Plant Science 233 (2015) 22–31

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Identification and analysis of the mechanism underlying heat-inducible expression of rice aconitase 1

Juan Li[a,b,1], Rui-Ying Qin[a,1], Hao Li[a], Rong-Fang Xu[a], Chun-Hong Qiu[a], Yi-Chen Sun[a], Hui Ma[a], Ya-Chun Yang[a], Da-Hu Ni[a], Li Li[a], Peng-Cheng Wei[a,*], Jian-Bo Yang[a,b,*]

[a] Key Laboratory of Rice Genetic Breeding of Anhui Province, Rice Research Institute, Anhui Academy of Agricultural Sciences, Hefei 230031, China
[b] Institute of Technical Biology and Agriculture Engineering, Chinese Academy of Sciences, Hefei 230031, China

ARTICLE INFO

Article history:
Received 9 November 2014
Received in revised form
23 December 2014
Accepted 4 January 2015
Available online 10 January 2015

Keywords:
Heat response
Aconitase
W-box
WRKY
Promoter truncation

ABSTRACT

Respiratory metabolism is an important though poorly understood facet of plant adaptation to stress. Posttranslational modification of aconitase, a component of the tricarboxylic acid cycle (TCA), may be involved in stress tolerance. However, such stress-related transcriptional regulation and its mechanism remain unknown. In this study, we found that expression of the rice *Aconitase* gene *OsACO1* is induced in a time-dependent manner by heat but not other typical abiotic stresses. To analyze the transcriptional regulation mechanism underlying the response to heat, the *OsACO1* promoter (P_{OsACO1}) was isolated and characterized in transgenic rice. Using qualitative and quantitative analyses, we found that the expression of the *GUS* reporter gene responded to heat in different tissues and at different stages of development when driven by P_{OsACO1}. A series of 5′ distal deletions of P_{OsACO1} was generated to delineate the region responsible for heat-induced gene expression. Transient expression analyses in tobacco leaves identified a 322-bp minimal region between −1386 and −1065 as being essential and sufficient for heat-induced expression by P_{OsACO1}. We screened for known heat response-related *cis*-elements in this 322-bp region; however, sequences correlating with heat-induced gene expression were not identified in P_{OsACO1}. Therefore, truncations and successive mutagenesis analyses were performed in this 322-bp region. By comparing the activities of promoter fragments and their derivatives, our results indicated that the heat response element resided in a 9-bp region between −1132 and −1124, a sequence that contains a W-box motif. Additional site-directed mutagenesis analyses eliminated the heat response activity of P_{OsACO1} via the W-box element, and an electrophoretic mobility shift assay (EMSA) indicated the binding of P_{OsACO1} by factors in the nuclear extracts of heat-stressed rice seedlings in a W-box-dependent manner. Our results illustrate the expression pattern of a key component of the TCA response to abiotic stress and establish a putative regulatory pathway in the transcriptional modulation of rice respiratory metabolism genes in response to heat.

1. Introduction

Rice is a major staple food in Asia and feeds more than half of the world's population. Rice is grown mainly in tropical and subtropical zones. High temperature (or heat) stress is a major limitation of rice growth and production. Indeed, the germination of rice seeds is interrupted by incubation under heat, and heat stress dramatically retards rice development in vegetative growth stages, resulting in severely abnormal root branching patterns and tillering durations [1]. Rice is most susceptible to heat injury during flowering [2]. Pollen grain swelling is particularly sensitive to heat stress, and swelling failure results in floret sterility and directly limits grain yield [2–4]. The presence of greenhouse gasses such as carbon dioxide, which have increased in the atmosphere

Abbreviations: TCA, tricarboxylic acid cycle; P_{OsACO1}, *OsACO1* promoter; EMSA, electrophoretic mobility shift assay; IRPs, iron-regulatory proteins; CSD2, Cu/Zn superoxide dismutase 2; MV, methyl viologen; 4-MU, 4-methylumbelliferone; HS, heat shock; HSE, heat shock element.

* Corresponding authors at: Key Laboratory of Rice Genetics Breeding of Anhui Province, Rice Research Institute, Anhui Academy of Agricultural Sciences, Hefei 230031, China. Tel.: +86 551 65160535; fax: +86 551 65145277.
E-mail addresses: weipengcheng@gmail.com (P.-C. Wei), yjianbo@263.net (J.-B. Yang).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.plantsci.2015.01.003
0168-9452/

following the worldwide industrialization over the last century, may be responsible for rising global temperatures [3]. Therefore, the generation of rice with high heat tolerance has become a breeding target in the near future [5].

Carbon metabolism and energy production are re-balanced in response to abiotic stress [6]. Aconitase, an important component of the tricarboxylic acid (TCA) cycle and cytosolic citrate metabolism, catalyzes the reversible hydration of *cis*-aconitate to either citrate or isocitrate. In animals, active aconitase contains an iron–sulfur (4Fe–4S) cluster and can be reversibly inactivated under certain stresses and signals, e.g., reactive oxygen species (ROS), nitric oxide (NO) and low iron concentration, through the loss of the iron–sulfur cluster, and switch to the structure of RNA-binding proteins (iron-regulatory proteins, IRPs) [7,8]. By specifically binding to iron-responsive elements (IREs) in the UTR of ferritin mRNA, IRPs play important roles in regulating iron metabolism. The stress response of aconitase has been primarily investigated in animals and microorganisms. Under stress, aconitase is regulated via one of two putative mechanisms. Stress may lead to the posttranslational inactivation of aconitase, resulting in a protective effect by blocking the TCA cycle, which consequently reduces electron flow through the mitochondrial electron transport chain, decreasing the generation of ROS during respiration [8,9]. Meanwhile, stress-inactivated aconitases could structurally switch to IRPs, which in *E. coli* are able to bind and stabilize *superoxide dismutase* (*SOD*) mRNA, and inactivated aconitases thus should enhance ROS scavenging activity [10]. Conversely, the selective inactivation of aconitase is thought to induce ROS accumulation due to the over-production of reduced materials in animal cells [11]. Therefore, aconitase should be transcriptionally induced to avoid excess ROS generation and response to the higher energy requirement of cells under stress [12].

Aconitases have been detected in various plant species and exhibit structural and biochemical characteristics similar to their animal counterparts [13]. *Arabidopsis aconitase* (*AtACOs*) genes have an amino acid sequence similarity of approximately 80% with human IRPs, and the tobacco gene *NtACO1* has a similarity of more than 75% with IRPs [14,15]. The inhibition of aconitase activity in *Arabidopsis* or mutations in *AtACO* result in disturbances in the TCA cycle or in cytosolic citrate metabolism [16,17]. Previous studies have also demonstrated the involvement of plant aconitases in stress responses. For instance, the inactivation of aconitase leads to significant increases in the expression of alternative oxidase [16], a major target and regulator of mitochondrial stress responses [18,19]. Similar to their homologs in animals, plant aconitases are targeted by stress signaling molecules [9,15], and several studies have revealed that the enzymatic activity of aconitase is rapidly inhibited by ROS or NO in different plant species [9,15,20,21]. Although the structural switch that is triggered by inactivation has not been identified in plants, it has been suggested that aconitase binds to mRNA due to the presence of highly conserved functional residues [21,22]. For example, AtACO is unable to regulate iron homoeostasis, but evidence suggests that it can bind to the 5′ UTR of chloroplastic *Cu/Zn superoxide dismutase 2* (*CSD2*) mRNA to enhance stress resistance [14,21].

Although the posttranslational modification of aconitase during environmental stress responses has been extensively studied, the transcriptional regulation of plant *Aconitase* genes and the underlying mechanisms are poorly understood. In this study, we identified a rice *Aconitase* gene (*OsACO1*, *LOC_Os08g09200*) that is upregulated in response to heat stress. Our results suggest that a W-box element located in its promoter participates in the regulation of heat stress, providing new insight into how *Aconitase* is transcriptionally modulated. These findings may help improve the understanding of the molecular regulation mechanisms underlying the changes in plant respiratory metabolism during stress responses.

2. Materials and methods

2.1. Plant materials and treatments

Rice seedlings (*Oryza sativa* L. ssp. *japonica*) were grown on 1/2 Murashige and Skoog (MS) medium and incubated under a light/dark cycle of 16 h/8 h at 28 °C. Seedlings (10 days after germination, DAG) were exposed to various stress treatments. For salinity, osmotic and oxidative stress treatments, seedlings were irrigated with 1/2 MS liquid culture containing 150 mM NaCl, 300 mM mannitol or 5 μM methyl viologen (MV), respectively. For cold and heat treatments, seedlings on agar plates were exposed to 4 °C, 37 °C or 42 °C. Treated seedlings were harvested at the indicated times and stored at −80 °C until RNA extraction.

2.2. RNA extraction and qRT-PCR analysis

Total RNA samples were extracted from plant materials using an RNAprep Pure Plant Kit (TIANGEN, China) in accordance with the manufacturer's instructions. Total RNA samples were extensively pre-treated with RNase-free DNase I to eliminate contaminating genomic DNA. First-strand cDNA was synthesized from 1 μg RNA using the FastQuant RT Kit (TIANGEN, China). qRT-PCR was performed using an ABI PRISM 7500 real-time PCR system (Applied Biosystems, USA) with SYBR Green (TIANGEN, China) according to the manufacturer's protocol. The rice gene *ACTIN* was selected as an internal standard. qRT-PCR assays were repeated for each gene at least twice, with three replicates for each repetition.

2.3. Vector constructs

A region of the putative promoter of *OsACO1* (P_{OsACO1}) was PCR-amplified from rice (*O. sativa* L. ssp. *japonica*) genomic DNA using the primers P_{OsACO1}-FP and P_{OsACO1}-RP. Cloned fragments were confirmed by restriction enzyme digestion and DNA sequencing and inserted upstream of the *GUS* gene in the binary vector pCAMBIA1391 using *Hin*dIII and *Sal*I sites.

To generate truncated promoters, different forward primers (F1, F2, F3 and F4) and the reverse primer P_{OsACO1}-RP were used to amplify fragments of P_{OsACO1}; these fragments were fused upstream of GUS through *Hin*dIII and *Sal*I digestion to generate the P_{Tru1}, P_{Tru2}, P_{Tru3} and P_{Tru4} constructs, respectively.

To construct chimeric promoters using the P_{OsACO1} fragment, we first inserted a 46-bp CaMV 35S minimal promoter [23] into the *Hin*dIII/*Bam*HI site of the pCAMBIA1391 vector; this construct was named P_{mini}. Fragments representing the regions −2000 to −1386 (615 bp), −1386 to −1065 (322 bp), −1255 to −1065 (191 bp), −1173 to −1065 (109 bp) and −1140 to −1065 (76 bp) of P_{OsACO1} were cloned into the *Hin*dIII/*Sal*I sites of P_{mini}, generating the P_{Tru5}, P_{Tru6}, P_{Tru7}, P_{Tru8} and P_{Tru9} reporter constructs, respectively. The fragment representing position −1108 to −1065 (44 bp) of P_{OsACO1} was synthesized and ligated into the binary vector P_{mini} using *Hin*dIII/*Sal*I digestion to produce the P_{Tru10} construct.

To generate block mutations in P_{Tru9} (P_{Tru9}-M1, P_{Tru9}-M2, P_{Tru9}-M3, and P_{Tru9}-M4), oligonucleotides with targeted nucleotide substitutions were synthesized and ligated into the binary vector using *Hin*dIII/*Sal*I digestion. To generate site-directed mutations in P_{OsACO1} and P_{Tru6}, the Quick Change site-directed mutagenesis kit (Transgene, China) was used. Mutagenic primer pairs were used to generate target mutations within P_{OsACO1} and P_{Tru6} according to the manufacturer's protocol. After mutagenesis was confirmed by sequencing, the obtained mutated fragments were digested with *Hin*dIII and *Sal*I and ligated into the binary vector. These constructs were designated P_{OsACO1}-MA, P_{OsACO1}-MB, P_{OsACO1}-MC, P_{OsACO1}-MD, P_{OsACO1}-ME and M-P_{Tru6}.