression, and the fusion expression of dCas9 and TET1cd was driven by UBQ1, and the carbenicillin resistance gene was added. The CRISPR / dCas9-TET1 demethylation system targeting the OsCTF 3 '- UTR was introduced into Agrobacterium,with the goal of reducing the methylation level of the OsCTF 3' - UTR.

3.1.2 *Agrobacterium Genetic Transformation*

(1) Seed disinfection: select full, mildew free mature rice seeds to be dehulled, by first rinsing them three times with sterile water and then treating them with 70% ethanol for 1 min with shaking from time to time; rinse with sterile water 3 times for 30s each with shaking; sterilizing with 0.1% mercury bichloride solution for 12 min with shaking from time to time; the above procedures can be operated outside the super net bench, and taking it to the super net bench after wiping the small triangular bottle containing the sanitized seeds clean with an alcohol cotton pellet about 12 min. Rinsing with sterile water 5 more times for 30 s each with shaking from time to time; The seeds were put on sterilized filter paper to blot dry water, and then clamped onto the induction medium with sterile forceps for the induction culture

(2) Callus subculture: the rice seeds began to germinate after 2 days, and small pieces of callus were produced from scutellum by 7-10 days, and the callus further expanded around 2 weeks. At the same time, the callus had a stiffer texture and became lumpy and yellowish. Embryogenic calli that were brightly colored, compact, and dry (those that were spread onto the medium) were picked and cultured on secondary medium

(3) Pre culture of callus: pick compact and dry embryogenic calli that are dark cultured on pre medium for 4-14 d (not more than 2 weeks) at 28 °C

(4) Agrobacterium infestation: pick embryogenic calli that are bright, compact and dry in color and transfer to a sterilized triangular bottle; agrobacterium suspensions were added and the calli was soaked for 30 min; transfer callus to sterilized filter paper and blow dry (over 1 h) on an ultra clean bench

(5) Coculture: calli were placed on coculture medium that had been plated with a layer of filter paper and cultured at 20 °C for 3 d

(6) Callus screening culture: the selection medium was supplemented with a certain amount of carbenicillin for screening

(7) Robusting seedling and transplanting: the calli grown at about 10 cm height with better root system, opening the cap and injecting sterile water into the flask and, after 2 d of exercise, washing out the residual medium on the roots (be careful not to injure the roots) and transplanting. During the first few days, coverslips were masked, protecting them from intense light exposure, and keeping them moist.

3.1.3 *determination of cadmium accumulation in transgenic plants*

3.1.3.1 stress detection

(1) Inductively coupled plasma mass spectrometry determination of cadmium accumulation in Rice: detection of cadmium accumulation in DNA demethylated rice and wild-type rice on a background of high cadmium ion concentration, determined using inductively coupled plasma mass spectrometry. Plant tissues are first ground, broken, or cut to increase the sample surface area, and then an appropriate amount of sample, usually ranging from a few to tens of grams, is taken into a clean container and digested using aqueous ammonia oxide, nitric acid, and hydrogen peroxide, which are dissolved and diluted before ion separation and detection

(2) DNA methylation analysis

① By sulfite sequencing: after the DNA of rice plants was extracted, it was subjected to sulfite treatment, the genomic DNA was treated with sodium sulfite modification, BSP primers were designed to amplify the fragment of interest, the unmethylated cytosine was changed into uracil, followed by PCR amplification, the converted uracil was changed into thymine. The amplified product ligation vector was then transferred into E. coli competent cells, and by sequencing, it was possible to identify where demethylation had occurred.

② Mcrbc PCR assay: degradation of methylated regions on DNA fragments into DNA fragments using McrBC-

PCR endonuclease Cleavage at the unmethylated SmaI site was again performed by using the SmaI restriction enzyme; digestion using a methylation sensitive HpaII restriction enzyme;DNA fragments were amplified by AP-PCR using specific MLG2 primers. Only selected DNA fragments containing methylated HpaII (CCGG) or SmaI (cccggg) site sequences were amplified.

(3) RNA transcriptome analysis

① Extract RNA: rice tissues are snap frozen to prevent RNA degradation, and RNA extraction buffer, such as chloroform, is added. Samples were centrifuged to separate into supernatant (containing RNA) and pellet (containing protein and RNA), and RNA was purified from the supernatant using tools such as the RNA Purification kit. RNA quality and concentration assays were performed.

② RNA quality detection: the isolated and purified RNA was checked for quality using UV absorbance and gel electrophoresis, respectively, by first zeroing the spectrophotometer with diluted TE solution. A small volume of the RNA solution was then taken and diluted with TE (1:100), and the absorption values at 260 nm and 280 nm on a spectrophotometer were read to determine the RNA solution concentration and purity. Gel electrophoresis prepare the apparatus by first rinsing it with 70% ethanol and air drying for later use, weighing the agar powder into a conical flask by adding DEPC water to heat the agar to dissolve completely, adding electrophoresis fluid, formaldehyde after less cooling, and pouring it into a glue tank with a comb inserted for sizing.

③ RNA-Seq sequencing experiments: RNA extraction was followed by opening the RNA secondary structure by heating, enriching for RNA using oligo (DT), fragmenting it under high temperature conditions, and synthesizing cDNA using the RNA at this time as a template. Finally, the synthesized cDNA was modified by magnetic bead purification followed by sequencing adaptors, and finally, the library was constructed by PCR amplification.

④ Calculation of the gene expression of OsCTF was performed by the RPKM formula.

3.1.3.2 Direct test:

(1) Chop-PCR assay: the total DNA from the genome was digested with MspI and then the target sequences were amplified with specific primers to detect the high and low methylation levels of the OsCTF 3 '- UTR sites based on the bright and dark of the PCR amplified bands

(2) Detection of OsCTF gene expression levels by real-time quantitative PCR

① Total RNA extraction: repeat step (3) - ①

② Reverse transcription: sample total RNA 1 μg; Random primers or oligo - dT(18-20) 100pmol; (Mmol / L each dNTP), 1 μl; Reverse transcription buffer (5 ×) four μl; RNase inhibitor (20-40 u/ μl) 1 μl; 20U of M- MLV reverse transcriptase; DEPC treated water was supplemented to 20 μl; The negative control replaced the RNA template with DEPC treated water and the remaining components were the same. Add the above reaction components to a 0.5 ml EP tube, mix, and centrifuge briefly. Place each reaction tube into the PCR amplicon and store at 37 ° C for 60 min, denaturation at 70 ° C for 15 min, and store the reverse transcripts at 4 ° C for further use.

③ PCR amplification: 1.0 U of Taq DNA polymerase; cDNA template 1.0 μl; One primer pair (10 pmol each/ μl) 1.0 μl; (Mmol / L each dNTP), 2.0 μl; PCR buffer (10 ×,Containing MgCl2) 2.5 μl; double distilled water was supplemented to 25.0 μl. Taking the reverse transcription negative control instead of cDNA template as PCR negative control, the above reaction components were added into 0.2 ml PCR tube, centrifuged briefly, and mixed. Each PCR reaction tube was put into a DNA amplifier, 94 ° C for 5 min, and then thermocycled with the following conditions: 94 ° C for 45 s, 56 ° C for 30 s, 72 ° C for 30 s, cycling 30 times, and a final extension at 72 ° C for 10 min.

④ RT-PCR products were detected by agar gel electrophoresis.

3.2 *Establishment of low Cd grown rice lines*

The amount of Cd accumulation under Cd stress was recorded in F1 and F2 generation rice plants by subjecting T0 generation rice plants to two successive generations of selfing, comparing the selfed F2 generation plants with untreated rice plants (as a control). And the methylation level of OsCTF gene in plants was examined by chop PCR to select rice lines in which the demethylation of OsCTF 3 '- UTR was stably inherited in the progeny.

3.2.1 *Inbreeding method*

① The anthesis period is strictly controlled and the bag is covered and closed when the flower is not open, collecting the pollen into the bag.

② Pour the pollen on other flowers and close, waiting for firmness.

③ Sowing, with grain formation followed by progeny identification.

④ Elite inbred lines were selected for preservation and promotion.

3.2.2 *Method of cadmium determination in Rice*

The above direct assay and cadmium stress assay were repeated to detect the amount of cadmium accumulation in rice in the progeny.

4. Conclusion

This study directly employed biotechnology CRISPR / dCas9-TET1 targeted demethylation to regulate OsCTF expression, and the targeted demethylation of OsCTF 3 '- UTR did not affect the uptake of other ions by rice, and the amount of OsCTF expression increased with OsCTF 3' - UTR demethylation, so that rice cultivation in a high Cd environment could also play a better role in self detoxification and present low Cd accumulation in rice. Precise demethylation of the OsCTF 3 '- UTR by CRISPR / dCas9-TET1 technology could be stably inherited in rice progeny through transgenic rice selfing, providing a research basis for creating new low Cd cultivars and forming new low Cd accumulation lines in rice. The new low Cd growing lines of rice can be grown normally in Cd ion contaminated soil, and the rice grain neither appears high Cd ion accumulation and thus damages the human body, nor can it make full use of land resources.

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