Indian Journal of Agricultural Sciences **83** (4): 386–90, April 2013/Article

CP-HSP gene transcribe in rice (*Oryza sativa*) under heavy metal stress

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Received: 18 May 2011; Revised Accepted: 5 February 2013

ABSTRACT

Chloroplast small heat shock protein (CP-sHSP) genes activate in different plants when under stress. Present investigation revealed that chlorophyll content decreased in rice (*Oryza sativa*) when under heavy metal stress. Total chlorophyll content decreased up to 36.61% and 42.6% when rice plants were grown at 20mM cadmium and nickel heavy metals, respectively. However, copper stress did not drop the chlorophyll content at threshold level. Transcriptome analysis shows that CP-HSPs gene transcribes in heavy metals stressed plants. Based on these observations, CP-HSPs gene was polymerized using standard primer and sequenced. The sequence alignment with CP-HSPs from gene bank divulges that *Oryza sativa* CP-HSP gene has 100% similarity with *Arabidopsis* CP-HSP 21 gene. While others; such as from other monocots and dicots, have varying resemblance. Translational sequence was proposed based on gene sequence. Phylogenetic analysis based on gene and protein sequences demonstrate evolutionary relationship of *Oryza sativa* CP-HSP with other monocots and dicots.

Key words: CP-sHSPs gene, mRNA expression, *Oryza sativa*, PCR, RT-PCR, Sequencing

Abiotic stresses adversely affect the growth and productivity of plants (Mathur *et al.* 2008). During abiotic stress, a group of genes turned on that increase the levels of secondary metabolites and proteins, which may be responsible for conferring a certain degree of protection to these stresses (Allen 1995). Heavy metal stress is a major cause of producing toxicity in plants. If they accumulate at high level in plant tissue, they can influence growth, senescence and metabolic processes (Maksymiec 2007). Their effect may vary species to species, e g copper negatively affects light reactions (Patsikka *et al.* 2002) and CO_2-fixation reactions (Moustakas *et al.* 1994). Cadmium is toxic to all organisms. It can be easily taken up by plants. The reduction of biomass by cadmium toxicity could be the direct consequence of inhibition of chlorophyll synthesis (Padmaja *et al.* 1990) and so on photosynthesis (Baszynski *et al.* 1980). Nickel is considered to be an essential micronutrient for plants; however at additional concentration this metal becomes toxic for many plant species. The most common symptoms of nickel toxicity are the inhibition of plant growth, wilting and necrosis (Nakazawa *et al.* 2004).

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Generally, at elevated level of heavy metal, plant increases the synthesis of different heat shock proteins (HSPs). There is evidence that HSPs function to protect cell membranes from this heavy metal damage (Hall 2002). The chloroplast small HSPs (CP-sHSPs) protect photosynthesis during stress conditions (Hamilton and Heckathorn 2001). The CP-sHSPs are produced when heavy metals accumulate in the chloroplast and damage photosynthesis process. The CP-sHSPs protect the photosynthesis from excess of heavy metals stress (Heckathorn *et al.* 2004). This response is mediated by heat shock transcription factor which bind to promoters of heat shock genes. The induction of gene encoding HSPs was one of the most prominent responses observed at molecular level of organisms exposed to high temperature (Vierling 1991).

When plants exposed to heavy metals stress, a decrease in their growth and reproduction occur (Heckathorn *et al.* 2004). The CP-sHSPs protects photosynthetic electron transport chain during heat, oxidative and photo inhibitory stresses. The CP-sHSPs synthesized in cytoplasm and transported to the chloroplast. The current study was to quantify the chlorophyll content and expression gene of CP-sHSP in *Oryza sativa* and its expression after heavy metals stress, i e cadmium, nickel and copper stress.

MATERIALS AND METHODS

Seeds of *Oryza sativa* variety KS-282 were collected from NARC (Pakistan). Germination of seeds was started on

sterilized wet filter papers in petriplates placed in growth chamber. After 3 days of germination, plantlets shifted to the pots and heavy metals treatment was carried out at 15th day of their germination in the pots.

Heavy metal stress was induced by using pot method (Heckathorn *et al.* 2004). According to this method, plants growing in pots with normal nutrients solutions (10mM/L NH_4NO_3, 2mM/L Na_2HPO_4, 3mM/L $CaCl_2$, 2mM/L $MgSO_4$, 1mM $MnCl_2$, 10µM/L $FeSO_4$, 1µM/L H_3BO_3, 1µM $(NH_4)_2MnO_4$, 2µM/L NaCl, 1mM/L EDTA) were directly exposed to different concentrations [0mM, 0.5mM, 2mM, 5mM, 10mM, 20mM] of cadmium, nickel and copper salts ($CdCl_2$ $2.5H_2O$, $NiCl_2$ $2H_2O$ and $CuCl._2H_2O$). After 5 days of treatment, 0.5g of leave tissues were taken and immersed in DMSO at 65°C for 1 hr, to extract the chlorophyll (Hiscox and Israelstam 1979). Optical density of the extracts were measured at 645 nm and 663 nm by Agilent spectrophotometer (Model-8453) and total chlorophyll contents were calculated (Arnon 1949). Statistical significance of the results was measured using ANOVA.

Total chlorophyll contents=$20.20(OD_{645}) + 8.02(OD_{663})$.

For RNA isolation, 100 mg of leaf tissue (treated with 0 mM, 5 mM and 20 mM cadmium) was homogenized with 1 ml TRI Reagent. The sample was incubated for 10 min at room temperature, then 200 µl chloroform was added, vortexed and centrifuged at 12000 g for 15 min. Supernatant was transferred to a fresh appendroff tube and 0.5ml isopropanol was added to precipitate RNA. The tubes were kept on ice for 10min and again centrifuged at 8000g for 8min. Supernatant was discarded and pallets were washed twice with 75% ethanol and air dried. Pellets were resuspended in DEPC-water and quantified at 260nm. Genomic DNA was removed from RNA sample by mixing it with 1µL 10X reaction buffer with $MgCl_2$ and 0.5µL of Dnase I. Mixture was incubated for 30min at 37°C. Then 1µL of 25mM EDTA was added and incubated at 65°C for 10min. RNA was separated as discussed above.

RNA transcripts were reverse transcribed into cDNA by Fermentas Kit (Fermentas Revert Aid M-MuLV Reverse Transcriptase kit, Cat. # EP0442), using real time PCR. The reaction mixture (template RNA, oligo dT primers, 5X reaction buffer, ribolock RNase inhibitor, dNTPs Mix and RevertAid M-MuL Reverse Transcriptase) was incubated at 42°C for 60min and then at 70°C for 10min. The reverse transcribed product was stored at –20°C and used for amplification reaction. Primers were designed for CP-sHSPs sequence based on cDNA sequence available at NCBI. PCR was carried out in T3 thermocycler (Biometra, Germany).

Genomic DNA of plant was extracted by using the modified method of Zhang and Stewart (Zhang and Stewart 2000). According to this method, 1g of plant leaves were homogenized in liquid nitrogen, 3ml extraction buffer (100mM T.HCl pH 8, 20mM EDTA, 1M NaCl, 2% PVP-40, 0.002% CTAB, 0.02% phenanthrolein, 0.2% ß-mercaptoethanol) was added in it and sample was incubated for 1 hr at 65°C. Then, 3ml of solution A (chloroform + isoamylalcohol in 24:1) and solution B (phenol) was added in ratio of 1:1 and samples were centrifuged at 12000g for 15min, this step was repeated twice and supernatant was transferred to fresh appendroff tubes. Equal volume of ice-cold isopropanol was added and mixture was kept overnight at –20°C. Thread like DNA was spooled out. The isolated DNA (5µL) was loaded in 1% agarose gel, run for 1 hr at 120 voltages and samples were analyzed by gel imaging system (Syngene Bio Image).

Forward and reverse primers, were designed for CP-sHSPs gene sequence of chloroplast available at NCBI. PCR was performed in 0.2ml PCR tube containing 25 µl total reaction mixture. The mixture contained 2µl DNA sample, 2.5µl 10X buffer (100mM Tris-HCl, pH 8.3, 500mM KCl, 25mM $MgCl_2$), 0.5 µl dNTPs (10mM), 0.5 µl of each forward and reverse primers (200ng/µl), 1.5 µl $MgCl_2$ and 0.5 µl Taq DNA polymerase (Fermentas, UK) in 17µl PCR water. The reaction mixture was vortexed and centrifuge for a few seconds. PCR was carried out in T3 thermocycler (Biometra, Germany). Thermocycling conditions were: 5 minutes of 95°C for denaturation followed by 30 cycles of amplification each consisting of 3 steps: 45 seconds at 94°C for denaturation, 45 seconds at 54°C for annealing and 5 min at 72°C for Taq polymerase to synthesize strands and final holding at 15°C for 15min. The 10µL of amplified product was analysed on 1.5% agarose gel along with 100bp DNA ladder (0 Range Ruler^{TM}, MBI Fermentas, UK). Gel was prepared by melting 0.45g of agarose in 30ml 1XTBE (0.89M Tris-Borate, 0.032M EDTA, pH 8.3). Then 1µl of ethidium bromide was added to stain DNA. Amplified products were analyzed by gel imaging system (Syngene Bio Image).

The PCR product was purified using Fermentas Kit (K05113). Agarose gel slice was dissolved by adding ½ volume of TBE conversion buffer and 4.5 volume of binding solution and incubation at 55°C for 5min. Then suspension of silica powder was added and incubated again for 20min at 55°C. The mixture was spin for 5sec and supernatant was removed. Then pellets were washed thrice and air dried. PCR product was eluted in TE buffer, incubated at 55°C for 5 min and small amounts of silica powder was removed. Purified product was checked again on 2% agarose gel. Sequencing PCR reaction mixture was made by mixing DTCS kit (3µL), primer (1.5µL), template (2µL), betaine (2µL) and water (1.5µL). Sequencing mixture transferred to 1.5ml appendroff tube in which 2.5µL of stop solution (1µL of 3M NaAc+1µL of 100mM Na_2EDTA+0.5µL of 20mg/ml glycogen) was added. Mixture was vortex and centrifuged at 13000 g for 15 min. Supernatant was removed and 70% ice cold ethanol (150µL) was added in each tube. Sample was again centrifuged at 13000g for 15min, supernatant removed and pellets were vacuum dried at 30°C. These pellets were re-suspended with 30µL of sample loading solution (SLS),

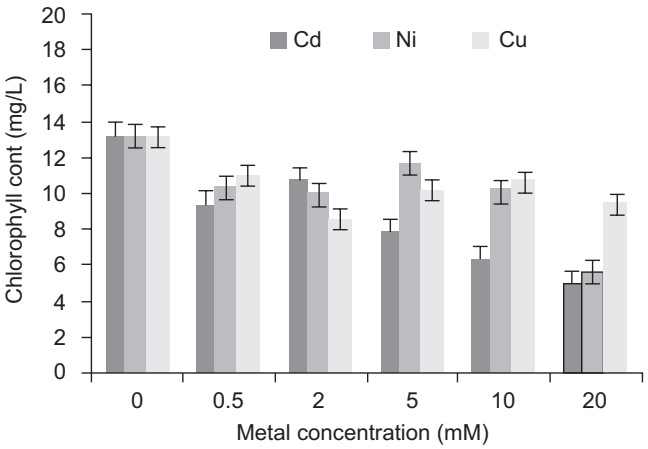

Fig 1 Chlorophyll contents measured under heavy metals stress.

transferred in sample plate (CEQ8800). Sequencing was carried out in Beckman CEQ8800 sequencer at 96°C for 1min, 96°C for 30min, 54°C for 30 sec, 70°C for 4min and 70°C for 10min. The sequences of nucleotides and proteins were compared with the reported sequences by using clustalW.

RESULTS AND DISCUSSION

Heavy metals stress effect the chlorophyll content of the plant (Patsikka *et al.* 2002, Padmaja *et al.* 1990) and impedes the plants growth and productivity by affecting its physiological processes (Mathur *et al.* 2008, Dimpk *et al.* 2009). Under stress, several genes turn on and increase the levels of several proteins. Heavy metal accumulation damages cell components directly by poisoning enzymes and indirectly by oxidative damage. Therefore, the production of certain HSPs might be predicted to be increased (Hall 2002). In current study, *Oryza sativa* plants were exposed to heavy metal stress by using pot method. There was a sharp decrease in chlorophyll contents with the increase of heavy metals concentrations. All three metals significantly affected the chlorophyll contents of the plant but cadmium showed a sharpest decline in chlorophyll content as shown in Fig 1.

The pot method of heavy metal treatment decreased the chlorophyll content in the leave tissues of the plant with respect to control. A bi-phase response of decrease in chlorophyll content was observed at increasing concentration of heavy metal stress. Where, cadmium stress decreased the chlorophyll content from 13.173 mg/L at 0 mM to 4.823 mg/L at 20 mM. This decrease was 36.61% with respect to control. While nickel stress decreased the chlorophyll content from 13.173 mg/L at 0 mM to 5.613 mg/L at 20 mM, showing 42.60% decrease. Comparatively, a less decrease in chlorophyll content took place under copper stress. The results were highly significant and in accordance with the findings of Heckathorn *et al.* (2004). They claimed the decrease in total chlorophyll contents of *Zea mays* in response to the nickel, zinc and copper stress when they used pot method of heavy metal stress. This decrease in chlorophyll contents has been attributed to disturbances in the synthesis of chlorophyll pigment (Stobart *et al.* 1985) as well as increase in its degradation process (Somashekaraiah *et al.* 1992). Cadmium stress decreased the chlorophyll content more sharply and similar finding was reported by another research group. They also reported that cadmium toxicity had reduced the photosynthetic rate in different plant species (Sawhney *et al.* 1990).

In metal stress experiment, cadmium greatly affected chlorophyll content of plants. So its two concentrations, 5mM and 20mM, were used for CP-sHSP gene expression study. These two concentrations were used to determine the total mRNA transcripts expressed under heavy metal stress. For this, first of all cDNA was synthesized and amplified by using consensus primers. The results showed that CP-sHSP transcripts were present only in treated plants. Higher number of transcripts was observed at cadmium stress of 20 mM while no transcripts was seen in control plant samples. The results showed that rice CP-sHSP production is regulated at transcription level, under metal stress. These results are in the agreement with another finding of another research group (Steiner *et al.* 1998). They reported, the level of CP-sHSPs mRNA was dependent on the metal ion concentration. All these reports suggested that metal stress treatment expressed the CP-sHSPs gene in plants. Our findings were also supported by the reports of some other laboratories which described that cadmium chloride increased the cellular HSPs (Somji *et al.* 2002).

The spooled out genomic DNA was amplified by using consensus primers (consensus 1 and Met-5), designed by downloading the CP-sHSP of some plants and aligned them on Bioedit software. Amplified product was sequenced, containing approximatelyv144bp as shown in Fig 2. Table 1 showed the similarity of CP-sHSPs sequence with many CP-sHSPs sequences present on NCBI database like that of *Seneio crataegifolius, Rhododendron simsii, Gossypium arboreum, Lycopersicon esculentum, Persea kusanoi, Capsicum annuum, Epilobium amurense, Nothaphoebe konishii, Triticum aestivum, Agrostis stolonifera, Cinnamomum philippinense* and *Machilus mushaensis.* The CP-sHSPs of *Arabidopsis thaliana* showed 100% identity with new rice CP-sHSP and 87% similarity with *Senecio crataegifolious*.

To examine the evolutionary relationship of CP-sHSPs sequence of *Oryza sativa* with other CP-sHSPs, already present

GCACGACGTCGCGACTAGGGGTTCCCCGCCACTCCGCGGACGTCGCCGGCACGG
GCGAGGTGCGGATGCCGTGGGACGTCATGGAGGACGACAAGGAGGTGAGGATG
CGGTTCGACATGCCGGGCCTGTCGCGGGAGGAGGTGA

Fig 2 Genomic sequence of *O. sativa* CP-sHSP fragment approximately 144bp.

Table 1 BLAST results of newly isolated CP-sHSPs gene of *Oryza sativa*

Accession	Description	Max. score	Total score	Query cov.	E value	Max. identity
EU289266.1	*Arabidopsis thaliana* isolate CS1622 CP-sHSP 21 (HSP21) gene, complete sequence; nuclear gene for chloroplast product	2111	2111	100%	0.0	100%
DQ337173.1	*Senecio crataegifolius* CP-sHSP gene, partial cds; nuclear gene for chloroplast product	1009	1009	69%	0.0	87%
AM392507.1	*Machilus mushaensis* partial CP-sHSP gene for chloroplast small heat shock protein, clone 2	87.8	87.7	67%	1e- 14	79%
AM392509.1	*Cinnamomum philippinense* partial CP-sHSP gene for chloroplast small heat shock protein, clone 2	87.7	87.7	67%	1e- 14	79%
AM392503.1	*Rhododendron simsii* partial CP-sHSP gene for chloroplast small heat shock protein	215	215	28%	3e-52	74%
AF097658.1	*Triticum aestivum* heat shock protein HSP26 (HSP26.6) mRNA, HSP26.6-i allele, nuclear gene encoding chloroplast protein, complete cds	60.8	119	11%	1e- 05	80%
DQ975269.1	*Gossypium arboreum* alpha-crystalline heat shock protein mRNA, complete cds	212	212	34%	4e-51	71%
AY153760.1	*Agrostis stolonifera* var. palustris chloroplast low molecular weight heat shock protein HSP26.8 gene, complete cds; nuclear gene for chloroplast product	134	134	86%	8e- 29	83%
U66300.1	*Lycopersicon esculentum* chloroplast heat shock protein (HSP21) mRNA, complete cds; nuclear gene for chloroplast product	183	183	33%	2e-42	70%
AM392514.1	*Nothaphoebe konishii* partial CP-sHSP gene for chloroplast small heat shock protein, clone 1	60.8	60.8	4%	1e- 05	83%
EF467641.1	*Epilobium amurense* chloroplast small heat shock protein gene, complete cds; nuclear gene for chloroplast product	73.4	116	14%	2e- 09	79%
AM392506.1	*Persea kusanoi* partial CP-sHSP gene for chloroplast small heat shock protein, clone 2	136	136	21%	2e-28	71%
EU311416.1	*Capsicum annuum* chloroplast small heat shock protein mRNA, partial cds; nuclear gene for chloroplast product	84.2	84.2	8%	1e- 12	78%

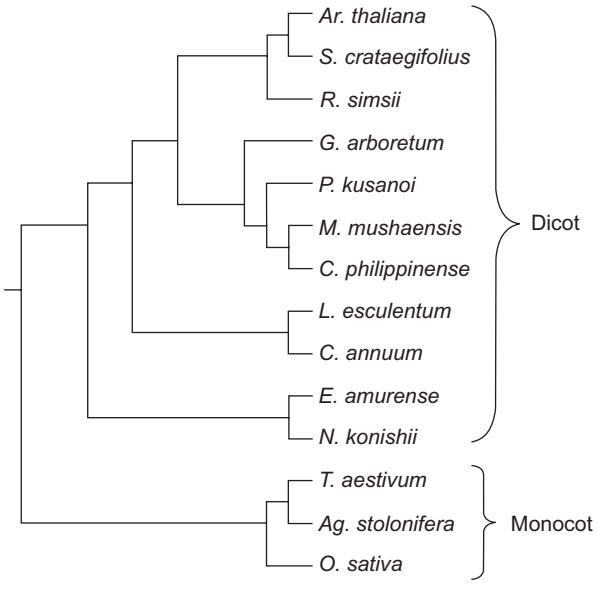

Fig 3 Phylogenetic tree based on nucleotide sequence of CP-sHSPs of *Oryza sativa* with selected sequences from NCBI database.

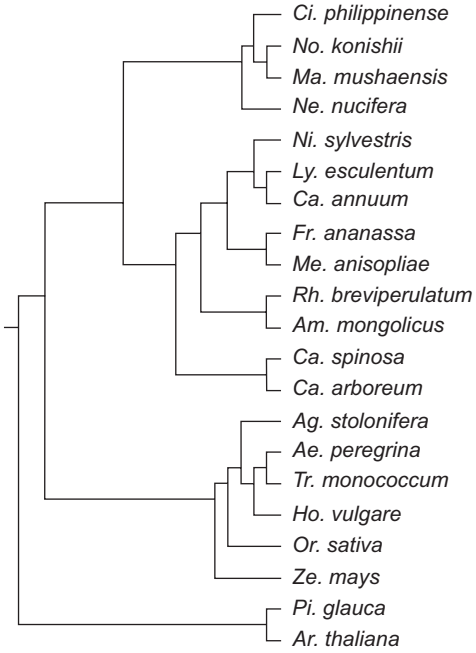

Fig 4 Phylogenetic tree based on amino acids of CP-sHSPs of *Oryza sativa* with selected proteins from NCBI database.

on database, a phylogenetic tree was constructed by using clustralW. The Fig 3 showed evolutionary relationship of new sequences CP-sHSP with already present CP-sHSPs sequences of NCBI database. It was cleared that *Oryza sativa* CP-sHSPs is place with other monocot like *Aegilops variabilis, Triticum aestivum, Agrostis stolonifera.* The amino acid sequence of CP-sHSPs gene was also used to construct phylogenetic tree by using clustralW. A phylogenetic tree of amino acids sequence was drawn as shown in Fig 4. The phylogenetic tree showed the evolutionary relationship of newly sequenced CP-sHSP with other sequences of NCBI database.

This study showed that cadmium and nickel stress significantly decreased the chlorophyll content of the leaves. In which cadmium decreased the chlorophyll content more sharply. Cadmium stress increased the transcripts of CP-sHSP gene at its increasing concentration as well. Furthermore, CP-sHSP gene was also sequenced to determine its similarity with sequence CP-sHSP gene of other plant present at NCBI database. A phylogenetic tree was constructed by using clustralW showing that *Oryza sativa* CP-sHSPs is placed with other monocot.

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