Cloning and Expression of Citrate Synthase Gene of *Sinapis alba* Under Drought Stress

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Abstract: *Sinapis alba* has many desirable agronomic traits, especially its tolerance to drought. Citrate synthase is a key rate-limiting enzyme of varieties of metabolic pathways in cells and a marked enzyme of metabolic changes which can be found in most organisms. We studied expression of citrate synthase gene of *Sinapis alba* when plants were under drought stress. The gene segment of citrate synthase was cloned from *Sinapsis alba*, which consists of 561bp, and 26% A, 22% C, 28% G, 24% T. It encoded 187 amino acid residues with a predicted molecular weight of about 2.057 KD. Additionally, the amino acid sequence deduced from the citrate synthase gene in *Sinapsis alba* shared 90.0%, 86.2%, 87.4%, 86.9%, 80.5%, 82.0%, 75.0%, 87.0%, and 89.5% homology with that of citrate synthase gene in *Arabidopsis thaliana*, *Brassica campestris* L., *Solanum tuberosum*, *Oryza sativa* , *Citrus reticulate* Blanco, *Amygdalus persica* L., *Daucus carota*, *Nicotiana tabacum* L., and *Malus xiaojinensis*, respectively. The results also showed that the gene expression of the citrate synthase in *Sinapsis alba* increased significantly when it was subjected to the treatment of transient and light drought stress, then it restored after 144 hours，which suggested the plant might be gradually adapt to this stress. However, when *Sinapsis alba* was treated under severe drought condition, the gene expression increased and could not restore, which suggested that *Sinapsis alba* was probably to be injured too seriously to restore itself.
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Introduction

Drought stress often affects the activities of key enzymes in the metabolic activities of plants. Research on these enzymes can help us reveal the resistance mechanism of plants (Schnarrenberger and Martin, 2002). Citrate synthase plays an important role in the processes of energy metabolism, vegetable fat metabolism, fatty acid oxidation and cellular detoxification (Liu et al., 2013). Previous studies found that citrate synthase in Arabidopsis thaliana play an important part during seed germination and fatty acid metabolism (Morgunov and Stere, 1998). In order to study plant's growth regulation and mechanism of anti-adversity, Citrate synthase gene of Brassica campestris L. was cloned (Tong et al, 2009). Citrate synthase gene in tobacco has been transferred into Alfalfa to improve the receiver's tolerance to aluminum toxicity; this technology has been widely used in agricultural production (Hu et al., 2009). It has been found that citrate synthase and organic acid content were positively correlated in the process of mature in pineapple, which means that organic acid accumulated when the activities of citrate synthase increased (Zhang et al., 2007).

Studies on citrate synthase mainly were focused on the fruit growth and development, and focused on the plant aluminum toxicity in recent years. Researches on the role of citrate synthase or reaction mechanisms were rarely reported when plants under drought stress. *Sinapis alba* can grow under arid environment, but it doesn't have a adaptive morphology to resist drought (Dong et al., 2012), we can infer that it has a particular mechanism of drought-resistance on the physiology, biochemistry and molecular level. Currently, the expression of citrate synthase gene under drought stress has not been reported in *Sinapis alba* yet. Citrate synthase gene of *Sinapis alba* was cloned, and the expression of this gene under drought stress was also analyzed in this paper, which can help identify the drought resistance mechanism of *Sinapis alba*.

Materials and methods

Experimental materials

Sinapsis alba seeds were purchased from Chinese herbal seeds company. Total RNA was extracted by using TakaRa RNAiso kit. Fluorescence quantitative PCR reaction kit was TakaRa one step SYBR⑧ Prime script TM RT-PCR kit. The primer was designed by using software Primer5, synthetized by Shanghai biological engineering company.

Experimental method

Drought stress treatment

Sinapis alba seeds were planted in trays filled with peat soil and vermiculite (2:1 ratio), and were placed in the culture room until those seeds germinated. After germination, seedlings were moved to greenhouse to grow, and watered enough. At 28 days when seedlings had four leaves, drought treatments were applied. Within indoor simulation of drought

conditions by water PEG 6000 solutions, *Sinapsis alba* seedings were treated with polyethylene glycol 6000 (PEG6000). There were two treatments of different PEG6000 concentrations 15%，20%. PEG solutions were poured （500ml, respectively） out near the root of *Sinapsis alba* seedlings only one time. At the same time, control treatment received water only.

Leaves were clipped from those seedlings treated by PEG solution 3 days (72 hours), 6 days (144 hours), and 9 days (216 hours), respectively, then treated in liquid nitrogen and stored in -80℃. Leaves from control treatment were clipped, treated and stored as well (0 hour).

RNA extraction and reverse transcription

RNA was extracted according to operating instructions of RNAiso. RNA quality and quantity were analyzed using spectrophotometer and electrophoresis. The RT-PRC kit was used in the reverse transcription test. The system of reverse transcription included 10ul sample: primer oligo dT 1 ul, reaction buffer 4ul, dNTP 1ul, RNA less than 5ug and RT enzyme 1ul. And then ddH2O was added to 20ul. A. RT enzyme was inactivated in water for 5 minutes at 42℃, and then 5 minutes at 95℃.

Primer design and synthesis

Degenerate primers were designed through software primer 5 according to citrate synthase gene sequences known in Arabidopsis thaliana, Brassica napus in the NCBI database, and the advanced position: 5'-CTG GTT TTG AGT TTT GGA GAA TG-3'，the backward position: 5"-GGC TTC TCT CTG AGT TGA AGT G-3', synthesized by Shanghai biological engineering company.

PCR amplification, gene cloning and sequence analyses

The total volume of PCR reaction was 20 ul, which includes template 1.0 ul, primer 0.5 ul, mix10 μl, then ddH2O was added to 20 ul. The reaction procedure was: predenaturing at 94 ℃ for 5 minutes, denaturing at 94 ℃ for 30 seconds, annealing at 55℃ for 30 seconds, and stretching at 72℃ for 2 minutes, 35 cycles. Amplified product was separated through 1%SDS-PAGE, then the target fragment was recovered, purified with Gel Extraction Kit and connected to cloning vector pTZ57R/T. Heat shock was given to the well-connected plasmid to transmit the prepared competent E coli DH5α. After positive colonies were cultured overnight at 37 ℃, the collection of the thallus was sent to Shanghai biological engineering company for sequencing.

The deduced amino acid sequence of citrate synthase gene in *Sinapsis alba* was aligned with other that from peach (Prunuspersica, AF367444.1), orange (Citrus junos, AY428532.1), rice (Oryza sativa, AK072950.1), potato (Solanum tuberosum, 75082.1), Arabidopsis (Arabidopsis thaliana, NM～180084.1) and carrot (Daucus carota, AB017159.1), etc. The homology was analyzed by the sequencing analysis software of DNAMAN6.0.

Semi-quantitative RT-PCR and fluorescence quantitative PCR.

Table 1 Reaction systems of Real time PCR for housekeeping gene and the objective gene

housekeeping gene			objective gene		
Number	Reagent	Dosage	number	Reagent	Dosage
1	SYBR Green 1	4.0μl	1	SYBR Green 1	4.0μl
2	Actin upstream primer F	0.2μl	2	Upstream primer	0.4μl
3	Actin downstream primer R	0.2μl	3	Downstream primer	0.4μl
4	dNTP	0.2μl	4	dNTP	0.4μl
5	Taq enzyme	0.4μl	5	Taq enzyme	0.8μl
6	Sample cDNA	2.0μl	6	Sample cDNA	2.0μl
7	ddH_2O	13.0μl	7	ddH_2O	12.0μl
8	Total volume	20.0μl	8	Total volume	20.0μl

To further study the expression of the citrate synthase gene in *Sinapsis alba* under drought stress, semi-quantitative RT-PCR was used to analyze the expression of the gene in *Sinapsis alba* under drought in different time, with β-actin gene as the inner control (Li, et al., 2006). Primers of the citrate synthase gene in *Sinapsis alba* were designed, and the upstream primer is 5 '-CAA TTA CTG AGT GAC CGA ACG-3', and the downstream primer is 5'-CCA TGA TCC AAC GAT TTA TCT G-3'. The universal primer of the housekeeping gene of β -actin: 5'-3'CTA CAA TGA GCT GCG TGT GG, and 3'-5'AAG GAA GGC TGG AAG AGT GC. The reaction procedure was: 94℃ for 5 minutes, followed by 30 cycles (94℃, 30 seconds; 55℃, 45 seconds; 72℃, 60 seconds) and ended with 72℃ for 5 minutes.

Quantitative PCR was performed using SYBR green PCR master mix (Takara) in optical 96-well reaction plates (Applied Biosystems) on an Eppendorf master cycler system. The SYBR Green fluorescent dye was used to detect the synthesized dsDNA. A total reaction volume of 20 μl contained 10 μl 2 X Power

SYBR Green Master Mix Reagent (Applied Biosystems), 2 μl of diluted cDNA and 1 μl of each gene-specific primer (10 μmol). PCR conditions: 95℃ for 2 minutes; 40 cycles of 95℃ for 20 seconds, 55℃ for 20 seconds,72 ℃ for 20 seconds; and then the melting curve. The data were expressed as the final cycle number necessary to reach a threshold fluorescence value (Ct). Data were normalized by the 2-ΔCt method and β-actin gene was used as control. Reaction systems of Real time PCR for housekeeping gene and the objective gene as seen in table 1.

Results

The cloning and homology analysis of citrate synthase gene in *Sinapsis alba*

As template, the total RNA isolated from leaves of *Sinapsis alba* was reversely transcribed to cDNA library. PCR primers were designed according to the cDNA sequence of citrate synthase gene in *Sinapsis alba*, and the fragment of the citrate synthase gene in *Sinapsis alba* was obtained by using the genomic DNA as PCR template.

The PCR product was separated by electrophoresis and purified by DNA gel recycling purification kit, then connected to the cloning vector. After PCR identification, the positive clones were sequenced. Sequence analysis showed that the citrate synthase gene segment in *Sinapsis alba* consisted of 561bp, with a predicted molecular weight of about 2.057 KD, which encoded 187 amino acid residues, and 26% A, 22% C, 28% G, 24% T (Fig.1).

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1       GACCAAAACTCAAAACCTCTTACATGGTGTTTCGCAGCGCGGCCTCTAGGCGGTCTCGCG
1        D  Q  N  S  K  P  L  T  W  C  F  A  A  R  P  L  G  G  L  A
61      AGGGTGAGGTCTCGCGTTGGGCAACAATCTTCGCTCAGCAATTCCGTTAGATGGATTCAG
21       R  V  R  S  R  V  G  Q  Q  S  S  L  S  N  S  V  R  W  I  Q
121     ATGTTGGGCAATCTTCGCTCAGCAATTCCGTTAGATGGATTCAGATGCAGAGCTCTACCG
41       M  L  G  N  L  R  S  A  I  P  L  D  G  F  R  C  R  A  L  P
181     ATACAGAGCTCTACCGATATGGACCTCAAGTCCCAGCTGCAAGAGTTGATTCCGGAACAA
61       I  Q  S  S  T  D  M  D  L  K  S  Q  L  Q  E  L  I  P  E  Q
241     CAGGACCGTTTGAAGAAACTGAAGTCAGAACATGGGAAGGTCCAACTGGGAAACATCACT
81       Q  D  R  L  K  K  L  K  S  E  H  G  K  V  Q  L  G  N  I  T
301     GTTGATATGGTAATTGGTGGGATGAGAGGGATGACTGGATTGCTCTGGGAAACCTCATTG
101      V  D  M  V  I  G  G  M  R  G  M  T  G  L  L  W  E  T  S  L
361     CTTTGGGAAACCTCATTGCTTGACCCGGAAGAGGGAATACGCTTTAGGGGCTTGTCAATT
121      L  W  E  T  S  L  L  D  P  E  E  G  I  R  F  R  G  L  S  I
421     CCTGAGTGCCAGAAAGTATTGCCTGCTGCTCAGTCTGGAGGAGAGCCGTTGCCCGAGGGT
141      P  E  C  Q  K  V  L  P  A  A  Q  S  G  G  E  P  L  P  E  G
481     CTTTTGTGGCTTCTTTTAACTGGAAAGGTACCTAGCAAAGAGCAAGTTGAAGCACTATCA
161      L  L  W  L  L  L  T  G  K  V  P  S  K  E  Q  V  E  A  L  S
541     CAAGACTTGGGCAAACCGTGC
181      Q  D  L  G  K  P  C
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Fig.1 Sequence of fragment of the citrate synthase gene and amino acid in *Sinapsis alba*

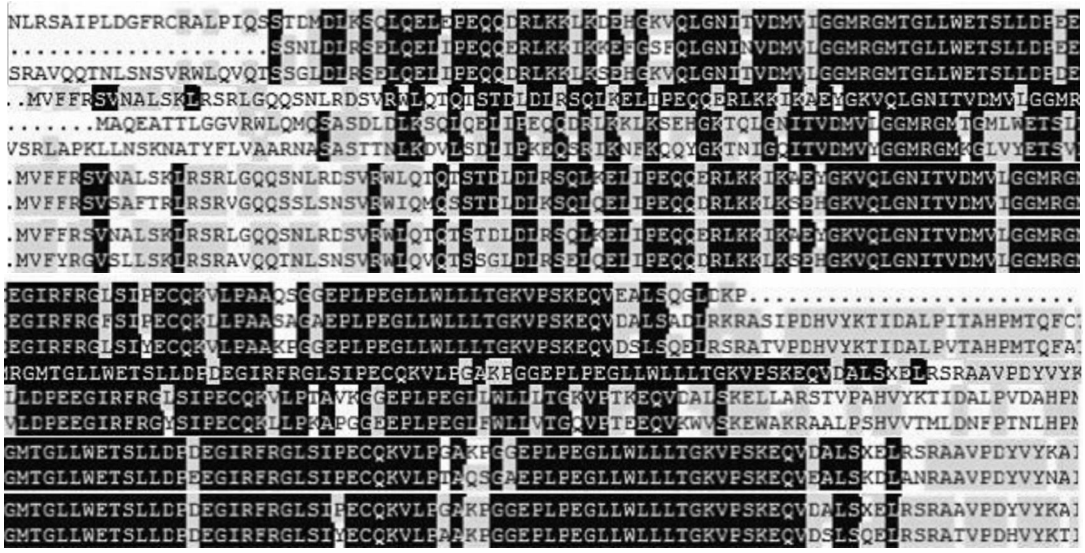

Fig.2 the homology analysis results by DNAman software based on amino acid sequence of citrate synthase from ten species. Species from top to bottom are *Sinapsis alba*, *Arabidopsis thaliana*, *Brassica campestris* L., *Solanum tuberosum*, *Oryza sativa*, *Citrus reticulate* Blanco, *Amygdalus persica* L., *Daucus carota*, *Nicotiana tabacum* L., and *Malus xiaojinensis*.

Citrate synthase genes of Arabidopsis thaliana, rapeseed, potato, rice, citrus, peach, carrot, tobacco, and Malus xiaojinensis in the genbank were searched, and the homology of those citrate synthase genes sequence was also analyzed by using DNAMAN software. Homologous analysis showed that the amino acid sequence deduced from the citrate synthase gene in *Sinapsis alba* shares 90.0%, 86.2%, 87.4%, 86.9%, 80.5%, 82.0%, 75.0%, 87.0%, and 89.5% homology with those of citrate synthase gene in *Arabidopsis thaliana*, *Brassica campestris* L., *Solanum tuberosum*, *Oryza sativa* , *Citrus reticulate* Blanco, *Amygdalus persica* L., *Daucus carota*, *Nicotiana tabacum* L., and *Malus xiaojinensis*, respectively (Fig. 2).

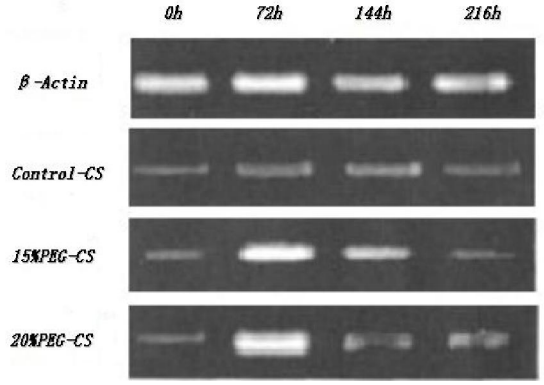

Fig.3 The expression of the citrate synthase gene in *Sinapsis alba*

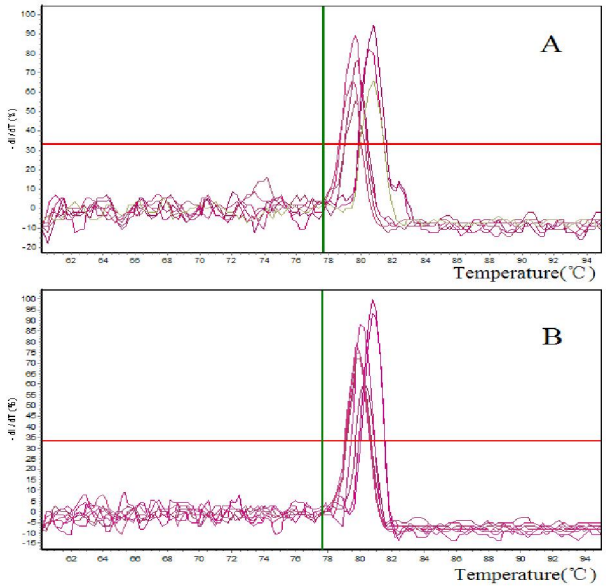

Fig.4 The dissolve curve of cDNA in *Sinapsis alba* by real-time PCR. A. treatment 1 with concentration of PEG 6000 at 15%; B. treatment 2 with concentration of PEG 6000 at 20%.

Tab.2 Expression amounts of template DNA contained in samples calculated according to the value of Ct at different PEG drought of 15% and 20% in different time

PEG6000 concentration	Objective gene cycle numbers	β-actin cycle numbers	ΔCt	ΔΔCt	2-ΔΔCt
15%PEG					
Control	28.14	13.74	14.41	0.00	1.00
72h	26.48	13.85	12.63	-1.78	3.43
144h	24.87	14.06	10.81	-3.60	12.13
216h	27.97	13.90	14.07	-0.34	1.27
20%PEG					
Control	28.25	14.05	14.20	0.00	1.0
72h	25.17	14.12	11.05	-3.15	8.88
144h	24.37	13.96	10.41	-3.79	13.83
216h	27.42	13.89	13.53	-0.67	1.59

Expression of the citrate synthase gene in *Sinapsis alba* under drought

First, expression of the citrate synthase gene in *Sinapsis alba* was examined under drought stress by semi quantitative RT-PCR and the results were shown in Fig. 3. The expression level of citrate synthase gene reached the peak value at 72 h after treated with 15% PEG or 20% PEG, and began to decrease at 144 h after the treatment.

In the process of quantitative fluorescence PCR, the fluorescence intensity increased in different degree, the melting profile was in single-peak (in Fig. 4) and it represents that there was nothing but specific amplification. Computational result showed that the curve slope absolute value of each gene was less than 0.1, that is there was no difference between the amplification efficiency of target gene and endogenous control gene. Therefore the amounts of template DNA

can be calculated by using the method of 2-△△ct (Zhang, et al., 2001). Table 2 shows the expression amounts of template DNA contained in samples which are calculated according to the value of Ct.

Discussions

Citrate synthase is one of the key enzymes in tricarboxylic acid cycle which is a common metabolic pathway in aerobic organisms. As a key enzyme of cell metabolisms, citrate synthase indicates the level of metabolism (Zhang, et al. 2008).Citric acid has become the research focus for its important role in plant metabolic response and environmental regulation (de la Fuente et al. 1997).

Suffered a slight drought stress, the citrate synthase expression in Sinapsis alba gradually increases, then the expression of the citrate synthase begins to decrease. When the plant gradually adapts to the drought stress, the expression returns to normal level. When suffered severe drought stress, the increasing expression of the citrate synthase in Sinapsis alba will no longer drop. That shows the damage on plant by severe drought is difficult to restore.

Generally, the expression of citrate synthase gene is closely related to plant stress tolerance. Citrate synthase gene in Arabidopsis thaliana was successfully transferred into carrot cells, the activity of citrate synthase in transgenic carrot cells is 19% higher than that of the wild type (Koyama et al. 1999). Transgenic cells grow 22%~40% faster than wild type in the medium containing aluminum, which shows that the higher expression of citrate synthase can improve the resistance of plant cells to aluminum toxicity. Tong et al. (2009) found that the citrate synthase gene in Brassica napus L. showed different expression patterns under different stress conditions.

The results in our study demonstrated that the expression of the citrate synthase in *Sinapsis alba* increased significantly when being subjected to transient and light drought stress, then the plant gradually adapted to the stress and the expression restored. However, under severe drought, the expression of the citrate synthase increased and did not drop. This shows seedling was seriously injured and was difficult to restore to normal level. The conclusion is consistent with the results of previous studies.

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