J Biosci (2022)47:71
DOI: 10.1007/s12038-022-00315-1

Review

Crosstalk between MAPKs and GSH under stress: A critical review

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MS received 25 March 2022; accepted 26 August 2022

Plants are frequently exposed to a plethora of unfavourable climatic catastrophes, be it abiotic or biotic stresses, viz., salinity, water (drought or water logging), extreme temperature, heavy metal, nutrient deficiency, ozone, pathogen attack, etc., which badly affect the yield and productivity of crops. Plants, as part of their defence machinery, employ different tolerance mechanisms to survive under adverse conditions. In addition to other stress responses, the mitogen-activated protein kinase (MAPK) signalling cascade and accumulation of glutathione (GSH) are two important aspects of plant defence response. Induction of the MAPK cascade is one of the earliest responses when a plant is under any environmental stress, and there is documentary evidence of this signalling pathway, in turn, regulating various phytohormone-signalling networks and other defence-related pathways during stress. Similarly, GSH being a low molecular weight metabolite also has a key role in environmental stress tolerance. It is known to be involved in multi-step interactions with various phytohormones, many signalling molecules, and redox molecules such as reactive oxygen species (ROS). This review provides an outline on GSH–MAPK crosstalk to better understand its role in the context of defence signalling in plants.

Keywords. Abiotic factors; biotic factors; defence response; GSH; MAPK; stress

Abbreviations: ASC, ascorbate; AsA, ascorbic acid; DMTU, dimethylthiourea; DPI, diphenyleneiodonium; HR, hypersensitive response; NEM, *N*-ethylmaleimide.

1. Introduction

Under homeostatic conditions, plants maintain a delicate balance between reactive oxygen species (ROS) production and their quenching. However, environmental stress results in the over-accumulation of ROS in plants, creating an oxidative stress condition that damages cellular and subcellular components. Plants have developed efficient enzymatic or non-enzymatic antioxidant and ROS scavenging systems that can protect them from such disasters (Mittler *et al*. 2004). Glutathione (γ-glutamyl-cysteinyl-glycine, or GSH) is one such important ROS scavenger/non-enzymatic antioxidant that is oxidized by reactive oxygen species (ROS) as part of the antioxidant barrier and prevents excessive oxidation of sensitive cellular components (Noctor *et al*. 2011). On the other hand, ROS signalling can influence the expression of a number of genes and signal transduction pathways, such as the mitogen-activated protein kinase (MAPK) cascade, suggesting that cells have evolved strategies to utilize ROS as signals that induce various biological pathways (Dalton *et al*. 1999). Therefore, apart from excessive ROS production, activation of the MAPK cascade and accumulation of GSH are two crucial aspects of plant defence response that help plants to mitigate environmental stress. Various defence signalling pathways stimulated by immunity receptors work via ROS and MAPKs, followed by transcriptional induction of many defence genes, accumulation of defence proteins as well as metabolites, reinforcement of cell walls, and apoptosis (Boller and Felix 2009).

Published online: 10 December 2022

GSH is a non-protein, water-soluble metabolite found in most plant species (Bergmann and Rennenberg 1993) and plays a key role in cell function and metabolism. GSH is widely used as a marker of oxidative stress in plants, although its role in plant metabolism is multifaceted (Grill *et al*. 2001). Since GSH is a pseudo-peptide, its biosynthesis does not follow the classical protein synthesis pathway. It is called a pseudo-peptide since glutamine forms a bond with the cysteine moiety via the carboxyl group side chain and not through the α-carbon carboxyl group. The GSH biosynthetic pathway is a two-step process involving two enzymes which require ATP (Meister 1988). The initial step includes conjugation of L-cysteine and L-glutamate to form γ-glutamylcysteine (γ-EC). The γ-glutamylcysteine synthetase (γ-ECS; EC 6.3.2.2) enzyme catalyzes the first step. Lastly, GSH synthetase (GS) catalyzes the incorporation of glycine into the C-terminal ends of γ-EC (GS; EC 6.3.2.3). An exception is in *Streptococcus agalactiae*, where GSH is biosynthesized in one step. Earlier reports have elucidated the importance of the master antioxidant GSH in plant defence, its multi-dimensional role in maintaining cellular redox homeostasis (Mou *et al*. 2003), its role in post-translational protein modification via glutathionylation (Zaffagnini *et al*. 2012) and detoxification of xenobiotics, and its function in the synthesis of sulphur-containing defence-related secondary metabolites (Geu-Flores *et al*. 2011; Su *et al*. 2011). Being an important non-enzymatic antioxidant and redox buffer, GSH takes part in the ascorbate–glutathione (AsA–GSH) cycle, which plays a vital role in the scavenging of H_2O_2 by reducing H_2O_2 to water and in recycling to the reduced forms of ascorbate (AsA) and GSH (figure 1). These functions are catalyzed by APX (ascorbate peroxidase), DHAR (dehydroascorbate reductase), MDHAR (monodehydroascorbate reductase), and GR (glutathione reductase) (Mahmood *et al*. 2010). GSH is also a vital metabolite for plant survival. According to early reports, GSH mutants of *Arabidopsis thaliana* were noted to die at the embryonic stage (Cairns *et al*. 2006), while other mutants of *A. thaliana* with a less severe reduction in GSH levels could survive, although they are inherently more sensitive to many biotic and abiotic stress factors (Xiang *et al*. 2001; Ball *et al*. 2004; Parisy *et al*. 2007). The redox state of GSH plays an important role in regulating the expression of defence genes. One of the most typical examples corresponds to the activation of a critical defence gene, viz., non-expressor of pathogenesis-related proteins (NPR1). The reduction of NPR1 requires an increase in GSH content, the NPR1 protein conformation being sensitive to cellular redox changes (Mou *et al*. 2003). Treatments with L-2-oxo-4-thiazolidine-carboxylic acid (OTC), a cysteine precursor, lead to a massive increase in GSH content, thereby inducing the expression of the *NPR1* gene in both healthy and Plum pox virus (PPV)-infected peach plantlets (Clemente-Moreno *et al*. 2010). In that regard, GSH seems to be one of the primary antioxidants involved in activating plant defence genes (Wingate *et al*. 1988; Ghanta *et al*. 2011).

One of the most important signalling cascades working in transmitting stress-related stimuli is the MAPK cascade. MAPKs, highly conserved signalling pathways, play a significant role in the signal transduction of diverse stress responses even in a combination of many stresses (figure 2). A typical MAPK cascade is composed of MAPK, MAPK kinase (MAPKK, MAP2K, MKK or MEK) and MAPK kinase kinase (MAPKKK, MAP3K or MEKK) (Ichimura *et al*. 2002). In a classical MAPK signalling cascade, MAPKKK is activated by stimulated plasma membrane receptors and transmits signals downstream (Wang *et al*. 2014). MAPKKK activates MAPKK by phosphorylating the conserved S/T-XXXXX-S/T motif in MAPKK (Rodriguez *et al.* 2010). Subsequently, MAPKK activates MAPK by phosphorylating the TXY motif in MAPK (Taj *et al.* 2010). Finally, MAPK activates downstream kinases, enzymes, transcription factors (TFs), and response factors, and transmits extracellular environmental signals into cells (Zhang *et al*. 2018). MAPK pathway genes and proteins, viz., MAPKs, regulate the expression of many other genes through the phosphorylation of proteins, especially the phosphorylation of many transcription factors (Morris 2001; Zhang and Klessig 2001). The MAPK cascade is important in mediating cell differentiation, cell development, hormonal activity, and abiotic and biotic stress responses (Komis *et al*. 2018). Interestingly, MAPKs are not only known to be activated by the perception of ligands but are also activated by ROS molecules. These phosphorylation cascades are found to work either upstream or downstream of ROS (Asai *et al*. 2002).

The MAPK signalling pathway plays essential roles in the transduction of extracellular signals during stress. In recent years, GSH has been known to play a critical role in conferring stress tolerance to plants. However, the importance of GSH–MAPK crosstalk to combat stress is yet to be explored in detail. Previous studies have pointed towards GSH-mediated *MAPK* gene regulation (Matern *et al*. 2015; Boro *et al*. 2022). This review will highlight this unexplored area of plant

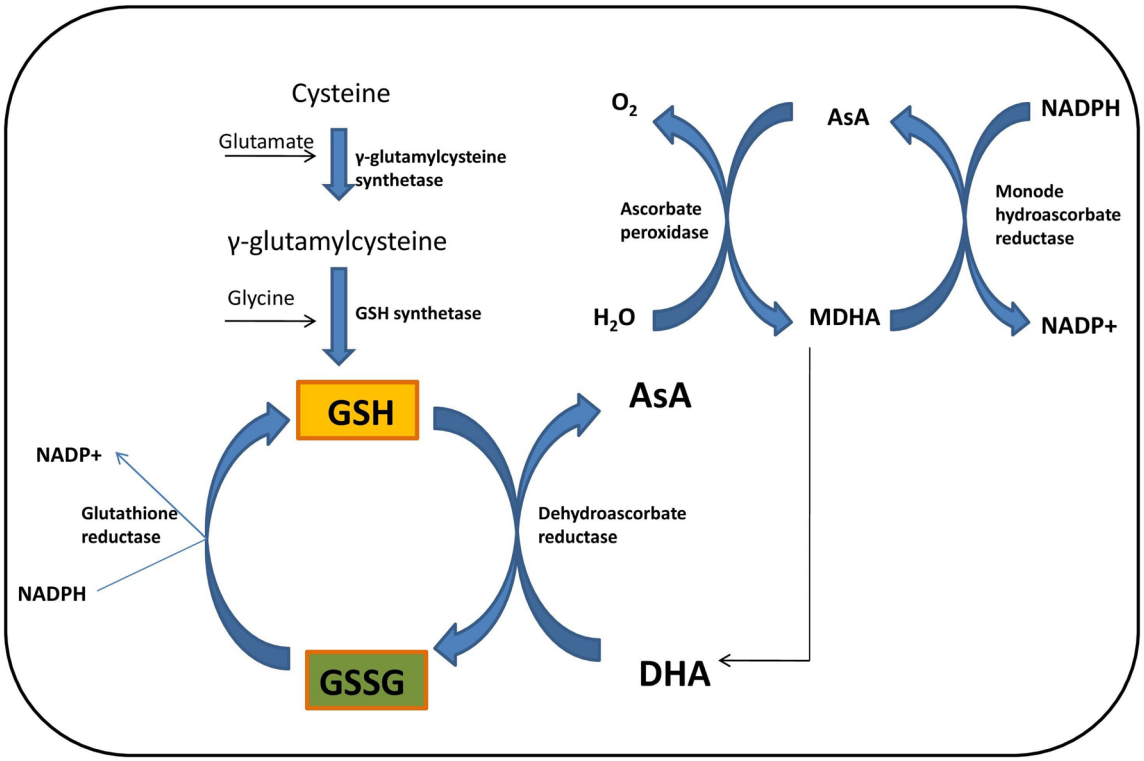

Figure 1. Biosynthesis and utilization of GSH AsA-GSH cycle for scavenging ROS under stress.

defence system, viz., GSH–MAPK interplay, and discuss its intricate mechanisms to mitigate environmental stress.

2. Biotic stress

In plant cells, when exposed to pathogen attack, activation of various types of defence machinery takes place, which includes host–pathogen interaction, viz., recognition of pathogen effectors by host cells or by the binding of non-host-specific pathogen-associated molecular patterns (PAMPs) to host receptors. Inducible plant defence responses include antimicrobial compounds, lytic enzymes such as chitinases and glucanases, oxidizing agents, cell wall lignification, and a number of pathogenesis-related proteins and transcripts of unknown function (Lamb *et al*. 1989; Dixon and Lamb 1990). For many of these responses, evidence that they are causally responsible for limiting pathogen growth has been obtained from studies of their toxicity *in vitro* and increased disease resistance displayed by transgenic plants constitutively expressing one or more pathogen-inducible defence-related genes (Glazebrook *et al*. 1997). Besides, as a part of plant defence response production of defence-related compounds, such as phytoalexins, camalexin, and scopoletin, as well as lignin (a structural barrier thought to restrict pathogen spread), takes place (Schenke *et al*. 2011). Among many defence-related compounds, phytoalexins, small molecules with antimicrobial activity, are synthesized by plants in response to pathogen attacks (Hammerschmidt 1999). Camalexin (3-thiazol-2-yl-indole), the main phytoalexin in *Arabidopsis*, are sulphur-containing tryptophan-derived secondary metabolites involved in biotic responses (Tsuji *et al*. 1992; Thomma *et al*. 1999) as well as abiotic responses (Tsuji *et al*. 1993) in plants. In the MAPK cascade, activation of MKK9 (upstream kinase of both MAPK3 and MAPK6) leads to the subsequent upregulation of multiple genes related to the camalexin biosynthetic pathway, and induces high levels of camalexin accumulation, thereby imparting stress tolerance in plants (Xu *et al*. 2008). Another study reported that MAPK3 and MAPK6 activities induce the production of GSH and camalexin upon *Botrytis cinerea* infection (Ren *et al*. 2008), suggesting a correlation between MAPK3/6 and GSH under biotic stress. In the MAPK signalling cascade, MKK4 and MKK5 function upstream of MAPK3 and MAPK6. Previous studies demonstrated that transient overexpression of the constitutively active, phospho-mimic double-mutant forms of MKK4/MKK5, named $MKK4^{DD}$ and $MKK5^{DD}$, leads to transcriptional induction of both *PAD2* and *PAD3* in *A.*

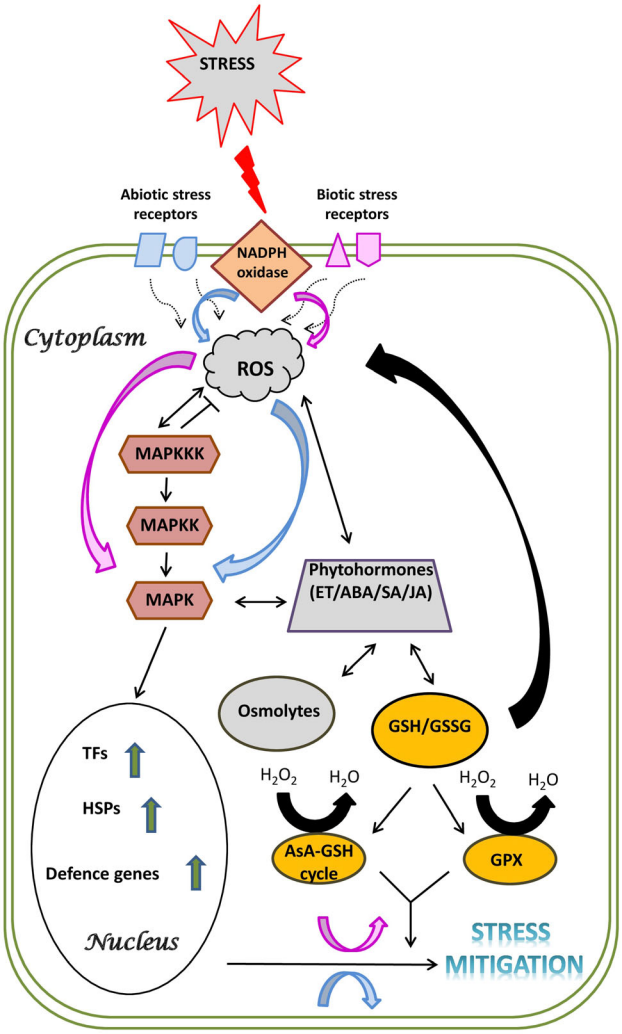

Figure 2. Overview of the crosstalk among ROS, MAPK cascade, phytohormone signalling pathways, etc., under environmental stress.

thaliana, suggesting a connection between GSH and MKK4/MKK5 (Rasmussen *et al*. 2012). The *PAD2* gene encodes for γ-glutamylcysteine synthetase (GSH1), one of the enzymes of the GSH biosynthetic pathway. PAD2 and PAD3 are necessary for camalexin production (Parisy *et al*. 2007; Ren *et al*. 2008). HR-like cell death induced by the activation of *Arabidopsis* MAPK3/MAPK6 or *Nicotiana* SIPK/WIPK/Ntf4 in dexamethasone (DEX)-inducible promoter-driven *NtMEK2DD* transgenic plants (GVG-NtMEK2^{DD}) is associated with ROS generation (Ren *et al*. 2002). Ren *et al*. (2008) also demonstrated that a pathogen-responsive MAPK cascade, MAPKKK/MEKK1-MKK4/MKK5-MAPK3/MAPK6, plays a positive role in regulating the biosynthesis of camalexin in *Arabidopsis*. Previous mutant screens identified *PAD2* (*PHYTOALEXIN DEFICIENT 1*) along with *PAD1*, *PAD3*, and *PAD4* as components of the GSH biosynthetic or other regulatory pathways that lead to pathogen-induced camalexin production (Glazebrook and Ausubel 1994; Glazebrook *et al*. 1997). Genetic analysis placed this MAPK cascade upstream of PAD2 (Ren *et al*. 2008). Additionally, it was also found that feeding the *GVG-NtMEK2^{DD}/pad2* (GVG-NtMEK2^{DD} crossed into *pad1*, *pad2*, *pad3*, and *pad4* backgrounds) seedlings with 1 mM GSH did not help in restoring camalexin production, revealing that the MAPK cascade comprising MAPKKK/MEKK1-MKK4/MKK5-MAPK3/MAPK6 interacts with *PAD2* (involved in GSH biosynthesis) and regulates the production of camalexin.

In *Nicotiana*, MAPK and salicylic acid (SA) signalling pathways may function separately, but both were reported as being regulated via the GSH redox potential (Matern *et al*. 2015). HGLs, transgenic lines overexpressing high GSH content, exhibited an oxidative change in the cytosolic redox potential and induction of the *Nicotiana* MAPKs, viz. wound-induced protein kinase (WIPK) and SA-induced protein kinase (SIPK). Similarly, rapid induction of WIPK and SIPK was triggered in wild-type tobacco when treated with exogenous GSH/GSSG (Matern *et al*. 2015). This observation again pointed towards GSH–MAPK crosstalk. It was also reported that *Nicotiana tabacum MAPKK* interacts with SA-induced protein kinase in tobacco, which may play a role in SA signalling through NPR1 (Liu *et al*. 2000). Further studies revealed that in the *N. tabacum* transgenic line exhibiting higher GSH content (*NtGB*) and in 100 µM GSH-fed BY2 cell lines of *N. tabacum*, upregulation of the *NtMAPKK* gene was noted along with other stress-related genes like *NtNPR1, NtPR1, NtPR4, NtGLS,* etc. (Ghanta *et al*. 2011).

Glutathione S-transferase (GST) is an enzymatic antioxidant that uses GSH as a co-factor and converts GSH to GSSG (reduced form of GSH), which is further reduced back to GSH, with glutathione reductase (GR) catalysis. A clearly recognized function of GSTs is their participation in antioxidative reactions with GSH to eliminate ROS and lipid hydroperoxides that accumulate in damaged plant tissues during stress (Gullner *et al*. 2018). MAPK is known to be a negative regulator of plant immune systems. Studies revealed that the MAPK4 mutants (*mpk4*) exhibited high expression levels of glutathione S-transferases and tended to have higher levels of SA and ROS (Petersen *et al*. 2000; Lin and Chen 2018).

3. Abiotic stress

There is documentary evidence of the crosstalk of GSH and MAPK signalling pathways regulating plant responses to abiotic stress factors. Most tested abiotic stresses have been shown to elicit increases in cytosolic free calcium levels (Ca^{2+}) and to involve protein phosphatases and kinases including MAPK cascades (Knight and Knight 2001). Under oxidative stress in *A. thaliana*, activation of MKK9 functioning upstream of MAPK3 and MAPK6 elevated the levels of phi-class GSTs like GST6, and this higher GST activity is related to higher camalexin levels (Tena *et al*. 2001). Besides, the GSH–MAPK crosstalk was well elucidated under heavy metal (HM) stress, which induced ROS accumulation in plant cells, triggering the MAPK signalling cascade, which induced antioxidant gene expression and elevated the GSH content (Sytar *et al*. 2013). In *A. thaliana*, cadmium (Cd) stress was reported to activate MAPK3 and MAPK6 in a dose-dependent manner in the roots compared with leaves, and the exogenous application of GSH effectively inhibited their activation. Further, it was observed that without Cd stress, GSH had no remarkable effect on the activities of MAPK3 or MAPK6. However, 1 h feeding with 200 μM GSH greatly reduced Cd-induced activation of MAPK3 and MAPK6 (Liu *et al*. 2010), which validated that GSH inhibits the induction of MAPK3/6 under stress. Similarly, it was also demonstrated that GSH itself had no notable effects on MAPK kinase activity in terms of myelin basic protein (MBP) kinase (artificial substrate for MAPK proteins) phosphorylation (Yeh *et al*. 2004). When copper-treated cells were fed with GSH, it strongly hindered the MAPK kinase activity in reducing levels of 42 kDa MBP kinase activity. Additionally, Cd-induced activation of 40 kDa MBP kinase activity was hampered by 200 μM GSH (Yeh *et al*. 2004). In another study, microarray analysis of 100 μM GSH-fed Col-0 seedlings in *A. thaliana* confirmed the upregulation of *MAPKKK19* compared with wild-type Col-0 (Sinha *et al*. 2015). Furthermore, upon exogenous SA application or upon induction of abiotic stress treatment that leads to increase in endogenous SA content, the MAPK signalling pathway is activated (Jonak *et al*. 2002). Under such circumstances, transcriptional activation of defence-related genes including GSTs takes place in a non-expressor of PR1 (NPR1)-independent manner, thereby increasing the content of GSH and making the plant stress tolerant (Kang *et al*. 2014).

It has also been documented that signal molecules such as ROS, MAPK, nitric oxide (NO), and Ca^{2+} play a significant role in controlling the AsA–GSH cycle in maize leaves. Treating plants with exogenous ABA or H_2O_2 led to the activation of MAPK and the upregulation of the expression of one of the antioxidant genes, viz., gluthathione reductase (*GR1*), which indirectly hinted at the relationship between MAPK and GSH. Likewise, the effects of pre-treatments with MAPKK inhibitors and ROS inhibitors or scavengers increased the expression and total activities of the antioxidant enzyme without having any significant effect on ABA-induced MAPK activation. Additionally, treatment with the NO donor, viz., sodium nitroprusside (SNP), also triggered the activation of MAPK and amplified the antioxidant defence system (Zhang *et al*. 2006, 2007).

Interestingly, phytohormones like jasmonic acid (JA) were reported to elevate H_2O_2 production, MEK1/2 phosphorylation, the transcript levels and activities of γ-ECS, contents of GSH, AsA, and total ascorbate, and ratios of GSH/GSSG and AsA/DHA, thereby establishing a connection between the MAPK cascade and GSH. Then, treatments with MEK1/2 inhibitors PD98059 and U0126 diminished the γ-ECS activity by increasing the phosphorylation level, because of which the level of ascorbate and GSH content in wheat leaves became significantly higher. Apart from this, for JA-induced modulation of the redox state of GSH, the protein kinase MEK1/2 controlled the GSH regeneration upon JA treatment (Shan *et al*. 2011). Treatments with exogenous H_2O_2 also increased the MEK1/2 phosphorylation, transcription levels, and activities of GSH as well as AsA metabolic enzymes, GSH and AsA contents, and the redox states of GSH and AsA (Dai and Gao 2016). Interestingly, the NO donor, viz., SNP, induced the phosphorylation of MEK1/2, which in turn upregulated the ascorbate and GSH metabolism in *A. cristatum* leaves (Shan and Dong 2017). Pre-treatment with PD98059 and U0126 significantly prevented the increases in the transcription and activities of γ-ECS, contents of GSH and AsA, total GSH and total ascorbate, ratios of GSH/GSSG, APX, GR, MDHAR, and DHAR, which were induced by SNP. However, pre-treatment with PD98059 and U0126 did not reduce the activity of γ-ECS induced by SNP. Besides, results showed that for GSH synthesis, MEK1/2 was involved in the regulation of the transcription of *γ-ECS*, but not the γ-ECS activity (Shan and Dong 2017). Similarly, another investigation unravelled the relationship between MEK1/2 and GSH in a JA-mediated NO-dependent manner (Shan and Sun 2018). This study investigated the relationship between MEK1/2 and NO in the JA-regulated metabolism of ascorbate and GSH in maize leaves. The results

showed that JA increased the activities of *APX*, *GR*, *MDHAR*, *DHAR*, *GalLDH*, and *γ-ECS*, the contents of AsA and GSH, and the production of NO. Together, it was summarized that JA-mediated NO production, and exogenous treatment with SNP and H_2O_2, activated MEK1/2 by increasing the phosphorylation level, which, in turn, resulted in the upregulation of ascorbate and GSH metabolism in maize and wheat leaves.

Some recent studies demonstrated the interplay between GSH and MAPK in some algal groups, viz., *Ulva compressa*, that were put under excess chronic Cu environment and treated with some MAPK inhibitors for 6 days. As a result, the amount of H_2O_2 and lipid peroxidation dropped significantly below the controls. Blocking of more than one MAPK resulted in the reduction of total glutathione content (GSH + GSSG) along with ascorbate on the 6th day (Rodríguez-Rojas *et al*. 2019). However, the level of total glutathione did not drop further when the whole MAPK pathway was blocked. Again, it was analyzed that Cu triggered the activation of MAPKs and other kinases, resulting in the modulation of glycolysis and carbon flux reprogramming, which led to an increase in GSH along with ASC and NADPH syntheses to mitigate copper-induced oxidative stress in green alga *U. compressa* (Laporte *et al*. 2020). The role of MAPKs in the accumulation of Cd and synthesis of GSH was also reported in *U. compressa* treated with 10 μM of Cd for 5 days (González *et al*. 2021). Lastly, GSH-mediated positive regulation of MAPK3 under combined abiotic cold and osmotic stress was studied. It was demonstrated that 100 μM of GSH feeding in *A. thaliana* increased the expression of MAPK3 at the gene and protein levels (Boro *et al*. 2022)

4. Conclusion

Plants being sessile in nature constantly face copious amounts of harsh environmental factors (biotic and abiotic stresses), and such environmental calamities impose serious threats to the productivity and yield of plants. Such climatic catastrophe has pushed many plant species to the verge of extinction.

Maintaining redox homeostasis among various signalling pathways is one of the simple and common mechanisms that are regulated by plant systems in various development and defence pathways. GSH, being a master antioxidant molecule, has a very crucial role to play in the plant's responses to various stress elements. GSH is known to have multiple functions modulating many defence-related proteins, phytohormones, transcription factors (TFs), etc., at various levels. Hence, they are ideal targets for ameliorating stress tolerance in plants. Consequently, there is an acute need for a comprehensive understanding of the mechanisms involved in such GSH-mediated regulatory pathways that are triggered under environmental stress.

The above discussion focuses on the pivotal role of GSH-mediated signalling and its accumulation in response to environmental stimuli. The MAPK cascade is a versatile signalling pathway that acts downstream of secondary messengers and hormones. It also plays a diverse role in intra- and extracellular signalling in plant systems. Recent reports reveal that the MAPK cascade is also regulated by GSH as part of the plant's defence machinery in mitigating stress. Sometimes this crosstalk is direct, where the *MAPK* genes (*MAPK*s) are activated due to the over-accumulation of GSH under stress or *vice versa*, and in other cases, the crosstalk is indirect, viz., via some TFs. The activated *MAPK*s also regulate the expression of various GSTs. Further studies are still required to explore the mechanisms underlying the GSH–MAPK crosstalk and to identify the factors responsible for such regulation. This will shed light on the significance of GSH and help us unravel a very interesting aspect of plant defence response.

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Corresponding editor: JYOTHILAKSHMI VADASERRY