Analysis of bHLH coding genes of *Cicer arietinum* during heavy metal stress using biological network

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Abstract bHLH family of transcription factors play important role in regulating many cellular and physiological functions in plants. These proteins are also known to be involved in response to several abiotic stress types. *Cicer arietinum* is an important source of protein in food across the globe. Considerable differential expression in the bHLH family of proteins during heavy metal exposure in *Cicer arietinum* was observed by microarray data analysis. The study aimed to construct a Pearson coefficient correlation based network of bHLH coding genes in the plant. Microarray data of *Cicer arietinum* recorded under cadmium and chromium stress (GSE86807) from GEO at NCBI was used for analysis. The network constructed from expression data set of the 85 bHLH coding genes revealed 10 hub genes that are connected with topological genes. These hub genes are stress responsive genes that may also be regarded as the marker genes for heavy metal response. Our analysis reported a new set of reference genes (hub genes) that have potentially significant role in development of stress tolerant crops.

Keywords Biological network · Microarray · Hub genes · Abiotic stress

Introduction

Transcription factors play significant role in enhancing the expression of genes that provide tolerance during abiotic stress (Udvardi et al. 2007; Agarwal and Jha 2010). It has been reported that bHLH, a family of transcription factors, has 133 members in *Arabidopsis thaliana* which is one of the largest genes family having key role in tissue development along with plant metabolism (Heim et al. 2003). The bHLH protein consist of two regions i.e. basic region and helix loop helix (HLH) region (Toledo-Ortiz et al. 2003). In *Arabidopsis* bHLH has 167 genes, in *Oryza sativa* 177 genes, in *Nietana tabacum* 190 and in *Vitis vinifera* 191 genes are identified as coding genes for the protein (Jaillon et al. 2007; Rushton et al. 2008; Carretero-Paulet et al. 2010). bHLH members are involved in light signaling (Roig-Villanova et al. 2007; Leivar et al. 2008), hormone signaling (Friedrichsen et al. 2002; Lee et al. 2006), shoot branching (Komatsu et al. 2001), stomata and root development (Ohashi-Ito and Bergmann 2007; Kanaoka et al. 2008) and abiotic stress response (Chinnusamy et al. 2003; Kiribuchi et al. 2004).

In tomato, bHLH is coded by different genes like FER which control the expression pattern of iron-regulated transporter (IRT) and the ferric oxidase reductase (FRO) during deficiency of iron (Ling et al. 2002). Certain orthologs of above mentioned gene have been identified in *Arabidopsis* which are differentially expressed during iron deficiency which induces synthesis of riboflavin reported in different plants (Yuan et al. 2008). It has been already reported that transcription factor bHLH expression varies in *Arabidopsis*, *Brassica*, and *Lycopersicum* during heavy metal stress and play important role (Yuan et al. 2008; Shameer et al. 2009). A study during abiotic stress in mulberry revealed that bHLH had intrinsically disordered

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regions which play important role in cell signaling as well as transcription regulation (Sajeevan and Nataraja 2016). During abiotic stress several genes are differentially expressed at transcription level and regulate the production of certain metabolites which provide tolerance towards heavy metal in plants (Kavar et al. 2008). Transcripts that regulates the genes are of two type, first one is those which codes for protein that provide defense mechanism during abiotic stress while second one are regulatory proteins that regulate the signal and expression of secondary metabolites, such transcripts are called transcription factor (Seki et al. 2002; Shinozaki and Yamaguchi-Shinozaki 2007). It has been reported by Wu et al. (Wu et al. 2012) that AtbHLH38 and AtbHLH39 interact with AtbHLH29 to increase the Cd tolerance of *Arabidopsis* seedlings via decreased cadmium transfer from roots to shoots and to improve the iron homeostasis and concentration of shoots.

Chickpea (*Cicer arietinum*) is one of the most commonly grown legumes in world which serves as an important source of nutrition for human. In many plants bHLH proteins are well characterized but in chickpea the bHLH coding genes are not well studied so far (Castillon et al. 2007). Our study involved 85 coding genes of bHLH transcription factor family in *Cicer arietinum*. Here, we have selected chickpea (ICC4958) grown under control and experimental condition i.e. cadmium (Cd(II)) and chromium (Cr(VI)) at 150 μ M concentration. Plant leaves of control and experimental condition were collected on 15th

day of treatment and microarray experiment has been performed. The genes which are coding bHLH was screened from total number of 34,760 probe present in gene matrix of GSE86807. The screened genes and their expression data was used to construct the biological network to find out most connected genes and their function in different biological processes. The graphical abstract showing full methodology is shown in Fig. 1.

Materials and methods

Microarray data collection

Microarray data of chickpea GSE86807 (Unpublished data) under chromium, cadmium and arsenic treatment at 150 μ M.

Selection of bHLH transcription factor gene from microarray data

Microarray data treated with different heavy metal/metalloids of chickpea had a total number of 34,760 probes. For different transcription factor there are more than one coding gene (Franco-Zorrilla et al. 2014). We have used online tool (<http://www.nipgr.res.in/ctdb.html>) developed by NIPGR, India to find out the coding genes of bHLH genes from 34,760 probes.

Fig. 1 Graphical abstract

Fig. 3 Biological network constructed on the basis of Pearson coefficient correlation using the expression data of chickpea treated with Chromium(VI) at 150 μ M concentration

Table 1 Different parameters of biological network obtained from microarray expression data of chickpea on the basis of Pearson coefficient correlation during exposure of Cd(II) using cytoscape software

Clustering coefficient	0.471
Shortest path	47%
Characteristic path length	1.039
Average no. of neighbors	74.916
Network density	0
No. of nodes	83

Table 2 Different parameters of biological network obtained from microarray expression data of chickpea on the basis of Pearson coefficient correlation during exposure of Cr(VI) using cytoscape software

Clustering coefficient	0.469
Shortest path	46%
Characteristic path length	1.082
Average no. of neighbors	68.86
Network density	0
No. of nodes	82

Hub gene identification and analysis

Top 5 hub genes obtained from biological network of chickpea data under Cd(II) and Cr(VI) treated shown in Tables 3 and 4 along with degree of connectivity. On the basis of connectivity of genes, function of gene can be also explored (Seki et al. 2002). The maximum number of connectivity i.e. degree of gene was 78 and 80 during Cr(VI) and Cd(II) stress respectively. Biological network and hub gene connectivity is shown in Figs. 4 and 5.

Table 3 Hub genes obtained from biological network of Cr(VI) treated data of chickpea along with number of connectivity and clustering coefficient of individual gene

Hub genes	Cluster coff.	Degree
TC03753	0.91341991	78
TC25557	0.91341991	78
TC03912	0.91341991	78
TC12109	0.91341991	78
TC15617	0.91341991	78

Table 4 Hub genes obtained from biological network of Cd(II) treated data of chickpea along with number of connectivity and clustering coefficient of individual gene

Hub genes	Cluster coff.	Degree
TC22481	0.95822785	80
TC19359	0.95822785	80
TC02853	0.95822785	80
TC07875	0.95822785	80
TC11252	0.95822785	80

The expression of observed hub gene was checked and differential expression pattern is shown in Figs. 6 and 7. During Cr(VI) stress in chickpea, among 5 coding gene of bHLH only one gene of transcript id TC2557 was up-regulated, while rest all four i.e. TC03753, TC03912, TC12109, TC15617 were down regulated. While in case of Cd(II) stress, three gene i.e. TC02853, TC07875 and TC19359 were over expressed and two TC11252 and TC22481 were down-regulated.

Fig. 4 Biological networks constructed from expression data of chickpea under chromium treatment along with hub gene i.e. most connected genes shown in red color (color figure online)

Fig. 5 Biological networks constructed from expression data of chickpea under cadmium stress along with hub gene i.e. most connected genes shown in red color (color figure online)

Gene set enrichment analysis

The hub genes during Cd(II) and Cr(VI) treatments in chickpea were used for finding homologous gene in *Arabidopsis* using nBLAST (Altschul et al. 1990) tool. The homologous gene of *Arabidopsis* plant and corresponding transcript id of chickpea are shown in Tables 5 and 6 and these genes were used to perform GSEA. Results of GSEA suggested that genes which are differentially expressed during Cr(VI) treatment are involved in transcription factor activity, DNA binding activity, regulation of microtubule biosynthesis process, regulation in cellular synthesis, regulation nitrogen metabolism at 5% level of significance, as shown in Fig. 8. Furthermore, the hub gene under Cd(II) treatment are mainly involved in transcription regulation, DNA binding activity, regulation of biological process and

Fig. 6 Differential gene expression patterns of hub genes obtained from biological networks constructed from expression data of chickpea under chromium treatment

Fig. 7 Differential gene expression patterns of hub genes obtained from biological networks constructed from expression data of chickpea under cadmium treatment

regulation of primary metabolic processes at 5% level of significance, as shown in Fig. 9.

Discussion

The transcripts of plant undergo differential expression during heavy metal exposure (Li et al. 2015). Our work includes the study of correlation between genes under Cd(II) and Cr(VI) treatment. During exposure of Cd(II) and Cr(VI) to chickpea, the expressions of bHLH transcription

Table 5 Hub genes obtained from biological network of Cr(VI) treated data of chickpea along with homologous genes of *Arabidopsis* and their function

Chickpea identifier	<i>Arabidopsis</i> ID	Gene name	Function
TC03753	AT3G57800	bHLH48 like transcription factor(LOC101503950)	Protein dimerization
TC03912	AT1G01260	bHLH13-like (LOC101493372)	Protein dimerization activity
TC12109	AT1G72210	bHLH96 like (LOC101495560)	Protein dimerization activity
TC15617	AT2G42280	bHLH130-like (LOC101496737)	Protein dimerization activity
TC25557	AT1G68810	bHLH30 (LOC101494151)	Protein dimerization activity

Table 6 Hub genes obtained from biological network of Cd(II) treated data of chickpea along with homologous genes of *Arabidopsis* and their function

Chickpea identifier	<i>Arabidopsis</i> ID	Gene name	Gene function
TC02853	AT2G16910	AMS ABORTED MICROSPORES-like (LOC101508137)	Protein dimerization activity
TC07875	AT4G37850	bHLH18-like (LOC101515651)	Protein dimerization activity
TC11252	AT4G34530	bHLH63-like (LOC101493214)	Protein dimerization activity
TC19359	AT1G51140	bHLH122 (LOC101497651)	Protein dimerization activity
TC22481	AT1G12860	ICE1-like (LOC101515675)	Protein dimerization activity

Fig. 8 Role of different hub genes in different biological processes along with the *p* value (decreasing order of *p* value from yellow to dark orange) retrieved from Cr(VI) treated microarray data of chickpea using gene set enrichment analysis (color figure online)

factor coding genes changes and this adjustment leads to increased tolerance level of chickpea. Biological networks were constructed on the basis of Pearson's coefficient correlation and analysis was done for both stress conditions. The aim of study is to understand the role of bHLH (transcription factor) coding genes in biological process and molecular functions during different heavy metal treatment on chickpea. Once biological network was constructed and visualized by cytoscape. In addition, comparison of different parameters of network was also performed. We have compared connectivity of hub genes in both conditions i.e. Cd(II) and Cr(VI). It was observed that maximum connectivity among genes during Cd(II) treatment was 80 with clustering coefficient 0.95 for TC22481 (ICE-1 like gene i.e. *inducer of CBF expression 1*) which participates in protein dimerization. The ICE-1 gene in *Arabidopsis* encodes MYC like bHLH transcriptional activator which activates the genes that provide chilling and freezing tolerance to the plants (Chinnusamy et al. 2003). It further hints that over expression of ICE-1 like gene in chickpea is meant to provide increased tolerance against Cd(II) stress. TC03912 (bHLH13-like), TC12109 (bHLH96 like), TC15617 (bHLH130-like) and TC25557 (bHLH30) are members of bHLH transcription factor which exhibit maximum connectivity during chromium exposure in chickpea. Comparative analysis revealed that homologs of the four genes in *Arabidopsis* are involved in protein dimerization, as mentioned in Table 6. One member of bHLH transcription factor gene, TC02853 which is up regulated during the

Fig. 9 Role of different hub genes in different biological processes along with the *p* value (decreasing order of *p* value from yellow to dark orange) retrieved from Cd(II) treated microarray data of chickpea using gene set enrichment analysis (color figure online)

treatment and resembles with AT2G16910 of *Arabidopsis*, which plays important role in protein dimerization and transcriptional regulation.

The GSEA identified five key genes TC03753, TC03912, TC12109, TC15617 and TC25557 during Cr(VI) exposure. TC03753 (bHLH48 like transcription factor), was predicted to have role in protein dimerization. Its homolog in soybean, the Basic Helix-Loop-Helix

Transcription Factor ORG3-Like, is known to enhance cadmium tolerance via increased iron and reduced cadmium uptake and transport (Xu et al. 2017). The homolog of TC03753 in *Arabidopsis* is AT3G57800 which is member of bHLH TF family and is still uncharacterized (Kazan and Manners 2013). TC03912, TC12109, TC15617 and TC25557 in chickpea were found to be the most connected members of bHLH TF family and all are

involved in protein dimerization activity during Cr(VI) exposure, as mentioned in Table 5.

The results of our work hint towards key role of bHLH transcription factor in protein dimerization in response to Cr(VI) and Cd(II) stress. It is pertinent to mention here that homodimers and heterodimers of bHLH proteins interact with DNA to regulate various processes involved in abiotic stress (Littlewood and Evan 1998). Possibly bHLH TFs regulate the gene expression by binding on E-box (Ephrussi-box) and G-box of RNA, as reported earlier in other plants (Toledo-Ortiz 2003).

Conclusion

This study reports that at least ten members of bHLH transcription factor family proteins are involved in response to during Cd(II) and Cr(VI) stress. Involvement of their homologs in other plants for similar type of stress response was also revealed by analytics. These findings will further help in characterization of individual proteins role against different abiotic stresses which in turn will be useful in screening stress tolerant varieties of the crop as well as marker based selection.

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