RESEARCH PAPER

Glutathione modulates the expression of heat shock proteins via the transcription factors BZIP10 and MYB21 in Arabidopsis

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Received 17 October 2017; Editorial decision 24 April 2018; Accepted 24 April 2018

Editor: Christine Foyer, Leeds University, UK

Abstract

The contribution of glutathione (GSH) in combating environmental stress in plants has long been known. Previous reports have pointed to the involvement of GSH in inducing various heat shock proteins (HSPs), but the molecular mechanism is yet to be explored. Here, we investigate how GSH induces the expression of important HSP genes in Arabidopsis. Expression of HSP genes BiP3, HSP70B, and HSP90.1 was positively regulated by GSH, and a promoter activation assay suggested a role for GSH in their induction. Lower expression of BiP3 and HSP70B in the GSH-fed Atmyb21 mutant and of HSP90.1 in the GSH-fed Atbzip10 mutant, in comparison with GSH-fed Col-0, revealed a role for GSH in activating their promoters through the transcription factors MYB21 and BZIP10. Co-transfection of transcription factor mutant protoplasts with transcription factor constructs and HSP promoters confirmed the results. Comparative proteomics also revealed proteins whose expression was controlled by MYB21 and BZIP10 in response to GSH feeding. A co-immunoprecipitation assay demonstrated a role for GSH in modulating the level of interaction of glutathione-S-transferase with HSP70. Collectively, our results demonstrate a role for GSH in activating the promoters of BiP3 and HSP70B via MYB21 and of HSP90.1 via BZIP10.

Keywords: Arabidopsis thaliana, glutathione, heat shock proteins, proteomics, protoplast, transcription factors.

Introduction

Plants consistently face various stress conditions that adversely affect cellular homeostasis and have harmful effects on their growth, fitness and productivity (Boyer, 1982; Peters et al., 2004; Mickelbart et al., 2015). To protect themselves from extreme environments, plant defense reactions come rapidly into play, within hours or days (Levitt, 1972; Jenks and Hasegawa, 2005). The plant response to different stress conditions is mediated by a complex system of transcription factors (TFs) and regulatory genes that control the stress and defense network (Bray et al., 2000; Cushman and Bohmert, 2000; Suzuki et al., 2005). Although the molecular mechanisms for the expression of stress- and defense-related genes and proteins have been extensively studied globally, with such a complex scenario there is a need for further investigation aimed at the identification of new molecular factors involved.

An important metabolite that plays multiple roles in plant protection and in modulating the stress response is glutathione (GSH), a tripeptide composed of Glu, Cys, and Gly (Marrs and Walbot, 1997; Edwards et al., 2000; Pieterse and Dicke 2007; Noctor et al., 2007; Noctor et al., 2012; Kovacs et al., 2015). GSH also acts as an

Abbreviations: BSO, buthionine sulfoximine; 2DE, two-dimensional gel electrophoresis; DaRed, Discosoma-specific red fluorescent protein; DTT, dithiothreitol; GFP, green fluorescent protein; GSH, glutathione; GST, glutathione-S-transferase; HSP, heat shock protein; β-ME, β-mercaptoethanol; TF, transcription factor; YFP, yellow fluorescent protein.

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important factor determining basal jasmonic acid (JA) gene expression in response to oxidative stress in Arabidopsis (Han et al., 2013). The role of GSH in plant growth and development is well established. Previous reports have indicated its key role in embryo development, meristem development, pollen germination, pollen tube growth, and cell cycle regulation as well (Vernoux et al., 2000; Cairns et al., 2006; Reichheld et al., 2007; Pellny et al., 2009; Zechmann et al., 2011). Earlier reports have shown that plants with an increased GSH level can develop significant tolerance to several environmental stress conditions (Noctor et al., 1998; Gomez et al., 2004; Mullineaux and Rausch, 2005; Ferretti et al., 2009). On the other hand, a reduced GSH level has a negative effect on the biological functions of plant cells, as shown by Arabidopsis plants with low GSH content (<10% of wild type) that were found to be hypersensitive to cadmium stress (Xiang et al., 2001). A GSH mutant line of Arabidopsis, cad2, was found to be hypersensitive to metal toxicity (Howden et al., 1995). Another GSH mutant line of Arabidopsis, pad2.1, was reported to be deficient in camalexin, which is considered a crucial defense metabolite with a role in limiting the growth of virulent bacteria (Glazebrook and Ausubel, 1994; Parsiy et al., 2007). All these GSH mutant lines were reported to have a susceptibility to both biotic and abiotic stress conditions (Ball et al., 2004; Schlaeppe et al., 2008; Kumar et al., 2015). Additionally, as a cofactor GSH plays a key role in the detoxification of methylglyoxal, a cytotoxic compound that is formed as a byproduct of glycolysis under stress conditions (Zang et al., 2001; Singla-Pareek et al., 2003).

In plants a role of GSH has been reported in combating stress through interaction with various stress- and defense-related signaling molecules and their modulation of their pathways (Noctor et al., 2012). It has been demonstrated that GSH mimics salicylic acid (SA) and induces the PR1 gene by reduction of NPR1 and promoting its recruitment to the nucleus (Mou et al., 2003). A role of GSH in mitigating biotic stress through an NPR1-dependent SA-mediated pathway has been suggested by our group (Ghanta et al., 2011). A recent study by our group has also revealed a role of GSH in regulating 1-aminoacyclopropane-1-carboxylate synthase to induce ethylene in response to salinity stress (Datta et al., 2015). GSH also modulates various stress- and defense-related genes by interacting with ABA and ethylene in response to abiotic stress conditions in plants (Yoshida et al., 2009; Wei et al., 2015; Kumar et al., 2016).

One of the effects of environmental stress is protein aggregation, which results in protein dysfunction (Lindquist, 1986). Sustaining proteins in their native conformations and preventing the aggregation of non-native proteins are essential for cell survival in response to stress. Heat shock proteins (HSPs) are important chaperones that play a key role in stabilizing proteins and membranes, thereby helping in protein refolding to combat environmental stress (Wang et al., 2004). The correlation between HSP synthesis and an increase in thermotolerance in plants is well known. Furthermore, previous reports have shown that expression of HSPs is associated with the intensity of the stress that they are naturally exposed to (Howarth and Oughham, 1993; Feder and Hofmann, 1999). The interaction of HSPs such as HSP60, HSP70, and HSP90 with a wide range of co-chaperone proteins that regulate their activity has been reported (Bukau and Horwich, 1998; Buchner, 1999; Frydman, 2001). HSP70-overexpression lines of Nicotiana and Arabidopsis showed tolerance to salt, water, and high-temperature stress (Leborgne–Castel et al., 1999; Sugino et al., 1999; Alvim et al., 2001; Ono et al., 2001; Sung and Guy, 2003). Plant HSPs acts in response to a broad range of environmental stresses, including heat, cold, drought, salinity, and oxidative stress (Wang et al., 2004). HSP90 activity is also responsible for brassinosteroid signaling via trafficking of BIN2–HSP90 complexes into the cytoplasm (Samakovli et al., 2014).

In our previous work we have reported an elevated level of HSP expression in a GSH-fed line of Arabidopsis (Sinha et al., 2015). In the GSH-overexpression line of Nicotiana, HSP70 was notably up-regulated as well (Ghanta et al., 2011). We have also found reduced levels of expression of the same members of the HSP family in combined stress-treated pad2.1, a GSH mutant of Arabidopsis, which were previously found to be up-regulated in GSH-fed Arabidopsis (Kumar et al., 2015). Together, previous reports have pointed towards the involvement of GSH in inducing HSPs, which plays a vital role in combating various stresses in the plant system. Here, we demonstrate the molecular mechanism through which GSH induces the expression of these HSPs. Our results unravel the function of GSH in modulating the efficiency of interaction of HSP70 with other proteins to combat environmental stresses in planta.

Materials and methods

Plant materials

All Arabidopsis seeds were obtained from the Nottingham Arabidopsis Stock Centre (NASC). SALK_106031:Atbzip10, N6241:AtAP2, SALK_042711C:Atmyb21, N8052:Atein3, and N3804:pad2.1 are mutants of BZI10, APETAL2, MYB21, EIN3, and GSH respectively. The pad2.1 mutant line of Arabidopsis has a mutation of the first enzyme, viz. γ-glutamylcysteine synthetase (γ-ECS), of the glutathione pathway, thus reducing the GSH content by 80% compared with the wild type (Parisy et al., 2007; Zechmann et al., 2008). Arabidopsis Col-0 ecotype, the wild type, and ΔECS1 (a γ-ECS overexpression line) were used also. Surface-sterilized seeds were grown in Murashige and Skoog (MS) medium, and maintained in a growth chamber (Eyela) at 22 °C under a 16 h light–8 h dark cycle. The collective leaf structures of 3- and 4-week-old seedlings were harvested for RNA and protein isolation.

Chemical treatment of seedlings

Three-week-old seedlings were used for all feeding experiments. Buthionine sulfoximine (BSO) is an important inhibitor of the first enzyme in GSH biosynthesis thus leading to a strong decrease in GSH level in Arabidopsis (Meyer and Fricker, 2002). For GSH and BSO feeding, seedlings were fed separately with 100 μM GSH and 1 mM BSO solutions for 24, 48, 72, and 96 h, as described previously (Sinha et al., 2015; Datta et al., 2015). Both dithiothreitol (DTT) and β-mercaptoethanol (β-ME) are strong reducing agents that produce a reducing environment in plant cells (Lindermayr et al., 2010). For DTT and β-ME feeding, seedlings were fed with 5 mM freshly prepared DTT and β-ME solutions.

RNA extraction and quantitative RT-PCR analysis

Total RNA from the collective leaf structures of Arabidopsis was isolated using the Trizol method. Quantitative RT-PCR was performed using the total RNA of both control and mutant lines. One microgram of total RNA
Promoter analysis using protoplast isolation and transfection assay

The promoter regions of the genes BiP3, HSP70B, and HSP90.1, i.e. the intergenic region upstream to 938, 420, and 693 bases, respectively, of the transcription start site were cloned into pCAMBIA1303 plasmid with the green fluorescent protein (GFP) gene under control of these promoters. As a result, proBiP3:GFP, proHSP70B:GFP, and proHSP90.1:GFP constructs were developed. Protoplasts were isolated from the leaves of 3-week-old Col-0, AtECS1, and mutant lines of Arabidopsis. The proCaMV35S:MYB21 vector, which resulted in the development of proCaMV35S:MYB21:DrRed constructs. The expression of both myb21 and DrRed was under the control of the 35S CaMV constitutive promoter. Isolation of protoplasts was performed from the leaves of 3-week-old Arabidopsis. By using the PEG–CaCl2 method, the proCaMV35S:MYB21:DrRed construct was co-transfected with proBiP3:GFP or proHSP70B:GFP in Col-0 and myb21 mutant protoplasts. GSH of concentration 100 μM was added to the protoplast-maintenance medium after the protoplast transfection and maintained at room temperature for about 6 h. In the same way hz10 CDS isolated from Arabidopsis was cloned in the pAM-PAT-YFP vector under the control of 35S CaMV constitutive promoter. The resulting construct was proCaMV35S:YFP-BZIP10. In the next step the protoplasts of Col-0 and Atbzip10 lines were co-transfected with proCaMV35S:YFP-BZIP10 and proHSP90.1:GFP. Again GSH of 100 μM was added to the protoplast-maintenance medium for about 6 h. By using a confocal laser scanning microscope and a fluorescence microscope, GFP, DsRed and yellow fluorescent protein (YFP) expression levels were examined.

Homology modeling for molecular docking

A 3D model of MYB21 was generated by using the homology modeling program Swiss-Model (Bauern et al., 2014; Guex et al., 2009). At the start, the potentially related amino acid sequences of MYB21 were searched. To evaluate the model folding, the resulting model was subjected to PROCHECK (Laskowski et al., 1993). The active-site groove volume of the enzyme was measured through CASTp calculation (Dundas et al., 2006).

Two-dimensional gel electrophoresis

One gram of total protein was isolated from Col-0, GSH-fed Col-0, GSH-fed myb21 and GSH-fed hz10 mutant lines of Arabidopsis. The extracted protein was then suspended in IEF buffer consisting of 7 M urea, 2 M thiourea, 4% β-mercaptoethanol, 1% (w/v) Bio-Lyte (3/10) ampholyte (Bio-Rad) as before. Quantification of protein expression was performed by using the Bradford method. Protein of approximately 600 μg was re-hydrated in an immobilized pH gradient strip (7 cm; pH 4–7; GE) for 12 h. Isoelectric focusing was performed as follows: 250 V for 30 min, 8000 V for 2 h, 8000 V for 26 000 V h, 750 V for 1 h on a Bio-Rad PROTEAN IEF Cell system. After 15 min equilibration of focused strips in calibration buffers I and II (Bio-Rad) second-dimension gels were run by using 12% SDS polyacrylamide gels and staining with colloidal Coomassie Brilliant Blue (CBB) G-250.

Image analysis

The gel images were analysed with a Versa doc image system (Bio–Rad) and PD Quest software version 8.0.1 (Bio–Rad). Spot detection was

In silico identification of promoter-sequence-binding TFs

Detection of various TFs that could bind to the promoter sequence of BiP3, HSP70B, and HSP90.1 was performed by using Plant Promoter Analysis Navigator (PlantPan; http://PlantPan2.itps.edu.tw; Chow et al., 2016).

Procurement of TF mutants, GSH feeding and checking the expression of HSPs

On the basis of in silico identification of HSP promoter sequence-binding TFs, we procured TF mutants from NASC and germinated them as mentioned above. Again, 3-week-old seedlings were fed with 100 μM GSH. HSP expression was determined in all the GSH-fed TF mutants at both gene and protein levels by using the method described above.

Protoplast transfection of TF mutant lines

Protoplasts of Atmyb21 and Atbzip10 mutants were transfected with proBiP3:GFP, proHSP70B:GFP, and proHSP90.1:GFP constructs. GFP expression was visualized in the GSH-fed transfected protoplast by confocal microscopy.

Protoplast co-transfection assay

From Arabidopsis cDNA, the coding DNA sequence (CDS) of myb21 was cloned in the pBI121 vector. As a result, the proCaMV35S:MYB21 construct was developed. The Diconosoma-specific red fluorescent protein (DrRed) sequence of the pDsRed-Monomer-N1 vector (Clontech) was cloned into the proCaMV35S:MYB21 vector, which resulted in the development of proCaMV35S:MYB21:DrRed constructs. The expression of both myb21 and DrRed was under the control of the 35S CaMV constitutive promoter. Isolation of protoplasts was performed from the leaves of 3-week-old Arabidopsis. By using the PEG–CaCl2 method, the proCaMV35S:MYB21:DrRed construct was co-transfected with proBiP3:GFP or proHSP70B:GFP in Col-0 and Atmyb21 mutant protoplasts. GSH of concentration 100 μM was added to the protoplast-maintenance medium after the protoplast transfection and maintained at room temperature for about 6 h. In the same way hz10 CDS isolated from Arabidopsis was cloned in the pAM-PAT-YFP vector under the control of 35S CaMV constitutive promoter. The resulting construct was proCaMV35S:YFP-BZIP10. In the next step the protoplasts of Col-0 and Atbzip10 lines were co-transfected with proCaMV35S:YFP-BZIP10 and proHSP90.1:GFP. Again GSH of 100 μM was added to the protoplast-maintenance medium for about 6 h. By using a confocal laser scanning microscope and a fluorescence microscope, GFP, DsRed and yellow fluorescent protein (YFP) expression levels were examined.
performed by matching the gels automatically with manual verification. The spots detected in at least two replicate gels were selected for annotation.

Tryptic in-gel digestion and mass spectrometry analysis
Manually excised differentially accumulated protein spots were digested with trypsin (in-gel trypsin digestion kit, Pierce) using the manufacturer’s protocol. The mass spectrometric analysis of the samples was performed by using Zip-Tip μ-C18 (Millipore) and a 4800 matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI TOF/TOF) analyzer (Applied Biosystems). By using STRING 10 software, the protein interactions between identified differentially accumulated proteins in response to GSH treatment were recognized. The expression of some differentially accumulated proteins was validated by immunoblotting.

Protein immunoprecipitation and western blot analysis
Total protein was isolated from Col-0, pad2.1, and AtECS1. About 3 mg of total proteins was inoculated in protein isolation buffer containing protein A (magnetic beads, Millipore) and rabbit polyclonal anti-HSP70 primary antibody (Agrisera) overnight at 4 °C. After that the protein A solution was centrifuged and the pellet was taken. Extracted pellet was washed three times by dissolving in protein isolation buffer. Twenty microliters of protein loading dye (2% SDS, 100 mM Tris–HCl pH 7.5, 10% glycerol, 0.5 mM EDTA, 10 mM DTT) was added to the washed pellet and heated at 70 °C for 10 min. From the upper eluted liquid samples, western blotting was performed by using rabbit polyclonal anti-glutathione-S-transferase (GST) phi primary antibody (Agrisera). As a control the expression of HSP70 was also checked by using the same eluted sample.

Statistical analysis
All the experiments were performed in triplicate and the data presented as the mean±standard error (SE) to compare the relative gene and protein expression profiles of Col-0 and mutant lines. For statistical analysis, ANOVA followed by the Student–Newman–Keuls multiple comparison test was used (GraphPad InStat software, v. 3.1). P<0.05, 0.01 or 0.001 was considered to be statistically significant.

Results

Altered GSH conditions regulate the expression of HSP genes
The expression of the HSP genes (HSPs) BiP3, HSP70B, and HSP90.1 was determined in the γ-ECS-overexpressing transgenic line AtECS1, GSH mutant line pad2.1, and wild type Col-0. Expression of BiP3, HSP70B, and HSP90.1 was found to be greater in the AtECS1 line, whereas a lower expression level was noted in pad2.1 in comparison with Col-0 at both gene and protein levels (Fig. 1A, D; Supplementary Figs S1, S2). A similar result with greater expression of HSPs in the GSH-fed line of wild type Col-0 and lower expression in BSO-fed Col-0 in comparison with untreated Col-0 was observed at both gene and protein levels (Fig. 1B, E; Supplementary Figs S1–S3). To check the effect of a reducing environment on the expression of these HSPs, wild type Col-0 was treated with β-ME and DTT; no significant change in the expression of these HSPs was noticed in the treated wild type Col-0 in comparison with untreated Col-0 (Fig. 1C; Supplementary Fig. S1C). These results point towards a role of GSH in inducing these HSPs.

GSH has a role in activating the promoters of HSPs
GFP expression was noticed in the cytoplasm of the protoplasts with the promoter activation of these HSPs. As a control for GFP expression, Col-0 protoplasts were transfected with the pCAMBIA1303 plasmid, which lacked an HSP promoter (−HSpro:GFP), and GFP expression was estimated by confocal microscopy (Fig. 2B). We observed the highest level of GFP expression in the AtECS1 protoplast transfected with proBiP3:GFP, proHSP70B:GFP, and proHSP90.1:GFP and observed the least expression of GFP in the pad2.1 protoplast transfected with these constructs (Fig. 2C–E; Supplementary Figs S4, S5; Supplementary Table S2). These findings further support a role of GSH in inducing these HSPs by activating their promoters.

Identification of HSP promoter sequence-binding TFs
By using PlantPan software (Chow et al., 2016) we found that TFs such as EIN3, APETALA2, BZIP10, and MYB21 had a large number of binding sites in the promoter sequence of BiP3, HSP70B, and HSP90.1 (see Supplementary Fig. S6). For BZIP10, MYB21, APETALA2, and EIN3, the conserved binding sequences of the promoters of HSPs were ACCTT/GAAC, ACCG/CGAC, and TACA/GTAT, respectively. On this basis, Arabidopsis mutant lines of these TF genes were used.

Determining the expression and promoter activity of HSPs in TF mutant lines in response to GSH feeding
TF mutant lines Atein3, Atapetala2, Atbzip10, and Atmyb21 were treated with 100 μM GSH for 72 h, and the expression of HSPs in these treated mutant lines was compared with GSH-fed Col-0. The results revealed lower expression of BiP3 and HSP70B in the GSH-fed Atmyb21 mutant in comparison with GSH-fed Col-0 at both gene and protein levels (Fig. 3A, B, G; Supplementary Fig. S7A). On the other hand, lower expression of HSP90.1 was noticed in the GSH-fed Atbzip10 mutant line in comparison with GSH-fed Col-0 at both gene and protein levels (Fig. 3F, H; Supplementary Fig. S7A). We found insignificant change in the expression of these HSPs in GSH-fed Atein3 and AtAP2 in comparison with GSH-fed Col-0 (Supplementary Fig. S8).

The protoplasts of Col-0 and mutant lines were transfected with proBiP3:GFP, proHSP70B:GFP, and proHSP90.1:GFP, and the expression of GFP in the cytoplasm was observed in the GSH-fed transfected protoplasts by confocal microscopy. The results revealed lower expression of GFP in the GSH-fed Atmyb21 mutant line protoplast transfected with proBiP3:GFP and proHSP70B:GFP in comparison with GSH-fed Col-0 protoplast transfected with these constructs (Fig. 4A, B). Similarly, we also observed lower GFP expression in the GSH-fed Atbzip10 mutant line protoplasts transfected with proHSP90.1:GFP in comparison with GSH-fed transfected protoplast of Col-0 (Fig. 4C). These results pointed towards a role for MYB21 in activating the promoters of BiP3 and HSP70B in response to GSH. On the other hand, these results
also suggested a role for BZIP10 in activating the promoter of HSP90.1 in response to the elevated level of GSH in the plant cells.

Co-transfection assay confirmed the function of MYB21 and BZIP10 in the activation of promoters of HSPs

The findings mentioned above encouraged us to perform a co-transfection assay in Atmyb21 and Atbzip10 mutant protoplasts.

As a complementation test for this result, myb21 and bzip10 genes were cloned in proCaMV35S:DsRed and pAM-PAT-YFP vector, respectively, under the control of the CaMV35S promoter. The resulting constructs were proCaMV35S:MYB21-DsRed and proCaMV35S:BZIP10-YFP. After transfecting the proCaMV35S:MYB21-DsRed and proCaMV35S:BZIP10-YFP constructs in the protoplasts of Atmyb21 and Atbzip10 mutants, respectively, we observed the restoration of the myb21 mutant by determining the expression of DsRed fluorescence. After co-transfecting the construct proCaMV35S:MYB21-DsRed
with proBiP3:GFP or proHSP70B:GFP in GSH-fed Atmyb21 protoplasts, we observed restored GFP expression, like the co-transfected protoplasts of Col-0 (Fig. 5B, C, E, F, H, I, K, L; Supplementary Fig. S9). This result suggested a role for MYB21 in activating the promoters of BiP3 and HSP70B in the GSH-fed condition. Similarly, when the protoplasts of Atbzip10 were co-transfected with proCaMV35S:BZIP10-YFP and proHSP90.1:GFP in the GSH-fed condition, the expression of GFP was restored in the bzip10 protoplast (Fig. 5D, G, J, M). This result further indicated that bzip10 plays a key role in activating the HSP90.1 promoter. Together, our results pointed towards a role for MYB21 in activating the promoters of BiP3 and HSP70B and for BZIP10 in activating the promoter of HSP90.1 in response to an elevated level of GSH.

GSH induces the expression of myb21 without executing its post-translational modification

In silico analysis of MYB21 revealed that there was a very low chance of its S-glutathionylation, as the cysteine residue was

**Fig. 2.** Promoter activation analysis of proBiP3, proHSP70B, and proHSP90.1 in response to altered GSH conditions. Leaf protoplasts of Col-0, AtECS1, and pad2.1 were transfected with proBiP3:GFP, proHSP70B:GFP, and proHSP90.1:GFP. Promoter activity was monitored by checking the fluorescence of GFP in the transfected protoplast under a confocal microscope. (A) Linear map of pCAMBIA1303 vector in which the promoter sequences of BiP3, HSP70B, and HSP90.1 were inserted upstream of GFP. LB-TR, left border T-DNA repeat. (B) Control: GFP expression in Col-0 protoplasts transfected with pCAMBIA1303 plasmid, which was devoid of the HSP promoter (−HSPspro:GFP). (C) Elevated GFP fluorescence was observed in AtECS1 protoplasts transfected with proBiP3:GFP. Reduced GFP expression was noticed in pad2.1-transfected protoplasts. (D) Higher GFP fluorescence was observed in AtECS1 protoplasts transfected with proHSP70B:GFP and less GFP fluorescence was found in transfected protoplasts of pad2.1. (E) Similarly, more GFP fluorescence was noted in AtECS1 protoplasts transfected with proHSP90.1:GFP and less fluorescence was observed in transfected protoplasts of pad2.1. GFP fluorescence was noted at 488 nm. Scale bar: 10 μm. GFP fluorescence in control protoplast has been normalized (100%). Data are presented as mean ± SE (n=3 at ×60 magnification, n≥10 at ×20 magnification). ANOVA followed by Student–Newman–Keuls multiple comparison test was used (GraphPad InStat software, v. 3.1). *P<0.05, **P<0.01, ***P<0.001.
found to be embedded inside the protein’s globular structure (Fig. 6A, B). Quantitative RT-PCR revealed elevated expression of \textit{myb21} in \textit{AtECS1} and its lower expression in \textit{pad2.1} in comparison with Col-0 (Fig. 6C).

There are two ways by which GSH can regulate the expression of \textit{BiP3} and \textit{HSP70B} through \textit{MYB21}. The first is by GSH activating \textit{MYB21} by post-translational modification (\textit{S}-glutathionylation) after which its capacity to interact with and activate the promoters of \textit{HSP70B} and \textit{BiP3} is enhanced. The second is by enhanced expression of \textit{MYB21}. The results indicated that GSH plays a key role in activating the promoters of HSPs in other TF mutant lines. Data are presented as mean ±SE (n=3); ANOVA followed by Student–Newman–Keuls multiple comparison test was used (GraphPad InStat software, v. 3.1). *P<0.05, **P<0.01, ***P<0.001. (G, H) Western blot analysis also revealed lesser expression of \textit{HSP70} and \textit{HSP90.1} in GSH-fed \textit{Atmyb21} and GSH-fed \textit{Atbzip10} mutants, respectively, in comparison with GSH-fed Col-0, which further validated the transcript result. Tubulin: loading control for normalization. (This figure is available in color at JXB online.)

**Identification of other proteins regulated by \textit{MYB21} and \textit{BZIP10} in response to the elevated level of GSH**

Proteins regulated by \textit{MYB21} and \textit{BZIP10} in response to GSH were identified by using two-dimensional gel electrophoresis (2DE) followed by MALDI-TOF/TOF tandem mass spectrometry (MS/MS) from the proteins isolated from Col-0, GSH-fed Col-0, \textit{Atmyb21} and \textit{Atbzip10} (see Supplementary Fig. S10). Overall mean coefficient of variation of differentially expressed proteins was 69.76 suggesting significant differences in protein expression. Total protein spots identified in Col-0, GSH-fed Col-0, \textit{Atmyb21}, and \textit{Atbzip10} were 490, 373, 250, and 378, respectively. There were 15 common spots that matched to every member. Among the differentially expressed proteins in the above-mentioned plant samples, glutathione-\textit{S}-transferase, peptidylprolyl isomerase (PPIase) ROC4, high chlorophyll fluorescence 136, oxygen evolving protein, photosystem II subunit O-2 (PSBO2) and glutamate ammonia ligase GS2 were observed to be up-regulated in GSH-fed Col-0 and the same proteins were observed to be down-regulated in GSH-fed TF mutant lines (Fig. 7A; Table 1). STRING 10 software analysis of differentially expressed proteins revealed a strong interaction between \textit{HSP70} and glutathione-\textit{S}-transferase (GST) in response to the elevated level of GSH (Fig. 7B). Amongst the above-mentioned proteins the expression of GST F2 was validated by western blot analysis (Fig. 8A). Together, the above results helped in the identification of important proteins other than HSPs that were regulated by \textit{MYB21} and \textit{BZIP10} in response to GSH feeding.

**GSH has a role in increasing the GST interaction with \textit{HSP70}**

Total proteins, isolated from \textit{AtECS1}, \textit{pad2.1}, and Col-0, were co-immunoprecipitated with rabbit polyclonal anti-\textit{HSP70}
primary antibody. Western blot was performed using co-immunoprecipitated eluted sample of total protein and rabbit polyclonal anti-HSP70 primary antibody with rabbit polyclonal anti-GST phi primary antibody. The results revealed highest expression of GST in co-immunoprecipitated eluted sample of \textit{AtECS1} and least expression in an eluted sample of \textit{pad2.1} (Fig. 8B). The results cumulatively demonstrated the key role of GSH not only in inducing GST biosynthesis but also in increasing the chaperonic effect of HSP70 towards GST. A proposed model for the regulation of HSPs through GSH is shown in Fig. 9.

Discussion

Various environmental stresses have an adverse effect on plant metabolism, cellular homeostasis, and major physiological processes, as is well-established and the subject of many studies. The important role of GSH in the development of stress tolerance in plants has been widely recognized (Dron et al., 1988; Wingate et al., 1988; Tausz et al., 2004; Ball et al., 2004; Noctor et al., 2012). Our previous investigations also pointed toward the importance of GSH in inducing various stress-related genes and proteins, especially HSPs, to combat both biotic and abiotic stress conditions in planta (Ghanta et al., 2011, 2014; Sinha et al., 2015; Datta et al., 2015; Kumar et al., 2015, 2016).

In this investigation, we probed the mechanism through which GSH regulates important stress modulators, viz. the HSPs BiP3, HSP70B, and HSP90.1. This study demonstrated the proteins that are induced by TFs such as MYB21 and BZIP10 in response to the elevated level of GSH. Our study also demonstrated the functional role of GSH in changing the interaction efficiency of HSP70 towards GST.

Most of the stress- and defense-related genes and proteins, especially HSPs such as BiP3, HSP70B, and HSP90.1, were found to be down-regulated under altered GSH conditions in Arabidopsis GSH mutant \textit{pad2.1}, whereas, interestingly, the same genes were found to be up-regulated in GSH-fed wild type Arabidopsis (Ghanta et al., 2011; Sinha et al., 2015; Kumar et al., 2016). Heat stress also has a role in the accumulation of GSH in the frost-sensitive wheat plant (Kocsy et al., 2004), and also it has been reported that in response to heat stress, \textit{HSP70B} was induced (Sung et al., 2001a), which may suggest a relation between levels of GSH and \textit{HSP70B}. The contribution of \textit{HSP90.1} in mitigating environmental stress through developing RPS2-mediated disease resistance is an established fact (Krishna and Gloor, 2001; Takahashi et al., 2003). BiP3 or HSP70 family proteins have also been reported to play an important role in mitigating both abiotic and biotic stress in \textit{Nicotiana} and soybean (Alvim et al., 2001; Wang et al., 2005; Valente et al., 2009). Elevated GSH levels in eukaryotic cells

![Fig. 4. Effect on the promoter activity of HSPs in GSH-fed \textit{Atmyb21} and \textit{Atbzip10} mutant lines. (A, B) Protoplasts isolated from \textit{Atmyb21} transfected with proBiP3:GFP and proHSP70B:GFP. (C) Protoplasts of \textit{Atbzip10} transfected with proHSP90.1:GFP. GFP expression was monitored by confocal microscopy with or without GSH. Reduced GFP fluorescence was observed in GSH-fed transfected protoplasts of \textit{Atmyb21} and \textit{bzip10} in comparison with transfected GSH-fed Col-0. Scale bar: 10 µm. GFP fluorescence in control protoplast (mentioned in Fig. 2B) has been normalized (100%). Data are presented as mean ±SE (n=3). ANOVA followed by Student–Newman–Keuls multiple comparison test was used (GraphPad InStat software, v. 3.1). *P<0.05, **P<0.01, ***P<0.001.](image_url)
Fig. 5. Promoter activity analysis of proBiP3, proHSP70B, and proHSP90.1 in Atmyb21 and Atbzip10 protoplasts. (A) Linear map of pBI121 vector in which sequences of MYB21 and DsRed were inserted under the control of CaMV35S promoter. RB-TR, right border T-DNA repeat; NOS, nopaline synthase. (B) Protoplasts of Atmyb21 were co-transfected with proCaMV35S:MYB21-DsRed and proBiP3:GFP. (C) Atmyb21 protoplasts were co-transfected with proCaMV35S:MYB21-DsRed and proHSP70B:GFP. See Supplementary Fig. S9 for fluorescence microscopy result. (D) Protoplasts of Atbzip10 co-transfected with proCaMV35S:MYB21-DsRed and proBiP3:GFP. (E, I, K) After co-transferring Atmyb21 protoplasts with proCaMV35S:MYB21-DsRed and proBiP3:GFP, significant increase in GFP fluorescence was observed in response to GSH treatment, indicating the restoration of GFP expression, like in cotransfected protoplast of Col-0. (F, I, L) Co-transfection of Atmyb21 protoplasts with proCaMV35S:MYB21-DsRed and proHSP70B:GFP significantly elevated GFP expression in response to GSH treatment. (G, J, M) Similarly, after co-transferring Atbzip10 protoplasts with proCaMV35S:Atbzip10-YFP and proHSP90.1:GFP, significant increase in GFP fluorescence was observed in response to GSH feeding. Scale bar: 20 μm. GFP fluorescence in control protoplasts (mentioned in Fig. 2B) has been normalized (100%). Data are presented as mean ±SE (n=3). ANOVA followed by Student–Newman–Keuls multiple comparison test was used (GraphPad InStat software, v. 3.1). *P<0.05, **P<0.01, ***P<0.001.
produce a reducing environment (Deponte, 2013) and this reducing environment could also induce these stress- and defense-related HSPs. Therefore, we determined the expression of these HSPs in β-ME- and DTT-treated Col-0 to mimic reducing conditions and observed insignificant changes in the expression of these HSPs in comparison with untreated Col-0. Previous studies of our lab reported a 2.24-fold higher yield of GSH in the AtECS1 transgenic line, overexpressing γ-ECS, the key enzyme in GSH biosynthesis, in comparison with Col-0. In GSH-fed Col-0, a more than 2.0-fold increase in GSH content has been recorded as well. On the other hand, in BSO-treated Col-0 and pad2.1, the GSH content was reduced by about 5.5- and 3.0-fold, respectively (Datta et al., 2015; Kumar et al., 2015). This result clearly indicated that the content of GSH has a key role in inducing these HSPs. In low-GSH conditions (BSO-treated Col-0 or pad2.1), the HSP expression is not as much inhibited by far, as expected from the very low (~10%) GSH levels obtained. This may be due to a basal level of GSH in the BSO-treated cells. This basal level of GSH can also play a role in the regulation of HSP expression, but is unable to exhibit the enhanced expression of HSPs. Similarly, in pad2.1 a basal level of GSH remains in the plant cell that can also regulate the expression of HSPs.

Induction of BiP3, HSP70B, and HSP90.1 through GSH can occur in several possible ways. For instance, GSH can induce expression of HSPs at transcript level via activation of their promoters. A second possible way, in response to stress, is via the accumulation of GSH in plant cells, which changes the cellular redox homeostasis and can result in the induction of these HSPs. Post-translational modification of these HSPs by GSH-glutathionylation is a third way to regulate the proteins’ stability, and a fourth possible way is by modulating the stability of their mRNA.

To obtain further in-depth insight, a promoter activation assay on Arabidopsis protoplasts was performed and the results revealed that the elevated GSH level induced the expression of HSPs by activating their promoters. It is obvious that GSH itself cannot bind to the promoter sequences of these HSPs to activate them. Consequently, the next task was to identify the transcriptional regulators that activate the promoters of these HSPs in response to GSH. Previous reports have indicated that various HSPs that had MYB binding site elements in their upstream region were also found to be induced in response to drought stress (Reddy et al., 2014). Relocalization of the TF complex UBL–5–DVE-1 and bZIP to the nucleus activated the expression of the chaperones HSP6 (mtHSP70) and HSP60 (mt chaperonin) (Kirstein-Miles and Morimoto, 2010). Our transcript analysis of the TF mutant lines showed no marginal change in the induction of BiP3 and HSP70B in the GSH-fed Atmyb21 mutant and of HSP90.1 in the GSH-fed Atbzip10 mutant line in comparison with GSH-fed Col-0. This observation was confirmed by a co-transfection assay of the BiP3 and HSP70B promoters with myb21 in Atmyb21 protoplasts and HSP90.1 promoter with bzip10 in Atbzip10 mutant protoplasts. Previous reports also demonstrated that GSH is an important factor in determining the basal JA-responsive gene expression and suggested that a GSH-dependent control point regulates JA signaling (Han et al., 2013). The contribution of BZIP10 to the oxidative stress response in Arabidopsis is known (Kaminaka et al., 2006). Homology modelling of MYB21 showed no site for glutathionylation, and transcript analysis revealed the up-regulation of myb21 in a GSH overexpression line. Altogether, it is evident that in response to stress, the cellular GSH content increases, which induces expression of myb21 and ultimately activates the promoters of BiP3 and HSP70B.

Results of comparative proteomics analysis on wild type Col-0 and TF mutant plants revealed other proteins, such as GST, PPlase ROC4, high chlorophyll fluorescence 136 (HCF 136), oxygen evolving protein, photosystem II subunit O-2 (PSBO2) and glutamate ammonia ligase, that were also regulated by MYB21 and BZIP10 in response to an elevated level of GSH. The function of GSH in enhancing the expression and activity of GST is an established fact (Mauch and Dudler, 1993; Loyall et al., 2000; Sinha et al., 2015). Down-regulation of PPlase ROC4 in stress-treated pad2.1 at both gene and protein levels shows a role of GSH in inducing this gene (Kumar et al., 2015). Previous investigation also suggested the importance of...
accumulation of glutamate ammonia ligase in the increased assimilation of ammonium in response to salt stress in potato (Teixeira and Fidalgó, 2009). These observations suggest a role of GSH in inducing these stress- and defense-related proteins along with HSPs through the TFs MYB21 and BZIP10 in response to environmental stress.
STRING 10 protein–protein interaction analysis pointed towards an interaction between HSP70 family proteins and other differentially expressed proteins in GSH-fed Col-0, Atmyb21, and Atbzip10 mutants. Among the differentially expressed proteins, GST showed maximum chance of interacting with HSP70 followed by PPIase ROC4, GS2, HCF, and PSBO2. HSP70 induced by stress plays an important role in mitigating the aggregation of stress-denatured proteins and helps in the refolding of non-native proteins for restoring their biological function through iterative cycles of adenine nucleotide hydrolysis-dependent peptide binding and release (De Maio, 1999; Sung et al., 2001b; Wang et al., 2004). A protein co-immunoprecipitation assay followed by western blot analysis revealed an enhanced level of interaction of GST with HSP70 in response to an elevated level of GSH in plant cells. Previous reports also pointed towards the higher affinity of HSP70 for GST under GSH-rich conditions (Robin et al., 2003). These observations suggest that GSH not only plays an important part in the induction of GST, but also modulates its interaction with HSP70 chaperone. GSTs are important in combating a wide range of abiotic and biotic stresses (Roxas et al., 1997; Dalton et al., 2009). In response to stress conditions, the chances of denaturation of stress-mitigating proteins such as GST increases. At that point different HSPs can interact with GST and act as a molecular chaperone to form an HSP–GST complex that can help in the restoration of function under stress conditions by maintaining its active form.

In this study, we have revealed a fascinating interplay of GSH and HSPs that execute important functions in combating environmental stress in the plant system. The expression levels of HSPs were positively regulated by GSH. We further suggest that GSH induces BiP3 and HSP70B by up-regulating the expression of myb21 (Fig. 9A), which plays a vital role in activating the promoters of both BiP3 and HSP70B. On the other hand, BZIP10 elevates the expression of HSP90.1 by activating its promoter in response to an increased level of GSH (Fig. 9B). We also observed an increased level of interaction of GST F2 with HSP70, which pointed towards its greater chaperone-like effect in response to the elevated level of GSH in plant cells (Fig. 9A). Together, our study has revealed the molecular mechanism through which GSH modulates the expression and

**Fig. 8.** GST protein expression and interaction study with HSP70 under altered GSH condition. (A) Western blot analysis of GST F2 in three Arabidopsis lines, viz. wild type Col-0, transgenic AtECS1, and mutant pad2.1, revealed its up-regulation in AtECS1 and down-regulation in pad2.1. (B) Protein co-immunoprecipitation followed by western blot demonstrated the elevated level of interaction of GST F2 with HSP70 in AtECS1. Input: loading control; protein A+HSP70: non-specific binding control.

**Fig. 9.** A suggested working model for GSH and HSP interplay. (A) Elevated GSH level in plant cell induces the expression of myb21 TF, which activates the promoters of HSP70 family genes, i.e. BiP3 and HSP70B, and up-regulates their expression. GSH also plays an important role in inducing GST F2 gene through MYB21. Downstream GSH also modulates the chaperonic effect of HSP70 by elevating its interaction with GST F2. (B) GSH also up-regulates the expression of HSP90.1 at both gene and protein level by activating its promoter through BZIP10. Blue arrow, up-regulation of genes and proteins; green arrow, elevated protein interaction. (This figure is available in color at JXB online.)
chaperone-like effect of HSPs. However, the possibility of an indirect regulation of the expression of HSPs brought upon by MYB21/BZIP10 cannot be ruled out assuming that these TFs can interact with or are modulated by several other entities. This may be another appealing area of investigation in the near future that will improve our understanding of the contributions of HSPs in stress tolerance and management. Furthermore, it will be interesting to explore the effect of interactions of different proteins with HSPs to mitigate stress.

**Supplementary data**

Supplementary data are available at JXB online.

Fig. S1. Transcript study of HSP genes in Col-0, *AtECS1*, *pad2.1*, and GSH-fed and BSO-fed Col-0 with tubulin as a reference gene.

Fig. S2. Transcript study of HSP genes in altered GSH condition.

Fig. S3. Western blot analysis of HSP70 and HSP90.1 protein expression in Col-0, *AtECS1*, *pad2.1*, and GSH-fed and BSO-fed Col-0.

Fig. S4. Promoter activation analysis of *proBiP3*, *proHSP70B*, and *proHSP90.1* in response to altered GSH conditions.

Fig. S5. GFP fluorescence analysis through ImageJ/Fiji software.

Fig. S6. Promoter sequences of HSPs.

Fig. S7. Western blot analysis of HSP expression in Col-0, GSH-fed Col-0, *Atmyb21*, GSH-fed *Atmyb21*, *Atbzip10*, and GSH-fed *Atbzip10*.

Fig. S8. Effect on the expression of HSPs in GSH-fed *AtAP2* and *Atin3* mutant lines.

Fig. S9. Co-transfection of the protoplasts *Atmyb21* in GSH-fed condition.

Fig. S10. 2DE gel picture of comparative proteomics analysis of Col-0, GSH-fed Col-0, GSH-fed *Atmyb21*, and GSH-fed *Atbzip10* mutants.

Table S1. List of primers used in the quantitative RT-PCR.

Table S2. Value of GFP fluorescence in the protoplasts of transected plant samples with altered GSH condition.

**Acknowledgements**

The authors thank the Director, CSIR-IICB for providing the necessary facilities. This work received financial support from the Council of Scientific and Industrial Research (CSIR), New Delhi (code: BSC/0107). DK acknowledges the CSIR for his fellowship and the Central proteomics facility of CSIR-IICB, Kolkata is acknowledged herewith.

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