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Molecular Cloning and Characterization of Citrate Synthase Gene in Rice (*Oryza sativa*)

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Abstract: The full-length *OsCS* encoding citrate synthase was isolated from rice (*Oryza sativa* L. subsp. *japonica*). *OsCS* is 1477-bp long and encodes a 474 amino acid polypeptide. Its putative protein sequence is highly identical to *Daucus carota*, *Nicotiana tabacum*, *Beta vulgaris* subsp., *Arabidopsis thaliana*, *and Citrus junos* (＞70%). The deduced amino-terminal sequence of *OsCS* showes characteristics of mitochondrial targeting signal. Southern blot analysis using ORF of the *OsCS* as the probe indicated that this gene exists in multiple copies in rice genome. The band with predicated size of 82 kD was detected by Western blot after being induced by 0.4 mmol/L IPTG.

Key words: citrate synthase; rice (*Oryza sativa*); gene; clone

Acid soils cover almost 40% of the earth's arable land [1]. Aluminum (Al) toxicity represents the main factor limiting plant production in such soils [2, 3]. To increase the tolerance of crops in acid soils by genetic engineering, isolation of new genes in response to Al toxicity is necessary.

One possible mechanism proposed for Al tolerance is the exudation of organic acids from roots [4]. Citrate and malate are commonly released organic acids that can be effective in chelating Al^{3+} in rhizosphere and protecting plants from Al toxicity [5]. Other organic acids such as succinate and oxalate also play important roles in different varieties and species of plants [6]. Exudation of organic acids is believed to be well related to Al tolerance in many plant species, such as *Arabidopsis thaliana* [7, 8], and *Triticum aestivum* [9].

Carbohydrate metabolism plays a key role in organic acid synthesis and possibly excretion. Due to the essential role of citrate exudation in response to Al toxicity, the citrate synthase (CS) is widely investigated [8, 10]. CS is an enzyme involved in combination of oxaloacetate (OAA) and acetyl CoA to produce citrate. Citrate plays an important role in the Krebs cycle, β-oxidation of fatty acids, photo-respiratory glycolate pathway, and in chelating toxic ions. The cDNA encoding mitochondrial CS in higher plants was first isolated in *Arabidopsis thaliana* [10] and then in several other plant species [8]. Overexpression of an *Arabidopsis* mitochondrial CS (At-mtCS) in carrot (*Daucus carota*) cell lines and a carrot mitochondrial CS in *Arabidopsis* led to enhanced citrate efflux from roots and thereby enhanced Al tolerance in transgenic lines [8, 10]. Despite present reports on this subject, a clear relationship between citrate synthesis and Al tolerance has not been proved in major crops. Aiming at developing the genomic function of *Oryza sativa*, we isolated the full-length sequence of a mitochondrial CS cDNA from *Oryza sativa* by RT-PCR and it was characterized by prokaryotic expression.

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MATERIALS AND METHODS

Plant material and treatment

Rice cultivar 'Eyi 105' (*Oryza sativa* L. subsp. *japonica*) seeds were sown in quartz sands. Germinated seeds were grown in a 1.5 L plastic vessel containing a nutrient solution and grown in greenhouse at temperature ranged from 20 to 30℃, under 16 h of light and 8 h of darkness. Seedlings at the stage of 3-leaf were harvested, and frozen in liquid nitrogen and stored at -80℃ for RNA isolation.

Bacteria strains used in this study included *E. coli* DH5α for cloning and *E. coli* BL21 for prokaryotic expression.

RNA isolation, cDNA synthesis and gene clone

Total RNA was extracted from rice leaves using

Trizol reagent system (Life Technologies). First-strand cDNA was synthesized from 3 μg total RNAs with M-MLW reverse transcriptase (Roche) using oligo $(dT)_{18}$ as primer, according to the manufacturer's protocol. cDNA encoding *OsCS* was obtained from cDNA of rice by PCR (94℃ for 45 s, 62 ℃ for 1 min, 72℃ for 2 min, 35 cycles). Based on the genomic DNA sequence of rice, Sense (5' GCTCTAG AATGGCGTTCTTCAGGGGC 3') and antisense (5'C GAGCTCTCAAGCAGCAACCTTCTTGCA3') primers were designed, and *Xba* I and *Sac* I sequence were added respectively to 5' ends of the primers above. The amplified cDNA fragments were purified from the agarose gel and ligated with pGEM-T vector for sequencing and thus we obtained plasmid T-OsCS-3.

DNA preparation and Southern blot analysis

Genomic DNA was extracted by CTAB method. The DNA was purified by TaKaRa agarose gel DNA purification Kit ver.2.0. Five microgramme of genomic DNA was digested with *Dra* Ⅰ, *Eco*R Ⅰ, *Hin*d Ⅲ respectively, and electrophoresed on a 0.8% agarose gel, then transferred to a hybond-N^+ membrane. Hybridization, wash and detection were performed according to Gene Images Random Prime Labeling and Detection module's instruments (Amersham Biosciences).

Vector construction

According to plasmid T-OsCS-3 with ORF sequence, *OsCS* cDNA added with *Eco*R I (sense primer 5' CGGAATTCATGGCGTTCTTCAGGGGC 3'), and *Sma* I (antisense primer 5'CCCCCGGGAG CAGCAACCTTCTTGCA 3') sequence to 5' ends, respectively, was obtained by PCR. For Western blot analysis, *Eco*R I-*Sma* I fragments were introduced into the pEGFP-N2 (Clontech) and designated as pEGPF-OsCS-3 in *E. coli* DH5α. The plasmid digested by *Eco*R I and *Not* I was introduced into pET-24a as pET-24a-OsCS-GFP-1 for prokaryotic expression in *E. coli* BL21.

Western blot analysis

E. coli BL21 containing pET-24a-OsCS-GFP-1 grew to logarithmic phase when OD_{600} is 0.4-0.6, then added with 0.4 mmol/L IPTG (isopropyl-beta-D-thiogalactopyranoside) and induced for 4 h under 37℃. Lysates from GFP-expressing BL21 bacteria were electrophoresed by SDS-PAGE using a 12.5%(*W*/*V*) gel. Separated polypeptides were then transferred from the gel onto NC membrane (Roche, USA) by semi-dry electrophoresis transfer (Bio-Rad, USA) using the instructions provided by the manufacturer and then incubated with rabbit anti-GFP antiserum (Clontech, #8367-2) diluted 1:1000. An alkaline phosphatase conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, USA) was used for visualization of the polypeptides, and detected by BCIP/NBT (Roche, USA). The molecular weight marker was from Bio-Rad.

RESULTS

Cloning of *Oryza* citrate synthase gene

First-strand cDNA was synthesized from total RNA, and cDNA encoding *OsCS* was obtained by RT-PCR. PCR product showed a clear band of 1500 bp in agreement with predicted size (concentration of 40 ng/μL) (Fig. 1).

Structure and organization of *Oryza* citrate synthase

The full-length cDNA is 1477 bp in length, including a 45 bp 5' untranslated region (UTR), a complete ORF of 1425 bp and a 3'-UTR of 7 bp. The first ATG located at 46 nt agreed well with the translation initiation consensus sequence (Kozak squence, A/GNNATGG) [11]. The predicted protein product of *OsCS* comprises 474 amino acid residues (molecular weight of 52.4 kD, PI 8.268).

On amino acid level, OsCS showed 77%, 75%, 77%, 79% and 76% identity with *Daucus carota*, *Nicotiana tabacum*, *Beta vulgaris* subsp., *Arabidopsis thaliana*, and *Citrus junos*, respectively (Fig. 2). Analysis of the sequence of *OsCS* identified mitochondrion-

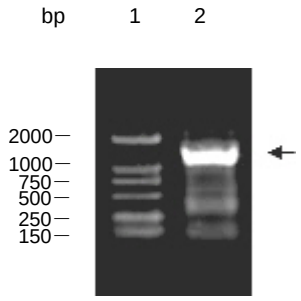

Fig. 1. Electrophoresis analysis of PCR product with agarose gel.
Lane 1, DL2000 DNA marker; Lane 2, *OsCS* of PCR product.

Fig. 2. Alignment of induced amino acid sequence of *OsCS* with that of *Daucus carota* (AB017159), *Nicotiana tabacum* (X84226), *Beta vulgaris* subsp. (X84228), *Arabidopsis thaliana* (AF387018), and *Citrus junos* (AY428532).

Identical amino acid residues are indicated by '*' and conserved substitutions are indicated by ':', a mitochondrion-targeting signal at amino terminus is highlighted and underlined.

targeting signals (mts), with predicated cleavage site after the first 4 N-terminal amino acids. The mts is rich in positive amino acids primarily Arg, residues with hydroxylated side chains primarily Ser, but has no acidic amino acid residues and hydrophobic stretches. The putative transit peptide sequence of RS(G)V(L)S(T)A(L)/S(T)R(K)LRSRA(V) is identical to some features of mitochondrion transit peptides described by Attardi and Schatz [12]. This fragment is also conserved in *OsCS* at positions 5–17 (RGLTAVSRLRSRV) (Fig. 2). So it can be inferred that *OsCS* gene must have been expressed in mitochondrion.

Based on the rice genome sequence, *OsCS* gene family comprises two members, and these two genes are located on two chromosomes in rice. One spans from 142 247 to 136 517 on chromosome 2 having 18 introns, the other from 8 776 to 93 509 on chromosome 11 having 17 introns. *OsCS* cDNA that we got arranged to chromosome 2 in *OsCS* gene family by a homology search of nucleotide sequence. The gene *OsCS* was deposited in GenBank with accession number AY753182.

Southern blot analysis

Hybridization with a 1425 bp fragment (*Eco*R I-*Sma* I) of the *OsCS* clone labeled with fluosescein-labelled probe, and detection was performed according to Gene Images. Southern blot of *Oryza sativa* genomic DNA digested with different restriction enzymes (*Dra* Ⅰ, *Eco*R Ⅰ, *Hin*d Ⅲ respectively) indicates the presence of probably two or three different related sequences in rice genome (Fig. 3).

Prokaryotic expression and Western blot analysis

Expression of *OsCS* was identified indirectly by Western blot using rabbit anti-GFP antiserum. For Western blot analysis, *Eco*R I-*Sma* I fragments were introduced into the pEGFP-N2 (Clontech) and designated as pEGPF-OsCS-3 in *E. coli* DH5α. The plasmid was cut by *Eco*R I and *Not* I, and a 2.1 kb fragment was introduced into pET-24a as pET-24a-OsCS-GFP-1 for prokaryotic expression in *E. coli* BL21. Restriction analysis identified the existence of the 2.1 kb band (*OsCS* 1.4 kb + *gfp* 717 bp) (Fig. 4).

Supernatant of *E. coli* BL21 containing pET-24a-OsCS-GFP-1 was electrophoresed by SDS-PAGE using a 12.5%(*W*/*V*) gel after induced by 0.4 mmol/L IPTG. Separated polypeptides were then transferred from the gel onto the NC membrane, and tested by rabbit anti-GFP antiserum. The result showed a clear protein band around 85.0 kD which is consist with predicted size (Fig. 5).

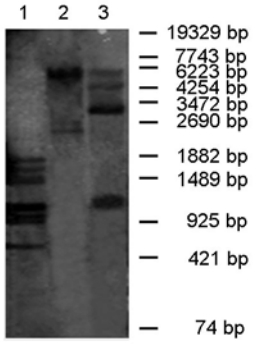

Fig. 3. Southern blot analysis of *Oryza sativa* genomic DNA using the probe of 1425 bp ORF sequence of *OsCS*.

5 μg genomic DNA was digested each with *Dra* Ⅰ (Lane 1), *Hin*d Ⅲ (Lane 2) and *Eco*R Ⅰ (Lane 3). λ-*Eco*T14 I digest marker is on the right.

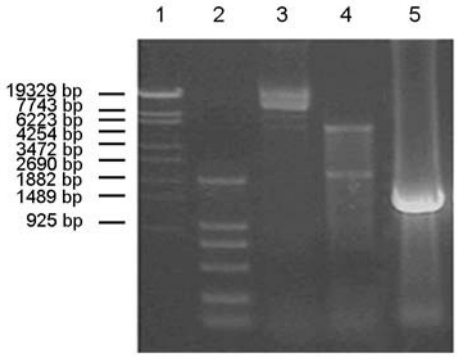

Fig. 4. Identification of pET-24a-ORCS-GFP-1 by restriction enzyme.

Lane 1, λ-*Eco*T14 I digest marker; Lane 2, DL2000 DNA marker; Lane 3, Plasmid pET-24a-OsCS-GFP-1; Lane 4, pET-24a-ORCS-GFP-1 digested by *Eco*R Ⅰ/ *Not* Ⅰ; Lane 5, PCR product using plasmid pET-24a-OsCS-GFP-1.

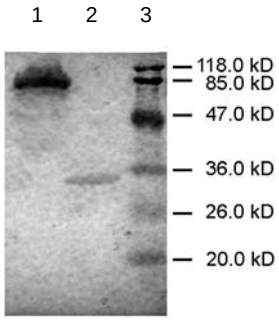

Fig. 5. Recombinant OsCS and GFP identified by Western blot.

Lane 1, Recombinant OsCS and GFP; Lane 2, Control; Lane 3, Molecular weight protein marker.

DISCUSSION

Aluminum (Al) is primarily in the form of Al^{3+} poisoning to many crops and limiting the plant productivity in acid soils. The exudation of organic acids such as citrate, malate, succinate from roots can be effective in chelating Al^{3+} to increase Al tolerance. So increasing the exudation of organic acids by genetic engineering appears to be an available method to enhance Al tolerance of plant. de la Fuente et al proved that organic acid excretion is indeed a mechanism of Al tolerance in higher plants and this trait can be engineered transgenically by introducing citrate synthesis gene from *Pseudomonas aeruginosa* into *Nicotiana tabacum* and papaya [13]. The similar results were reported in other papers [14]. By contrast, Delhaize et al showed transgenic tobacco lines expressing more citrate synthase protein did not show increased accumulation of citrate in roots or increased Al-activated efflux of citrate from roots, and they concluded that expression of the *Pseudomonas aeruginosa* citrate synthase gene in plants is unlikely to be a robust and easily reproducible strategy for enhancing the Al tolerance of crop and pasture species [15].

We have cloned citrate synthase gene from *Oryza sativa*. Based on a homology search of the predicted amino acid sequence in GenBank database and analysis of mitochondrion-targeting signals of *OsCS* gene, the *OsCS* gene encoding citrate synthase appears to be expressed in mitochondrion. Similarly, prokaryotic expression and Western bolt analysis indirectly identified the correct expression of citrate synthase. Further research will be focused on the gene expression mechanism by the method of *in situ* RNA hybridization and functional analysis in yeast.

ACKNOWLEGEMENTS

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