Plant Physiology and Biochemistry 49 (2011) 9–12

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

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

Research article

# Cycloheximide-mediated superinduction of genes involves both native and foreign transcripts in rice (*Oryza sativa* L.)

Manu Agarwal [1], Amanjot Singh, Dheeraj Mittal, Chandan Sahi [2], Anil Grover*

*Department of Plant Molecular Biology, University of Delhi South Campus, New Delhi 110021, India*

ARTICLE INFO

*Article history:*
Received 17 March 2010
Accepted 22 September 2010
Available online 1 October 2010

*Keywords:*
Cycloheximide
Heat shock
Promoter
Rice (*Oryza sativa* L.)
Superinduction
Transcripts

ABSTRACT

Rice seedlings subjected to heat shock show rapid and transient induction of *Oshsp17.4-CI*, *Oshsp17.9A-CI* and *OsClpB-cyt/hsp100* transcripts. When the seedlings were pre-treated with protein synthesis inhibitor cycloheximide, levels of the above transcripts during heat shock were more elevated than those seen with heat shock alone. Heat stress and cycloheximide co-treatment resulted in higher transcript accumulation in comparison to cycloheximide pre-treatment followed by heat stress. In transgenic plants raised with *OsClpB-cyt/hsp100* promoter driving expression of the reporter *gus* gene, expression of the *gus* transcript was subjected to similar superinduction event as was seen with native *OsClpB-cyt/hsp100* transcripts in untransformed plants. Yeast cells transformed with variably-sized rice *ClpB-cyt/hsp100* promoter driving expression of the *lacZ* reporter transcript showed that specific sequences of the promoter region may be implicated in superinduction.



## 1. Introduction

Superinduction of gene expression refers to a phenomenon in which protein synthesis inhibitors (such as cycloheximide, anisomycin, α-amanitin, etc) accentuate and prolong the induction of genes that are normally only transiently induced by other factors [11,14]. In mammals, cycloheximide (CHX) and anisomycin are shown to super-induce several stress responsive protein kinases [3,21]. Protein synthesis inhibitors also elevate and prolong the normally transient induction of the proto-oncogenes *c-jun* and *c-fos* in mammalian cells [5]. In plants, Franco et al. [6] reported that small auxin-up RNAs (*SAURs*) are super-induced if 2,4-D is added under conditions where protein synthesis is inhibited. Auxin induced *par* gene in tobacco mesophyll protoplasts also belongs to the category of superinduction genes [19]. ABA induced *hva22* gene is induced by either ABA or cycloheximide and addition of both the inducers to barley aleurone layers have a synergistic effect on the expression [16]. Horvath and Chua [7] noted that *IS10a* cDNA clone (protein encoded by *IS10a* shows sequence similarity to UDP-glucose:flavanoid glycosyl transferases) is expressed in response to salicylic acid (SA) as well as CHX. In combination, CHX and SA caused superinduction of *IS10a* mRNA levels that was also sustained. Berberich and Kusano [2] showed that *ZmCDPK1*, *mlip15* and *Adh1* transcripts are induced at low temperature as well as by sub-inhibitory concentrations of CHX, in maize. Recently, Kimura and Nambara [8] showed in *Arabidopsis thaliana* that down-regulation of *ABI2*, *ABI5*, *CP29*, *RD29B* and *At2g42560* (*At2g42560* mRNA corresponds to a LEA domain containing protein) genes by imbibition was partially alleviated by CHX treatment while up-regulation of *BME3*, *CYP707A2*, *GSTU22*, *HSP23.5* and *NPR3* genes by imbibition was not affected by CHX. These authors argued that the response of varied genes to CHX may be related to the ability of the CHX to inhibit translational machinery of the cells, i.e. inhibition of translation precociously activated transcription possibly using stored transcriptional machinery in case of imbibition down-regulated genes and induction of imbibition up-regulated genes at specified time points may not be requiring *de novo* protein biosynthesis.

Detailed studies to understand superinduction phenomenon have mostly been carried out in animal systems [10]. The superinduction phenomenon has been attributed to the loss of mRNA degrading enzymes and labile transcriptional repressors as a consequence of inhibition of protein synthesis in these systems. In plants, Li et al. [9] showed that superinduction of *SAURs* occurs through increase in stabilities of these mRNAs. Transcripts of *CBF* genes have been seen to accumulate rapidly in response to low temperature in *Arabidopsis*. Importantly, Zarka et al. [20] identified two elements called ICEr1 (inducer of *CBF* expression region 1; CACATG) and ICEr2 (inducer of CBF expression region 2; AGAAACTCG) in *CBF2* promoter which were shown to be responsible for CHX mediated regulation of the downstream gene.

* Corresponding author. Tel.: +91 11 24115097; fax: +91 11 24115270.
*E-mail address:* anil.anilgrover@gmail.com (A. Grover).
[1] Present address: Department of Botany, University of Delhi, New Delhi, India.
[2] Present address: Department of Biochemistry, University of Wisconsin-Madison, WI, USA.

0981-9428/$ – see front matter © 2010 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.plaphy.2010.09.010

Heat shock (HS) genes (*hsp*) are rapidly induced in response to heat as well as a host of other stresses, in organisms ranging from bacteria to higher eukaryotes [18]. In this study, we show that heat shock genes in rice are regulated by CHX. We further show that the CHX mediated regulation is applicable to both native as well as foreign genes. We further note that the presence of specific elements in the promoter region may have a role in this process.

## 2. Materials and methods

### 2.1. Rice tissues, stress treatments, RNA isolation and Northern analysis

Rice [*Oryza sativa* L. var. Pusa169] seeds were surface-sterilized by washing with a mild detergent, treating with 70% ethanol (for 2 min), washing thoroughly with water, subsequently treating with 1.2% sodium hypochlorite solution (for 30 min) and finally rinsing several times with water to remove traces of sodium hypochlorite. Seeds were sown on wet cotton, in plastic trays which were placed in dark (for 2 d) and then in light for varied periods under culture room conditions ($26 \pm 2$ °C). Six to seven days old intact uniform-sized rice seedlings were selected and transferred from plastic trays to 100 ml glass beakers. Seedlings were placed on a cotton pad soaked with distilled water. These beakers were placed for 24 h in a culture room for acclimatization. Seedlings were treated with different concentrations of CHX as per the experimental design. During CHX pre-treatment, the above beakers were kept under the culture room conditions. Seedlings were subjected to HS for various specified temperatures and time intervals by placing the beakers in water baths maintained at the requisite temperatures.

Rice has three ClpB/Hsp100 proteins namely Clp-B cytoplasmic (ClpB-cyt), ClpB-mitochondrial (ClpB-m) and ClpB-chloroplastic (ClpB-c) [17]. We previously made transgenic plants employing a plasmid construct containing 724 bp *OsClpB-cyt/hsp100* promoter sequence (from the transcription initiation site; lacking 5′UTR region of the *OsClpB-cyt/hsp100* except 10nt from the 5′end of the 5′UTR) driving expression of the reporter gene *gus* (724bpPro$_{OsClpB\text{-}cyt/hsp100}$::Gus; [1]). Analysis of the transgenic $T_1$ 724bpPro$_{OsClpB\text{-}cyt/hsp100}$::Gus plants showed heat inducible *gus* transcript [1]. For analyzing role of promoter in CHX application, leaf segments of 724bpPro$_{OsClpB\text{-}cyt/hsp100}$::Gus plants were floated on CHX solution in a Petri dish. HS was given by placing the Petri dish in an incubator shaker.

Total RNA was isolated from the control and stressed tissues from Pusa 169 as per the standard protocol [4]. For Northern blotting, 10 μg of RNA was transferred to nylon membrane and probed with labeled $\alpha P^{32}$-dCTP probes from different genes as indicated in the text.

### 2.2. Deletion constructs with 696bpPro$_{OsClpB\text{-}cyt/hsp100}$ promoter and transformation in yeast

To reveal the role of the predicted ICEr1 sequence present in *OsClpB-cyt* promoter in CHX mediated superinduction, 696 bp region upstream of translation initiation codon was PCR-amplified (primer sequences shown in Supplementary data 1) cloned upstream of CYC1 minimal promoter in EcoRI and XhoI sites in pLacZi vector. The construct was linearized using NcoI and transformed into yeast strain A2279 (YGRC, Japan) and transformants (696 bp-CYC1::lacZ) were selected on a medium lacking uracil. Similarly, 600 bp and 500 bp fragments of *OsClpB-cyt/hsp100* promoter amplified by PCR were also cloned into pLacZi and transformed into A2279 strain (to yield 600 bp-CYC1::lacZ and 500 bp-CYC1::lacZ cells, respectively). For examining the expression levels, culture of transformed cells were initiated and overnight cultures were diluted to an OD of 0.1 and fresh cultures were induced in duplicate. In one set, 20 μg ml$^{-1}$ CHX (concentration used was same as Nakagawa et al. [13]) was added while the control set lacked CHX. Cultures were grown for 16 h, subsequently cells were pelleted and RNA was isolated as per standard protocols [15]. RNA was quantified and 5 μg RNA was used for cDNA synthesis using polyT primer mediated by MuMLV RT (MBI Fermentas). For subsequent PCRs, primers specific to *lacZ* and yeast *actin* were used. Amplicons were resolved on 1% agarose gel.

## 3. Results

### 3.1. Effect of pre-treatment of CHX on induction of hsp genes

Intact rice seedlings pre-treated with 5 μg ml$^{-1}$ or 10 μg ml$^{-1}$ CHX (as against $H_2O$ control) were exposed to HS at 42 °C for 2, 4 and 8 h. Levels of *Oshsp17.4-CI*, *Oshsp17.9A-CI* and *OsClpB-cyt/hsp100* were examined by Northern blotting. Expression of all 3 transcripts was noted to be highest at 2 h sampling stage, followed by a continuous decline thereafter. At all these time points, levels of *hsp* transcripts were appreciably higher in seedlings pre-treated with CHX than $H_2O$ control. At higher concentration of 10 μg ml$^{-1}$ CHX, levels of all three transcripts were more elevated than 5 μg ml$^{-1}$ CHX or $H_2O$ treatments (Fig. 1A). CHX concentration as low as 5 ng ml$^{-1}$ also showed elevated levels of the *hsp* transcripts as against $H_2O$ control (Fig. 1B).

### 3.2. Effect of co-treatment of CHX on induction of hsp genes

Rice seedlings were exposed to 42 °C for varied time intervals in the presence of CHX (treatment initiated 2 h prior to HS) of CHX. As control, seedlings given only pre-treatment of CHX or placed in water during pre-treatment as well as during stress were analyzed. Levels of Oshsp17.9A-CI transcripts at different time points showed highest levels in seedlings subjected to CHX pre-treatment and

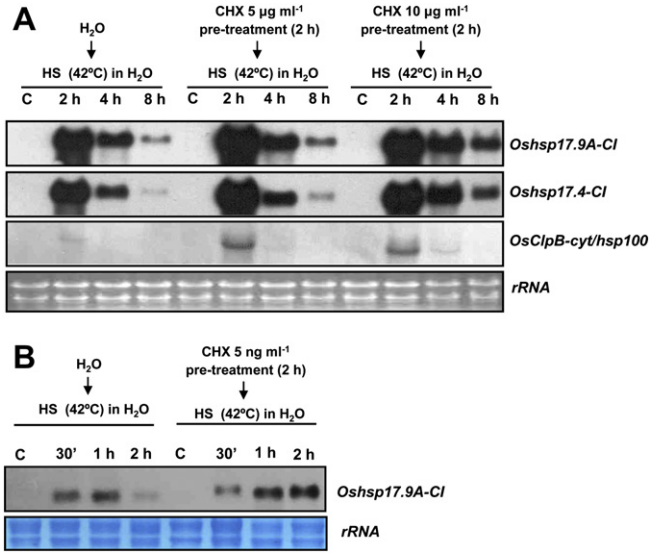


**Fig. 1.** Assay of the superinduction effect by Northern blotting, in response to pre-treatment of seedlings with cycloheximide. Treatment details are shown in the panels. CHX treatment was given for for 2 h. HS was given at 42 °C. Ten micrograms of RNA were resolved on 1% denaturing gel and transferred to nylon membrane. The membrane was probed with full-length labeled probes of *Oshsp17.9A-CI*, *Oshsp17.4-CI* and *OsClpB-cyt/hsp100* separately. C: control seedlings placed under culture room conditions. Equal quantity of RNA loading is depicted by comparable intensities of ethidium bromide stained bands of rRNA as shown in the lowermost panel. Cycloheximide concentrations were different in A and B panels as indicated. The membrane was probed with 3′ UTR probe of *Oshsp17.9A-CI* in case of panel B.