Increased glutathione contributes to stress tolerance and global translational changes in Arabidopsis

Mei-Chun Cheng¹, Ko Ko¹, Wan-Ling Chang¹, Wen-Chieh Kuo¹, Guan-Hong Chen² and Tsan-Piao Lin¹,*

¹Institute of Plant Biology, National Taiwan University, 1 Roosevelt Road, Section 4, Taipei 10617, Taiwan, and
²Institute of Plant and Microbial Biology, Academia Sinica, Taipei 11529, Taiwan

Received 3 February 2015; revised 19 June 2015; accepted 9 July 2015; published online 25 July 2015.
*For correspondence (e-mail tpl@ntu.edu.tw).

SUMMARY

Although glutathione is well known for its reactive oxygen species (ROS) scavenging function and plays a protective role in biotic stress, its regulatory function in abiotic stress still remains to be elucidated. Our previous study showed that exogenously applied reduced glutathione (GSH) could improve abiotic stress tolerance in Arabidopsis. Here, we report that endogenously increased GSH also conferred tolerance to drought and salt stress in Arabidopsis. Moreover, both exogenous and endogenous GSH delayed senescence and flowering time. Polysomal profiling results showed that global translation was enhanced after GSH treatment and by the induced increase of GSH level by salt stress. By performing transcriptomic analyses of steady-state and polysome-bound mRNAs in GSH-treated plants, we reveal that GSH has a substantial impact on translation. Translational changes induced by GSH treatment target numerous hormones and stress signaling molecules, which might contribute to the enhanced stress tolerance in GSH-treated plants. Our translatome analysis also revealed that abscisic acid (ABA), auxin and jasmonic acid (JA) biosynthesis, as well as signaling genes, were activated during GSH treatment, which has not been reported in previously published transcriptomic data. Together, our data suggest that the increased glutathione level results in stress tolerance and global translational changes.

Keywords: glutathione, GSH1, abiotic stress, polysomal profiling, translatome, translational control, Arabidopsis.

INTRODUCTION

Glutathione (γ-glutamylcysteinylglycine) is an essential metabolite with multiple functions in plants. It is an important thiol antioxidant as well as a scavenger of reactive electrophilic compounds that functions with glutathione-S-transferases (GSTs; EC 2.5.1.18) to detoxify a range of herbicides (Marrs and Walbot, 1997; Edwards et al., 2000) by tagging electrophilic compounds for removal during oxidative stress. In both animals and plants, glutathione is synthesized from L-Glu, L-Cys and Gly in an ATP-dependent two-step pathway catalyzed by the enzymes γ-glutamylcysteine synthetase (γ-ECS; EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3). In Arabidopsis thaliana, three genes involved in glutathione synthesis have been identified: one coding for plastidial γ-ECS (GSH1) and two glutathione synthetases (GSH2), a cytosolic protein and a chloroplast-targeted protein (Wachter et al., 2005). Knocking out the expression of GSH1 causes lethality at the embryo stage (Cairns et al., 2006), whereas the knock-out of both GSH2 genes results in a seedling-lethal phenotype (Pasternak et al., 2008). Similar to γ-ECS from other organisms, the plant enzyme catalyzes a key regulatory step in glutathione biosynthesis, and may be controlled at the level of enzyme activity, protein synthesis or transcription (Xiang and Oliver, 1998; Noctor et al., 2002). Glutathione, primarily in its reduced form (GSH), is present at concentrations of 2–3 mM in various plant tissues (Creissen et al., 1999; Meyer and Fricker, 2002; Noctor et al., 2002). Because glutathione is a major cellular antioxidant, it is regarded as a determinant of cellular redox state and may indirectly influence many fundamental cellular processes (Schafer and Buettner, 2001; Cooper et al., 2002; Noctor et al., 2002).

Recently, glutathione was shown to play a role in NADPH-dependent glutathione/glutaredoxin (Grx) system (NGS)-dependent thioredoxin signaling. The NGS, together with the NADPH-dependent thioredoxin (Trx) system (NTS), transfers a redox signal to target proteins with reversible dithiol/disulfide exchange (S–S → 2HS), in a process known as thiol redox regulation (Foyer and Noctor, 2011; Noctor et al., 2012). Through such signaling, the biological
activity of a given protein (e.g. enzymatic activity) is tightly regulated, usually in response to environmental, physiological and developmental cues.

Glutathione has multiple physiological functions through its ability to maintain intracellular redox homeostasis in addition to functions in various defense reactions. Glutathione is essential for plant growth and development. For example, it has critical functions in embryo and meristem development (Vernoux et al., 2000; Cairns et al., 2006; Reichhold et al., 2007; Frottin et al., 2009; Bashandy et al., 2010), and is required for pollen germination, pollen tube growth (Zechmann et al., 2011) and cell cycle regulation (Pellny et al., 2009; Díaz-Vivancos et al., 2010). In Arabidopsis, GSH has long been implicated in secondary metabolism and pathogenesis responses. Exogenous GSH can mimic fungal elicitors in activating the expression of defense-related genes (Dron et al., 1988; Wingate et al., 1988), including PATHOGENESIS-RELATED 1 (PR1) and NONEXPRESSOR OF PR GENES 1 (NPR1; Senda and Ogawa, 2004; Gomez et al., 2004). Crucial information has been generated by the analysis of GSH-deficient mutants. The GSH-deficient mutants cad2 and rax1-1 were reported to have increased susceptibility to avirulent Pseudomonas syringae (Ball et al., 2004). Similar to the cad2 mutant, the pad2-1 (phytoalexin deficient 2-1) mutant was identified as a mutant allele of GSH1 and showed enhanced susceptibility to various bacterial, fungal and oomycete pathogens because of a deficiency in camalexin, an indole phytoalexin that is produced by Arabidopsis in response to infection with a bacterial pathogen (Parisy et al., 2006). A conditionally important role of GSH occurs in response to excessive levels of heavy metals. GSH is the precursor of phytochelatins ([β-Glu-Cys][nGly]), compounds that are synthesized in response to cadmium and other heavy metals. Phytochelatins sequester the metal to form a complex that is then transported into the vacuole (Grill et al., 1987, 1989; Cobbett and Goldsbrough, 2002; Rea et al., 2004). Transgenic upregulation of GSH synthesis has been reported to increase heavy metal tolerance (Zhu et al., 1999). GSH can react chemically with reactive oxygen species (ROS), which are produced during various stress responses, such as oligogalacturonide elicitor treatment and pathogen infection (Ball et al., 2004; Dubreuil-Maurizi et al., 2011; Noctor et al., 2012), as well as upon genetic inhibition of H₂O₂-metabolizing enzymes (Smith et al., 1984; May and Leaver, 1993; Queval et al., 2007).

Despite the widely assumed involvement of GSH in abiotic stress signaling in plants, few examples of GSH-mediated drought and salinity stress tolerance in plants have been reported in the literature. In our previous study, the enhanced drought and salt stress tolerance conferred by exogenous GSH was unequivocally shown in A. thaliana (Chen et al., 2012). Primary root lengths were increased by 62% in 2-week-old wild-type plants growing in 25 and 50 μM GSH. Whereas plants growing in water containing 400 μM GSH could recover and resume growth following drought and salt stress, no plants could resume growth in water only.

To further understand the mechanism of GSH involvement in the abiotic stress response, we studied the physiological effects of altered exogenous and endogenous GSH levels. Here, we report the protective role of both endogenous and exogenous GSH in abiotic stress tolerance and senescence. We used gain-of-function and loss-of-function GSH synthesis mutants as well as plants treated with reduced GSH for various phenotypic analyses. Transcriptomic profiling has been frequently used to examine how changes in GSH levels regulate mRNA levels on a genomewide scale (Koprivova et al., 2010; Li et al., 2013), but few of these studies have revealed the relationship between GSH and abiotic stress. By performing transcriptomic analyses of steady-state and polysome-bound mRNAs in GSH-treated plants, we reveal that GSH has a larger impact on growth, development and the stress response than we have learned from total mRNA profiling studies. Translational changes induced by GSH treatment target numerous hormones and stress-signaling molecules, which might contribute to the enhanced stress tolerance in GSH-treated plants.

RESULTS

Increased concentration of endogenous GSH improves abiotic stress tolerance in Arabidopsis

In our previous report, we showed that exogenously applied GSH conferred drought and salt stress tolerance (Chen et al., 2012; Figure S1). To further study the exact role of GSH in abiotic stress in Arabidopsis, we tested whether endogenously increased GSH levels also lead to abiotic stress tolerance. We performed drought and salt stress tolerance assays using transgenic plants expressing the GSH1 gene driven by the stress-inducible RD29A promoter (RD29A:GSH1; Kasuga et al., 1999) and the pad2-1 mutant, which contains only 22% of the wild-type (WT) level of glutathione. Expression of GSH1 in the RD29A:GSH1 transgenic plants was verified by qRT-PCR assays before further analysis (Figure S2a). Under drought conditions, the GSH and GSSG levels in RD29A:GSH1 transgenic plants (RD12 and RD15) were significantly higher than those in WT plants (Figure 1a,b). The transgenic and mutant plants grown in pots together with WT plants were not watered for 2 weeks (Figure 1c). Approximately 70% of the transgenic plants survived within this 2-week period and continued to grow when watering resumed, whereas only 30% of WT plants survived this level of drought stress. In contrast, the pad2-1 mutants had a lower survival rate (46%) compared with WT plants (Figures 1c and S3). To examine the tolerance of the transgenic plants to salt stress, we performed salt stress tolerance tests. After salt

© 2015 The Authors
stress treatment for 18–24 days, approximately 71–80% of RD29A:GSH1 plants survived, whereas only 37–49% of WT plants survived. Conversely, only 48% of the pad2-1 mutants survived, whereas 78% of WT plants survived (Figure 1d).

To further confirm the salt tolerance phenotype of RD29A:GSH1 plants, we used different concentrations of sodium chloride (100 and 200 mM NaCl) to test germination rates under high salinity. After 3–4 days of incubation, the germination rates for RD29A:GSH1 plants were higher.
than those of WT and pad2-1 mutant plants (Figure 1e,f). After 3 days of incubation in 100 mM NaCl, the germination of WT seeds was inhibited, and only approximately 62% of the seeds germinated, whereas the germination rate of RD29A::GSH1 reached 85–95%. Additionally, after treatment with 200 mM NaCl treatment for 4 days, approximately 20% of WT seeds germinated, but the RD29A::GSH1 seeds had a 30–50% germination rate. In all conditions tested, pad2-1 mutants exhibited similar germination rates as WT seeds (Figure 1e,f). We also observed the root elongation of these transgenic plants both under drought and salt stress. As shown in Figure 1(g,h), RD29A::GSH1 (RD12 and RD15) exhibited greater root elongation under both salt and drought stress. Taken together, these results indicate that GSH has a positive effect on tolerance to abiotic stresses, including drought and salt, in Arabidopsis.

Increased level of GSH delayed senescence and flowering time

To investigate the potential role of GSH in senescence and leaf longevity, we added GSH to 10-day-old WT plants grown in pots. GSH content was measured every 2 days after treating the plants with 400 μM GSH. As shown in Figure 2(a), plants treated with GSH had higher GSH and GSSG contents compared with plants treated with mock solution. Analysis of leaf aging revealed that the plants treated with GSH exhibited delayed senescence and prolonged leaf longevity (Figure 2b). Similar patterns were also observed with chlorophyll leaf content (Figure 2c), and the expression of the cysteine protease-encoding SAG12 (SENESCENCE-ASSOCIATED GENE 12) and SEN4 (SENESCENCE 4) senescence marker genes (Figure 2d,e). SAG12 and SEN4 exhibited delayed induction in the GSH-treated plants. We also observed a higher expression level of the RBPCS gene, which yields sufficient rubisco for photosynthetic capacity, in GSH-treated plants (Figure 2f).

To test whether endogenous GSH accumulation also affects senescence and flowering time, we grew 35S::GSH1 transgenic plants and pad2-1 mutants together with WT plants. As shown in Figure 3(a), 35S::GSH1 plants (7-5 and 13-6) exhibited delayed flowering, whereas pad2-1 mutants showed an earlier flowering phenotype. We also added the glutathione biosynthesis inhibitor buthionine sulfoximine (BSO) to WT plants as a negative control (Figure 3b). BSO inhibits GSH1 and results in the depletion of cellular GSH levels (Griffith and Meister, 1979; May and Leaver, 1993). Our BSO treatment also resulted in decreased GSH levels (Figure S4). By contrast, BSO-treated plants exhibited the earlier flowering phenotype. The number of rosette leaves at bolting was higher in 35S::GSH1 than in WT, pad2-1 mutants and BSO-treated plants (Figure 3c). We also examined mRNA levels of the flowering-time genes FT and FLC in the transgenic plants and mutant. The expression of the flowering repressor FLC was strongly increased in 35S::GSH1 and decreased in pad2-1 mutants, in agreement with the late-flowering phenotype (Figure 3d); however, the expression of the flowering pathway integrator FLOWERING LOCUS T (FT) was lower in 35S::GSH1 (Figure 3d). Consistent with the flowering phenotype, SAG12 exhibited delayed induction in the two 35S::GSH1 lines (7-5 and 13-6), but accelerated induction in pad2-1 mutants and in the BSO-treated plants (Figure 3e). This result is most likely linked to the ROS scavenging property of GSH.

GSH enhances global translation

To investigate the effect of GSH on mRNA translation, 10-day-old Arabidopsis seedlings were transferred to media containing no GSH (control) or 100 μM GSH for 0.5, 4, 8 and 24 h. The root and shoot samples were then extracted separately and fractionated by centrifugation through a sucrose gradient. The translational status of the samples was examined by polysome profiling analyses, which allows for quantifying the levels of monosomes and large polysomes. Translating complexes with more than three ribosomes were designated as polysome (PL) fractions (Liu et al., 2012), whereas fractions with fewer than two ribosomes were designated as non-polysomal (NP) fractions (Figure 4a). The spatiotemporal translational profiling revealed that GSH treatment enhances the translation activity in roots after 8 h and in shoots after 24 h (Figure 4a), whereas the polysome profiles at other time points did not differ from the control conditions. Based on the results in Figure 4(a), we chose 8 and 24 h as time points for further studies of the GSH-triggered translational activation. To provide a more quantitative calculation of the ribosome loading efficiency, we used PL% (percentage of total RNA in the polysomal fraction, designated by Liu et al., 2012) to infer the proportion of RNAs engaged in active translation. At the 8-h time point, GSH treatment did not change the PL% in the shoot, but it promoted the PL% in the root by 5%. By contrast, after 24 h of GSH treatment, PL% increased approximately 6% in the shoot but remained the same as the control sample in the root (Figure 4b).

To further examine the effects of GSH treatment, we assayed the total glutathione content and glutathione redox status by measuring reduced (GSH) and oxidized glutathione (GSSG) levels in both roots and shoots of samples after GSH treatment for 8 h (8R and 8S) and 24 h (24R and 24S). GSH treatments slightly enhanced the total GSH content in all conditions, but significantly enhanced total GSH in roots and shoots after 8 h and in shoots after a 24 h of treatment (Figure 5a); however, the GSSG contents in these two particular samples (8R and 24S) showed no differences relative to control samples (Figure 5b). We also used GRX1-reduction oxidation sensitive GFP (GRX1-roGFP2; Meyer et al., 2007; Gutscher et al., 2008) to examine the glutathione redox status after GSH treatment.
in vivo. GRX1-roGFP2 can be excited at 405 and 488 nm in its oxidized and reduced states, respectively; thus, the 405:488 ratio reflects the glutathione redox status. Ratio-metric imaging showed that after GSH treatment, 405:488 ratios were increased in roots after 8 and 24 h, and in shoots after 24 h, of GSH treatment (Figure S5c). These results implied that the translational changes observed after GSH treatment might be triggered by the higher glutathione content and a more reduced glutathione redox status.

To further prove that GSH could enhance translation, we used salt stress (150 mM NaCl) to induce the production of GSH in 10-day-old RD29A:GSH1 transgenic seedlings, and compared the polysomal profile with Col-0 seedlings at 0, 3 and 6 h. GSH1 expression was induced by salt stress in RD29A:GSH1 transgenic seedlings, and GSH levels were also increased (Figure S6a,b). As shown in Figure S7(a), the translation activities in RD29A:GSH1 after 3 and 6 h of salt stress treatment were enhanced, whereas the polysome profiles of the control conditions did not differ from those of Col-0. PL% calculation also showed that more RNAs were engaged in active translation in RD29A:GSH1 after salt stress (Figure S7b). These results indicate that GSH1 could influence polysomal mRNA levels during salt stress.
Genome-wide analyses of the transcriptome and translatome in response to GSH

To understand the global translation regulated by GSH, we performed transcriptomic analyses of steady-state mRNAs (mRNA$_{SS}$) and polysome-bound mRNAs (mRNA$_{PL}$) by microarray assay using an Agilent Arabidopsis V4 Oligo 4x44K Microarray (http://www.agilent.com; for detailed data analyses procedures, see Appendixes S5). Two biological replicates were performed to reveal changes in mRNA$_{SS}$ and mRNA$_{PL}$ in both root and shoot samples after GSH treatment for 8 h (8R and 8S) and 24 h (24R and 24S). At the 8-h time point, we identified 3764 mRNAs that displayed significant two-fold changes at the mRNA$_{PL}$ level in the roots, whereas we identified only 676 mRNAs induced at the mRNA$_{SS}$ level; however, at the 24-h time point, 3491 mRNAs were two-fold upregulated in the shoot at the mRNA$_{PL}$ level, whereas 526 mRNAs were induced at the mRNA$_{SS}$ level (Figure 4c; data in Appendices S1 and S2). Thus, a longer delay was observed before the occurrence of transcriptional and translational changes in shoots compared with roots in response to GSH treatment, which is consistent with our polysomal profiling data. This result also suggests that GSH has a greater impact on translational changes than on transcriptional changes, and GSH-mediated translational activation results primarily from the increased translation of pre-existing mRNAs. We have also performed quantitative real-time PCR (qRT-PCR) to detect the levels of selected transcripts in total RNA and PL populations. We examined each seven genes in total RNA and mRNA PL, and these selected genes were highly relevant to abiotic stress, biotic stress and hormone synthesis (Figure S8). At both the mRNA$_{PL}$ and mRNA$_{SS}$ levels, qRT-PCR results showed consistent patterns with the microarray results for most of the 13 genes (Figure S8). We also performed western blot analysis to validate some of the proteins corresponding to the mRNAs that were enriched in the translational state. As shown in Figure S9(a), ABF3, ERF1, DREB1A and IRT1 showed increased protein levels at 8R, whereas HSP90.1 showed increased enrichment at 24S. These results agreed with the fold change values obtained from our microarray analysis (Figure S9b). To see whether the stress-related genes were also active in trans-

**Figure 3.** Senescence in wild-type (WT), GSH1 overexpressor and pad2-1 mutant plants.
(a) Phenotypes of 36-day-old WT, independent 35S:GSH1 transgenic lines (7-5 and 13-6) and pad2-1 mutant plants.
(b) Phenotype of 36-day-old WT plants treated with or without buthionine sulfoximine (BSO). Ten-day-old plants grown in soil under long-day (LD) conditions were watered with or without 50 μM BSO every other day for 16 days.
(c) Number of rosette leaves at bolting for WT, 35S:GSH1 transgenic plants, pad2-1 mutants and BSO-treated plants under LD conditions. Bars indicate SEs. An asterisk indicates significant differences from the WT: P < 0.05 (Student’s t-test).
(d) Expression levels of FLC and FT in 35S:GSH1 plants and pad2-1 mutants. Bars indicate SEs. An asterisk indicates significant differences from the WT: P < 0.05 (Student’s t-test).
(e) Expression of SAG12 in WT plants, GSH1 overexpressors, pad2-1 mutants and BSO-treated plants. The fourth leaves of approximately 30 plants grown in soil and harvested at the indicated time points were used for total RNA extraction. Transcript levels were determined by qRT-PCR. The Actin2 gene was included in the assays as an internal control for normalizing the variations in cDNA levels used. Biological triplicates were averaged. Bars indicate SEs. An asterisk indicates a significant difference from the WT: P < 0.05 (Student’s t-test).
lation after salt stress induction of GSH in RD29A:GSH1 seedlings, we selected 13 genes to examine their abundance in total RNA and PL mRNA. As shown in Figure S10, in Col-0 treated with salt stress for 3 and 6 h, the mRNA abundance of the stress-related genes showed little difference in total RNA and PL mRNA; however, in RD29A:GSH1 treated with salt stress for 3 and 6 h, the mRNAs were more abundant in the polysomal fraction. This provides interesting insight into stress-induced upregulation that a plastid-targeted enzyme has an impact on mRNA translation.

To investigate the biological impact resulting from gene expression regulated at different levels and in different tissues, we performed k-means cluster analysis to group the upregulated genes at 8R and 24S, and reveal three expression patterns (Figure S11a,c, as described by Liu et al., 2015).
mRNAs in cluster 1 had a concordant induction at the mRNA_{SS} and mRNA_{PL} levels in response to GSH. The translational increase of mRNAs in cluster 1 may simply reflect their increase at the mRNA_{SS} level, and thus, this cluster was designated the ‘RNA’ group (Figure S11a,c). mRNAs in the RNA group are primarily regulated at the mRNA_{SS} level. mRNAs in cluster 2 showed a moderate but clear induction at the mRNA_{SS} level and a more prominent increase at the mRNA_{PL} level, and were designated the ‘RNA+Protein’ group (Figure S11a,c). mRNAs in the RNA-protein group exhibited increased transcript levels and were preferentially associated with polysomes. mRNAs in clusters 3 showed enrichment at the mRNA_{PL} level, but their mRNA_{SS} levels remained unchanged. Therefore, the mRNAs in cluster 3 are mainly regulated through their close associations with the polysomes, regardless of their steady-state mRNA abundance, causing efficient translation after GSH treatment; this cluster was designated the ‘Protein’ group (Figure S11a,c).

To address whether translational control regulates specific aspects of cellular functions, genes showing regulation at the RNA, RNA+Protein and Protein levels were examined for over-representation of any specific biochemical pathway or gene ontology (GO) assignment. The results in Figure S11(b) and d show that pathways or processes for hormone signaling and transcriptional activity are largely regulated at the mRNA_{SS} level. Translational control appears to mainly apply to genes involved in hormone biosynthesis, the cell cycle, cell wall metabolism and redox homeostasis. Intuitively, transcripts of these genes receive higher priority in engaging translational machinery before the increase in their transcript levels, if any.

GSH-mediated translational changes regulate a wide range of cellular activities and biological processes

When comparing the similarities between 8R- and 24S-upregulated genes at the polysomal level, we found that approximately 20% of 8R-regulated genes were also regulated at the 24S level. After performing a hierarchical cluster analysis of the 8R and 24S co-regulated genes at the polysomal level, we found that 8R-regulated genes showed a pattern similar to the 24S-regulated genes, but that 8R- and 24S-regulated genes showed opposite expression patterns as the 8S- and 24R-regulated genes (Figure 5a, cluster analysis combining genes regulated at the steady-state level; see Figure S12). In addition, our correlation results showed that many genes were co-regulated at 8R and 24S, with a high correlation ($R = 0.79$), as shown in the scatter plot (Figure 5b). Among the 3764 8R upregulated genes, 756 genes were also regulated at the 24S level (Figure 5c). We then performed GO analysis and functionally clustered the results using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) resource (Huang et al., 2009). This analysis revealed that the 8R-24S co-regulated genes are preferentially associated with hormones, the stress response, oxidation-reduction and sulfur assimilation (Table 1). Distinct GO terms are also revealed in 8R and 24S. 8R-activated genes are enriched for carotenoid, proline metabolism and root development genes, whereas 24S-activated genes are enriched for cell cycle, $\alpha$-linolenic acid metabolism and transcription genes (Table 1). Our results show that genes co-regulated at the 8R and 24S levels have overlapping GO terms and yet are distinct. Thus, we further classified the genes regulated at the 8R and 24S levels into four categories: genes specifically regulated at the 8R level (8 h-root only); genes specifically regulated at the 24S level (24 h-shoot only); genes regulated at the 8R level with similar GO terms as 24S (8R); and genes regulated at the 24S level with similar GO terms as 8R (24S). Representative GSH-regulated genes with various GO terms based on Table 1, including cellular functions, development stages, environmental responses and hormone pathways, are shown in Figure 5(d).

A large number of genes that are involved in cellular processes and hormone responses are upregulated at the 8R-24S levels, and are likely to mediate the internal developmental transitions during GSH treatment (Figure 5d). Many genes involved in environmental responses, including biotic and abiotic stress responses, are also regulated at the 8R-24S levels, which is likely to contribute to the stress tolerance in Arabidopsis treated with exogenous GSH or induced to contain higher endogenous GSH concentrations. Genes involved in the response to water deprivation are also overrepresented, including DREB2A and DREB1A, two key regulators of stress responses to drought, heat and cold (Gilmour et al., 2000; Sakuma et al., 2006), dehydration-responsive genes such as ERD5, COR47, RAB18, ABF3 and other abscisic acid (ABA)-responsive genes. Increased expression of the transcription factors (TFs) WRKY33 and WRKY38, which are induced by hydrogen peroxide and exhibit enhanced plant defense responses (Kim et al., 2008), were also upregulated by GSH treatment, independently of stress.

Interestingly, at the 8R level, genes related to proline (Pro) synthesis, $\Delta^{1}$-PYRROLINE-5-CARBOXYLATE SYNTHASE 1 (P5CS1) and P5CS2, are upregulated. Pro is a compatible osmolyte that contributes to drought tolerance through the protection of cellular structure and through the role of Pro metabolism in redox buffering (Szabados and Savouré, 2010; Verslues and Sharma, 2010; Sharma et al., 2011). To determine whether GSH treatment affected Pro accumulation, we measured the Pro content in WT plants treated with or without GSH. GSH-treated plants accumulated higher Pro levels than mock-treated plants (Figure S13a). To determine whether GSH1 overexpressors also accumulate high levels of Pro, we measured the Pro contents in WT, 35S:GSH1 transgenic plants and pad2-1 mutants; however, GSH1 overexpressors, which contain
more glutathione than WT plants, exhibited significantly lower levels of Pro, whereas pad2-1 mutants exhibited higher levels of Pro than WT plants (Figure S13b). Given that both Pro and GSH share the same precursor, glutamate, excess GSH resulting from GSH treatment might serve as a glutamate reservoir for Pro synthesis.

Cell walls are dynamic structures and key determinants of overall plant form, growth, development, and responses to environmental and pathogen-induced stresses (Farrokhi et al., 2006). At the 8R and 24S levels, genes involved in cell wall biogenesis, such as the glycosyl transferases IRREGULAR XYLEM 9 (IRX9) and PARVUS, are upregulated. Other upregulated genes encode UDP-arabinose mutase, REVERSIBLY GLYCOSYLATED POLYPEPTIDE 1 (RGP1) and MURUS, which are involved in the biosynthesis of plant cell wall arabinoxylan components.

---

**Figure 5.** 8R-regulated genes showed a similar pattern as the 24S-regulated genes at the polysomal level.

(a) Hierarchical cluster analysis of the differentially expressed genes at the polysomal level after 8 and 24 h of GSH treatment. R, root; S, shoot.

(b) A genome-wide comparison by scatter plot of values with log₂-fold changes between 8R- and 24S-regulated genes. The diagonal line represents the correlation between 8R- and 24S-regulated genes analyzed by GENESPRING 12.5.

(c) Venn diagram showing the overlaps between 8R- and 24S-regulated genes.

(d) Representative 8R- and 24-upregulated genes with known functions in various cellular, developmental, environmental response and hormone pathways. Key activated genes involved in stress responses are red. The groupings of the genes are mainly based on the gene ontology terms listed in Table 1.
enzymes may play a role in controlling the flexibility and structural properties of cell wall components needed for desiccation (Moore et al., 2006). Interestingly, we also found that two iron binding-related genes, IRON REGULATED TRANSPORTER 1 (IRT1) and IRT2, are overexpressed at the 8R level but not at the 24S level. There is a strong relationship between heavy metal homeostasis and immunity in plants. Aznar et al. (2014) reported that strong iron scavengers such as siderophores can activate immune responses in Arabidopsis via IRT1-mediated alterations in iron distribution. Additionally, plants ectopically expressing the iron-binding protein ferritin are tolerant to oxidative damage and pathogens (Deák et al., 1999). Therefore, iron binding-related genes might contribute to the defense response induced by GSH treatment.

Compared with previous transcriptomic profiling data (Koprivova et al., 2010; Li et al., 2013), translational control appears to have much wider effects on gene expression regulation. It is presumed that the ability to selectively translate mRNAs that encode proteins required for physiological or morphological responses to stress facilitates a rapid and energy-conserving response (Bailey-Serres, 1999). We believe that the selective translation caused by GSH treatment allows for the quick production of proteins needed for the GSH-mediated stress response and environmental adjustment.

Translational control activates distinct hormone biosynthesis genes at the 8R and 24S levels

GSH redox is known to have impacts on auxin transport, signaling and even accumulation (Bashandy et al., 2010). Mhamdi et al. (2010) also reported that altered GSH redox status would affect salicylic acid (SA) accumulation and jasmonic acid (JA) signaling. Our previous findings also suggested that GSH treatment could enhance ABA accumulation (Chen et al., 2012). In our microarray data of the translatome, we found that among the genes regulated at both the 8R and 24S levels, genes involved in the synthesis of JA and ABA are clearly overrepresented (Table 1; Appendix S4). As shown in Figure 6(a), JA-related genes upregulated at 8R and 24S include genes required for JA biosynthesis, including two lipoxygenases, LOX3 and LOX5, an allene oxide cyclase, AOC3, three 12-oxophytodienoate reductases, OPR1, OPR2 and OPR3, and JASMONATE RESISTANT 1 (JAR1), which catalyzes the formation of a biologically active jasmonyl-isoleucine (JA-Ile) conjugate. Additionally, genes implicated in controlling JA responses are upregulated, including many MYB TFs, and the jasmonate ZIM-Domain gene, JAZ5 (Figure S14). This observation substantiates previous reports that blocking GSH accumulation strongly attenuates the expression of genes involved in JA synthesis and signaling (Ball et al., 2004; Han et al., 2013). A key SA biosynthesis gene, enhanced pseudomonas susceptibility 1 (EPS1), which

Table 1 Biochemical pathways and gene ontology (GO) terms for genes regulated at the mRNA level and at different spatiotemporal levels upon treatment with GSH

<table>
<thead>
<tr>
<th>Regulatory group</th>
<th>8 h-root (3008)</th>
<th>8 h-root + 24 h-shoot (756)</th>
<th>24 h-shoot (2735)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KEGG pathway</td>
<td>Arginine and proline metabolism</td>
<td>Biosynthesis of plant hormones</td>
<td>α-Linolenic acid metabolism</td>
</tr>
<tr>
<td>Carotenoid biosynthesis</td>
<td>Cell wall biogenesis</td>
<td>Response to abiotic stimulus</td>
<td>Cell cycle</td>
</tr>
<tr>
<td>Response to metal ion metabolism</td>
<td>Oxidation response</td>
<td>Transcription</td>
<td>α-Linolenic acid metabolism</td>
</tr>
<tr>
<td>Root system development</td>
<td>Hormone biosynthetic process</td>
<td>Sulfur metabolic process</td>
<td></td>
</tr>
<tr>
<td>Carotenoid biosynthesis</td>
<td>Response to hormone stimulus</td>
<td>CYP707A2</td>
<td>CYP707A3</td>
</tr>
</tbody>
</table>

The indicated gene numbers are referred to in Figure 5(c); only pathways and GOs with $P < 1.0E-04$ are listed.

Figure 6. Hormone biosynthesis genes translationally regulated after 8 and 24 h of GSH treatment.

(a-c) Heat map showing the normalized expression levels of genes involved in JA biosynthesis (a), auxin biosynthesis (b) and ABA biosynthesis (c). Extreme red and green colors indicate fourfold upregulation and downregulation, respectively. The signaling genes that are translationally regulated by JA, auxin and ABA are shown in Figures S14–S16.
encodes a member of the BAHD acyltransferase superfamily, is also upregulated at the 8R level.

At both the 8R and 24S levels, we found that many auxin biosynthesis genes are upregulated at the translational level. As shown in Figure 6(b), the expression of several auxin biosynthesis-related genes, including YUCCA 2, NITRILASE 1 (NIT1) and NIT2, SUPERROOT 1 (SUR1), which encodes a carbon-sulfur lyase, IAA LEUCINE RESISTANT-LIKE 1 (ILL1) and ILL2, were upregulated at the 8R level; however, at the 24S level, the expression levels of several other auxin biosynthesis-related genes, including CYP79B2, CYP83B1 and ILL6, were upregulated (Figure 6b). CYP79B family members are known to convert Trp to indol-3-acetaldoxime (IAOx), a precursor to indole acetic acid (IAA) and indole glucosinolates (Mashiguchi et al., 2011). Auxin-responsive genes are also highly upregulated at the 8R and 24S levels compared with the 8S and 24R levels (Figure S15). Among the auxin-related genes upregulated at the 8R level, ANTHRANILATE SYNTHASE BETA SUBUNIT 1 (ASB1), SUR1, IAA28 and IAA31 are known to function in lateral root formation (Boerjan et al., 1995), and may contribute to the more developed root structure of GSH-treated plants (Chen et al., 2012). Nikiforova et al. (2003) reported that sulfur-assimilation pathways are tightly connected with JA and auxin biosynthesis via the sulfur-induced transcription of OPR1 and the biosynthesis of indole glucosinolates, respectively. In our translatome data, several sulfur metabolism-related genes, including ATP SULFURYLASE 1 (APS1) and APS4, which are involved in glucosinolate biosynthetic processes, are upregulated. Therefore, it is possible that GSH treatment triggers metabolic changes and hormone biosynthesis by way of sulfur assimilation.

The most significant observation is that GSH has a direct effect on ABA biosynthesis and signaling that only exists at the translational level. ABA biosynthesis genes, including a zeaxanthin epoxidase gene, ABA DEFICIENT 1 (ABA1), NCED4 and NCED5 for 9-cis-epoxy-carotenoid dioxygenase (Figure 6c) are specially upregulated at the 8R level, but not at the 24S level. The production of ABA uses products of the carotenoid biosynthesis pathway, with ABA1 acting upstream of NCEs, which perform the rate-limiting step (Finkelstein and Rock, 2002; Tan et al., 2003). Conversely, ABA-responsive genes are upregulated at the 8R level; these genes include OPEN STOMATA 1 (OST1/SnRK2.6), ARABIDOPSIS THALIANA PROTEIN PHOSPHATASE 2CA (PP2CA), the ABA co-receptor AOS, ACID RESPONSIVE ELEMENTS-BINDING FACTOR 2 (ABF2) and ABF3 (Figure S16). We were also curious how ABA degradation might be translationally regulated. CYP707A2 and CYP707A3, members of the cytochrome P450 subfamily that direct the conversion of ABA to phaseolic acid in the root, were also upregulated. This result most likely occurred because the ABA synthesis and degradation pathways are tightly co-regulated (Kushiro et al., 2004; Saito et al., 2004). To further test whether ABA content is indeed affected by GSH treatment, we measured the ABA content in roots and shoots separately after treatment with GSH for 8 and 24 h. As shown in Figure S17, GSH treatment did not change the ABA levels in either roots or shoots after an 8-h treatment, but significantly enhanced the ABA levels in shoots after a 24-h treatment. This result suggests that the increase in ABA levels in roots occurs shortly after the 8-h time point, and that ABA synthesized in roots after 8 h of GSH treatment might be transported to shoots at 24 h. ABA has previously been reported to be transported from roots to shoots under drought stress (Sauter et al., 2001; Hartung et al., 2002).

It is interesting that ABA and auxin were the earliest hormones to be detected when plants were treated with GSH, followed by JA, indicating that the abiotic stress response is activated before the biotic stress response. We believe that stress tolerance and many other biological impacts resulting from GSH treatment might be linked to these increased hormone levels. Furthermore, hormone synthesis was transcriptionally altered by GSH signaling.

**EXPERIMENTAL PROCEDURES**

**Plant materials, growth conditions and transgenic plant construction**

*Arabidopsis thaliana* ecotype Columbia-0 was surface-sterilized, sown on a nylon mesh floating on half-strength liquid Murashige & Skoog (MS) medium (pH 5.7), and kept at 4°C in darkness for 3 days for stratification. For the GSH treatment, 10-day-old seedlings grown on a nylon mesh were transferred to soil with or without 400 µM GSH. To prevent the degradation or oxidation of GSH, the water solution was replaced every other day with newly prepared chemicals. For the senescence and flowering-time experiments, 10-day-old seedlings grown in soil were supplemented with newly prepared water solution containing GSH or BSO every other day, and then incubated under regular growth conditions. For polysomal profiling and microarray analysis, 10-day-old seedlings grown on a nylon mesh were directly transferred to half-strength liquid MS medium with or without 100 µM GSH. For the measurement of ABA levels, roots and shoots of 100 µM GSH were harvested after 24 h. GSH treatment did not affect the ABA levels in either roots or shoots after an 8-h treatment, indicating that the abiotic stress response is activated before the biotic stress response. We believe that stress tolerance and many other biological impacts resulting from GSH treatment might be linked to these increased hormone levels. Furthermore, hormone synthesis was transcriptionally altered by GSH signaling.

---

© 2015 The Authors

construct. The plasmid was introduced into Agrobacterium tumefaciens GV3101 cells, and Arabidopsis transformation was performed by the floral-dip method.

**Seed germination and root elongation assay**

Mature seeds were harvested at the same time and were stored under the same conditions for the seed germination assay. Imbibed seeds were cold-treated at 4°C in the dark for 3 days, and moved to 22°C with a 16-h light/8-h dark photoperiod. Germination was defined as a 1-mm protrusion of the radicle. For the root elongation assay, sterilized seeds were sown on half-strength MS agar medium and grown for 3 days. Seedlings were then transferred to square plates containing MS medium (control) or MS medium supplemented with 150 mM NaCl. The initial position of the root tip was marked on the plate. The plants were grown in a vertical position at 22°C with a 16-h light/8-h dark cycle for 5 days. The primary root lengths were scored.

**Drought and salt tolerance tests**

For the drought tolerance test, 15 plants of each line were initially grown in soil under a normal watering regime for 3 weeks. Watering was then halted, and observations were made after a further 12–16 days without water. This time range is dependent on the survival of the weaker plants. When the weaker plants exhibited lethal effects of dehydration, watering was resumed, and the plants were allowed to grow for a subsequent 5 days. For the salt tolerance test, 3-week-old plants were watered for 12 days at 4-day intervals with increasing concentrations of NaCl of 100, 200 and 300 mM. Survival was scored by examining the inflorescence base to determine whether it remained green.

**Isolation of polysomal RNA and total RNA**

The isolation of polysomal RNA was performed as described by Liu et al. (2012). For details on the isolation of polysomal and total RNA, see Appendix S5.

**Agilent GeneChip hybridization and data analyses**

For details on the microarray analysis, see Appendix S2.

**Quantification of chlorophyll and GSH contents**

Chlorophyll was extracted with 80% acetone, and the extracted solution was incubated for 10 min in complete darkness. Chlorophyll contents were assayed by measuring absorbance at 652, 665 and 750 nm, using a diode array spectrophotometer (WPA Bio-wave; Biochrom, http://www.biochrom.co.uk).

The methods used to measure GSH and GSSG were as described by Griffith (1980). Leaf tissues (200 mg) were ground with a mortar and pestle in liquid nitrogen. Subsequently, 2 mL of 1 mM EDTA and 6% (v/v) metaphosphoric acid, pH 2.8, were added and mixed, and then centrifuged at 15,000 g for 20 min. The supernatant was neutralized with 0.2 M NaOH. The final pH of the neutralized acid extracts was between 5 and 6. Glutathione disulfide (GSSG) was measured using glutathione reductase and 2-vinylpyridine. Glutathione levels were determined using a kinetic assay in which the reduction of 5,5-dithiobis(2-nitrobenzoic acid) was spectrophotometrically measured at 412 nm.

**ACKNOWLEDGEMENTS**

This work was supported by the Ministry of Science and Technology, Taiwan (grant nos. 103-2311-B-002-002-), and by the National Taiwan University (grant nos. 104R8920 and 104R892001 to T-PL). The 35S:GSH1 transgenic Arabidopsis plants were obtained from Dr Kuo-Chen Yeh (Academia Sinica).

**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** Effects of exogenous GSH on drought and salt tolerance.

**Figure S2.** Expression levels of GSH1 mRNA in transgenic plants.

**Figure S3.** Drought tolerance of pad2-1 mutants.

**Figure S4.** GSH and GSSG contents in wild-type plants treated with BSO.

**Figure S5.** Determination of GSH (a) and GSSG (b) levels and cellular glutathione redox (c) in shoots and roots after 8 and 24 h of GSH treatment.

**Figure S6.** GSH1 expression (a) and GSH levels (b) in RD29A: GSH1 transgenic seedlings after salt stress treatment.

**Figure S7.** Salt stress-induced GSH1 enhance translation activity.

**Figure S8.** Confirmation of increased ribosome occupancy by qRT-PCR.

**Figure S9.** Experimental validation of GSH-responsive genes regulated at the translational level.

**Figure S10.** Changes in polysome-bound mRNA and steady-state abundance between Col-0 and RD29A:GSH1 seedlings treated with salt stress for 3 and 6 h.

**Figure S11.** Categorization of genes regulated by GSH treatment at the mRNA or protein levels.

**Figure S12.** Cluster analysis of the differentially expressed genes at the steady-state level after 8 and 24 h of GSH treatment.

**Figure S13.** Pro content in GSH-treated plants (A) and 35S:GSH1 (OE7-5 and OE13-6) transgenic plants (B).

**Figure S14.** Heat map showing the normalized expression of JA-responsive genes translationally regulated after 8 and 24 h of GSH treatment.

**Figure S15.** Heat map showing the normalized expression of auxin-responsive genes regulated translationally after 8 and 24 h of GSH treatment.

**Figure S16.** Heat map showing the normalized expression of ABA-responsive genes regulated translationally after 8 and 24 h of GSH treatment.

**Figure S17.** Effect of exogenous GSH on ABA accumulation in root and shoot tissues.

**Appendix S1.** Genes with twofold or greater changes at the mrNApl level.

**Appendix S2.** Genes twofold or greater changes at the mrNAess level.

**Appendix S3.** Expression ratios of genes regulated at 8R and 24S organized by k-means clustering and GO analysis results.

**Appendix S4.** Gene lists and GO analysis results of genes regulated in 8R and 24S at the mrNApl level.

**Appendix S5.** Supplementary experimental procedures.

**REFERENCES**


GSH-mediated translational change

939


© 2015 The Authors