Oct-Dec 2022 - Volume 13 - Issue 4

Abstracts

Abstracts of

5th Asian Congress of Radiation Research (5th ACRR)

and

3rd Biennial Meeting of the Society for Radiation Research

Organised by

Bio-Science Group, Bhabha Atomic Research Centre, Mumbai

in association with

Society for Radiation Research-India
and Asian Association for Radiation Research (AARR)

DAE Convention Centre, Anushaktinagar, Mumbai

Nov. 17-20, 2022

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Nrf-2 Inhibition Leads to Radio-Sensitization of Human Lung Cancer Cells via Induction of Oxidative Stress, Reduced DNA Damage Repair and Ferroptosis

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Introduction: Ionizing radiation exerts its cytotoxicity primarily through the generation of reactive oxygen species (ROS). Nrf-2responds to oxidative stress through co-ordinated induction of battery of antioxidant genes by binding to antioxidant response element (ARE). Constitutive activation of Nrf-2-ARE pathway leads to radio-resistance. Pharmacologic inhibition of Nrf-2combined with radio/chemo-therapy is emerging as a promising strategy for better therapeutic gain. **Materials and Methods:** Effect of Clobetasol Propionate (CP), a known Nrf-2 inhibitor, on radiation induced cytotoxicity in cancer cells was studied using clonogenic, propidium iodide and MTT assays. Molecular mechanism of CP mediated radio-sensitization was investigated by using TEM, Western blotting, immunofluorescence assay and free iron measurement. **Results:** Treatment of A549 cells with CP prior to radiation exposure led to radio-sensitization. Combination of CP and ionizing radiation (IR) resulted in elevated ROS levels and reduced DNA damage repair. Transient over-expression of Nrf-2 abolished CP mediated radio-sensitization confirming the involvement of Nrf-2.Combination of CP with IR led to loss of mitochondrial membrane potential. Visualization of mitochondrial ultrastructure under TEM, revealed swollen and ruptured mitochondria along with loss of cristae which are strongly indicative of ferroptosis. Combination of CP with IR resulted in release of higher levels of free iron compared to those treated with either CP or radiation alone, which was abrogated by using an iron chelator, deferoxamine (DFO). Treatment of cells with DFO or Liproxstatin-1 (Ferroptosis inhibitor) prior to CP treatment abolished CP mediated radio-sensitization indicating a pertinent role of ferroptosis. Here, we show that pharmacologic inhibition of Nrf-2 leads to enhanced iron release, oxidative stress, reduced DNA damage repair and subsequent cell death through ferroptosis in radio-resistant human lung cancer cells. **Conclusions:** Our studies clearly demonstrate that Nrf-2 could be a potential target during radiotherapy of cancers that overexpress this pro-survival transcription factor.

Archives of Biochemistry and Biophysics 704 (2021) 108890

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Role of protein S-Glutathionylation in cancer progression and development of resistance to anti-cancer drugs

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ARTICLE INFO

Keywords:
Glutathione
S-Glutathionylation
Critical cysteine residues
Oxidative stress
Cancer
Redox balance

ABSTRACT

The survival, functioning and proliferation of mammalian cells are highly dependent on the cellular response and adaptation to changes in their redox environment. Cancer cells often live in an altered redox environment due to aberrant neo-vasculature, metabolic reprogramming and dysregulated proliferation. Thus, redox adaptations are critical for their survival. Glutathione plays an essential role in maintaining redox homeostasis inside the cells by binding to redox-sensitive cysteine residues in proteins by a process called S-glutathionylation. S-Glutathionylation not only protects the labile cysteine residues from oxidation, but also serves as a sensor of redox status, and acts as a signal for stimulation of downstream processes and adaptive responses to ensure redox equilibrium. The present review aims to provide an updated overview of the role of the unique redox adaptations during carcinogenesis and cancer progression, focusing on their dependence on S-glutathionylation of specific redox-sensitive proteins involved in a wide range of processes including signalling, transcription, structural maintenance, mitochondrial functions, apoptosis and protein recycling. We also provide insights into the role of S-glutathionylation in the development of resistance to chemotherapy. Finally, we provide a strong rationale for the development of redox targeting drugs for treatment of refractory/resistant cancers.

1. Introduction

Glutathione, originally named "philothion" by its discoverer J. de Rey-Paihade, is a sulphur seeker true to its original name [1]. Structurally, it is the tripeptide γ-l-glutamyl-l-cysteinyl-glycine, with cysteine sulphur at its reactive centre [2]. It is the most abundant thiol in eukaryotic cells with concentrations ranging from 1 to 10 mM [3,4]. The majority of GSH is present in the cytoplasm (up to 90%), where it is synthesized, but significant amounts are also found in the mitochondria and the endoplasmic reticulum, while the nucleus holds a small fraction [2].

Although glutathione is best known for its antioxidant [2,5–7] and detoxification functions [8], its other properties are slowly coming to the fore [9–13]. Interestingly, it is the redox regulatory property of glutathione that is garnering significant attention. The high reactivity of glutathione, the reversible nature of its binding, and the fact that it exists as a redox pair of reduced/oxidized form (which partly contributes to the cellular redox potential) make it a very important player in redox signalling [10].

Redox homeostasis is extremely critical for the survival and differentiation of a cell. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are constantly generated as by-products of various cellular processes and contribute towards oxidative and nitrosative stress [9]. Cells have elaborate mechanisms to ensure that they maintain redox equilibrium, and glutathione plays a key role in this process. Besides being the most important thiol-containing buffer, it also plays a pivotal role in executing the cellular response to oxidative stress by altering the structure and functionality of redox-sensitive proteins, through a post-translational modification process known as S-glutathionylation [14].

It is important to note that disruption of redox balance is evident in the development and progression of many pathological conditions including cancer, neurological disorders, metabolic disorders, inflammation and autoimmunity, and more [12,15,16]. The role of glutathione

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https://doi.org/10.1016/j.abb.2021.108890
Received 26 November 2020; Received in revised form 15 April 2021; Accepted 16 April 2021
Available online 21 April 2021
0003-9861/

in such redox-altering disorders has been extensively studied during disease pathogenesis and progression. Cancer, being the second leading cause of death, accounts for one out of every six deaths globally, and an aberrant redox state is often a characteristic feature of cancerous growth [17]. In this review, we address the role of protein S-glutathionylation, a form of redox-dependent post-translational modification, in influencing the initiation and progression of cancer, and also discuss its impact on the effectiveness of anti-cancer drugs.

2. Altered redox balance in cancer and its role in driving S-glutathionylation

In his pioneering studies during the 1920s, Otto Warburg clearly demonstrated that even under normoxic conditions, most cancer cells undergo metabolic reprogramming and preferentially use glycolysis to sustain their high rate of ATP production [18]. Such metabolic reprogramming not only allows increased synthesis of building blocks for membranes, nucleic acids, and proteins but also results in increased production of intracellular ROS, including H_2O_2 and superoxide [19–21]. Hydrogen peroxide, singlet oxygen, superoxide, and hydroxyl radicals are also generated in cancer cells as a consequence of hyper activation of multiple oncogenes such as C-myc, K-ras etc [22,23]. Similar ROS production is observed due to hypoxia in the core of solid tumours with aberrant neo-vasculature [24]. Finally, alterations in signalling pathways associated with oncogenic transformation and cancer metastasis are also linked to increased H_2O_2 production [25] (Fig. 1). Cancer cells can acclimatize themselves to this altered redox balance by multiple mechanisms, including the enhancement of their antioxidant capacity. In addition to increased antioxidant capacity, redox-driven post-translational changes play a pivotal role in the adaptation of cancer cells to enhanced oxidative stress (occurring due to inherent metabolic reprogramming as well as the effect of certain chemotherapeutic drugs).

Post-translational modifications (PTMs) refer to any covalent change in the protein after its synthesis, which adds functional flexibility and also allows it to be tightly regulated. PTMs allow reversible activation/deactivation of targets through covalent addition of moieties, for example phosphorylation and acetylation [26]. S-glutathionylation is a unique redox-driven post-translational modification in which the cysteine of glutathione binds to the –SH (thiol) group of a target cysteine in the protein through a disulphide bond, and thereby oxidizes the target cysteine. This is reversible in nature and can result in temporary changes in the structure and/or function of the target protein [14]. High throughput techniques have enhanced the rate at which we can detect new S-glutathionylation events (discussed in Section 4) [27]. Bioinformatics-based approaches are being used to compliment the experimental detection process [28,29]. dbGSH maintains a repository where up-to-date information regarding experimentally reported S-glutathionylated proteins can be found [30].

In cancer cells, redox imbalance is the primary driving force behind S-glutathionylation events, which helps them cope with the increased oxidative burden. Synthesis of glutathione takes place through enzyme-mediated reactions to produce the reduced form of the moiety (GSH) (Fig. 2). γ-glutamylcysteine ligase catalyses the joining of glutamic acid and cysteine by an atypical γ-linkage through the γ-carboxy group of glutamate. The resultant moiety, γ-glutamylcysteine (GGC), is acted on by glutathione synthetase to carry out the condensation reaction with glycine to form γ-l-glutamyl-l-cysteinyl-glycine (reduced glutathione) [31]. The atypical γ-bond makes glutathione resistant to most peptidases. A specific membrane surface peptidase, gamma-glutamyltransferase (GGT), can cleave the γ-amino bond and release cysteinyl-glycine (CG) which can be further cleaved by

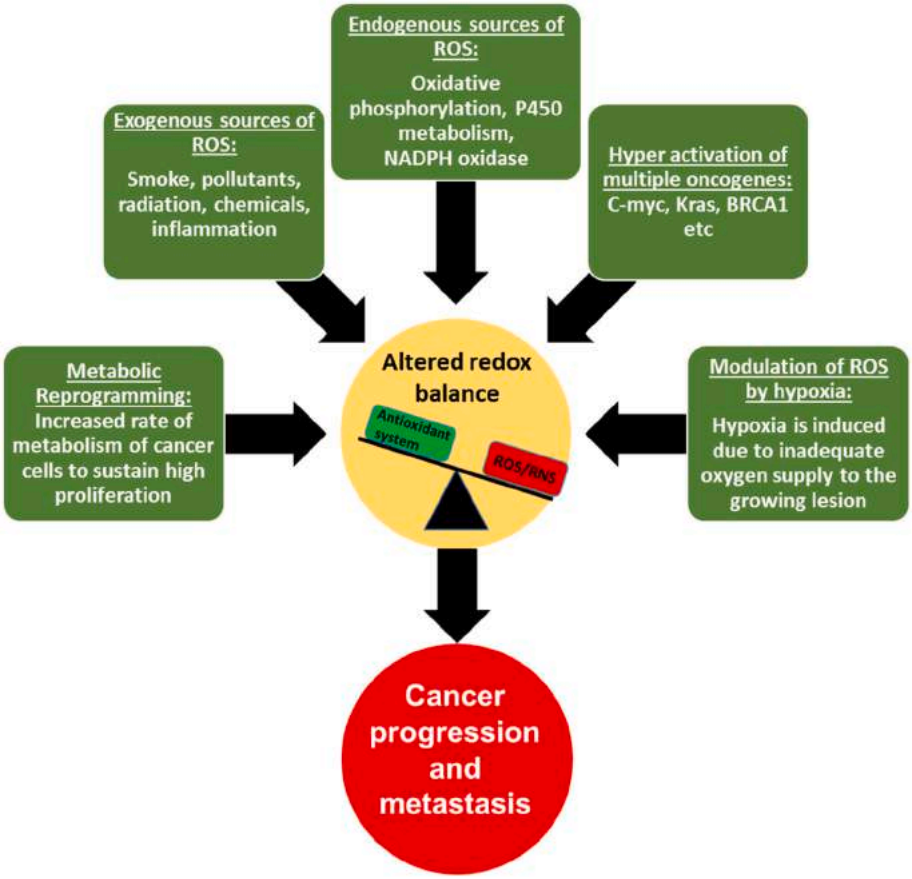

Fig. 1. Extracellular and intracellular sources contributing to redox imbalance in cancer cells.

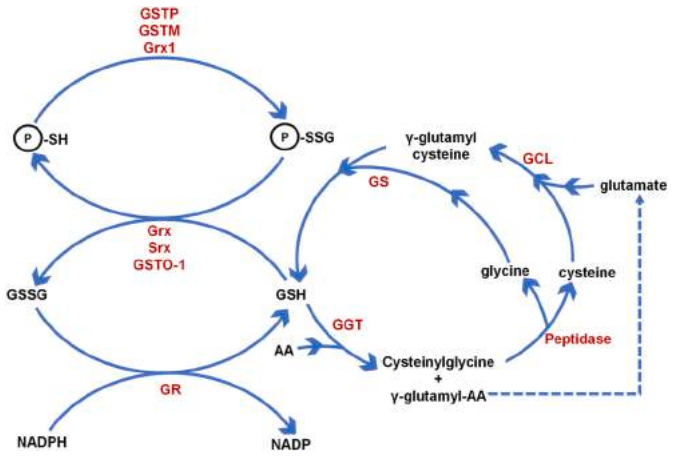

Fig. 2. Enzymatic control of glutathione synthesis, interconversion and S-glutathionylation events. GST = Glutathione S-transferase, Grx = glutaredoxin, Srx = sulfiredoxin, GR = glutathione reductase, GCL = gamma-glutamylcysteine ligase, GGT = gamma-glutamyltransferase, GS = glutathione synthetase, P-SH = Protein-SH, P-SSG = Glutathione bound protein.

peptidases to release cysteine in extracellular space.

Glutathione exists inside the cell as a redox couple of reduced (GSH) and oxidized (GSSG) glutathione. GSSG contains 2 molecules of reduced glutathione attached by a disulphide linkage. The GSH/GSSG ratio is dependent on the redox state of the cell. Under conditions of oxidative stress, as encountered in cancer cells, GSH counteracts oxidative stress by donating electrons to various moieties and thereby gets converted to GSSG, consequently shifting the ratio towards GSSG. Glutaredoxins are notable users of GSH as reducing equivalent. Oxidized GSSG can be reduced back by glutathione reductase, using NADPH as the reducing equivalent. Being the most abundant redox couple, GSH/GSSG contributes significantly to the cell's redox potential. The cell's overall redox potential has a vital role in determining whether a cancer cell will proliferate, differentiate, or survive [6]. A cell type's capacity to resist changes in redox potential depends on its content of reduced GSH, which determines its redox capacity. By altering the expression of enzymes involved in glutathione synthesis and metabolism, cancer cells can control the amount of GSH and thereby influence the extent of change in redox potential in response to a certain amount of oxidative stress [32].

The protein thiol pool is much more abundant compared to the GSH thiol pool [33]. As a result, the protein thiol pool exists in a dynamic equilibrium with the glutathione pool and can serve as a buffer to regenerate GSH. Reactions between the protein thiol group and glutathione can take place by a multitude of possible pathways (Fig. 3) [11]. Thiol disulphide exchange (reactions 1.1, 1.2, 1.3) can lead to protein S-glutathionylation, but for formation of a significant amount of PSSG, there is a need for a considerable shift of GSH/GSSG redox couple ratio towards GSSG. Such a drastic shift is unlikely under normal physiological conditions barring severe oxidative stress, which means this mechanism doesn't serve as the primary pathway for glutathionylation [34]. The estimated values of the second-order rate constants for the reaction of different thiols with GSSG are on the lower side (4.9×10^2–$1.2 \times 10^4\ M^{-1}\ s^{-1}$) [35], which makes thiol disulphide exchange an unlikely event. Certain proteins may be an exception to this norm by possessing high thiol redox potential, which allows them to undergo significant glutathionylation under physiologically achievable GSH/GSSG ratios.

$$PSH + GSSG \rightarrow PSSG + GSH \ldots. \quad (1.1)$$

$$PSSG + P'SH \rightarrow PSSP' + GSH \ldots. \quad (1.2)$$

$$PSSG + P'SH \rightarrow P'SSG + PSH \ldots. \text{ (second order rate constant (k) = } \sim 10^{-3}\ M^{-1}\ s^{-1}) \text{ [36].} \quad (1.3)$$

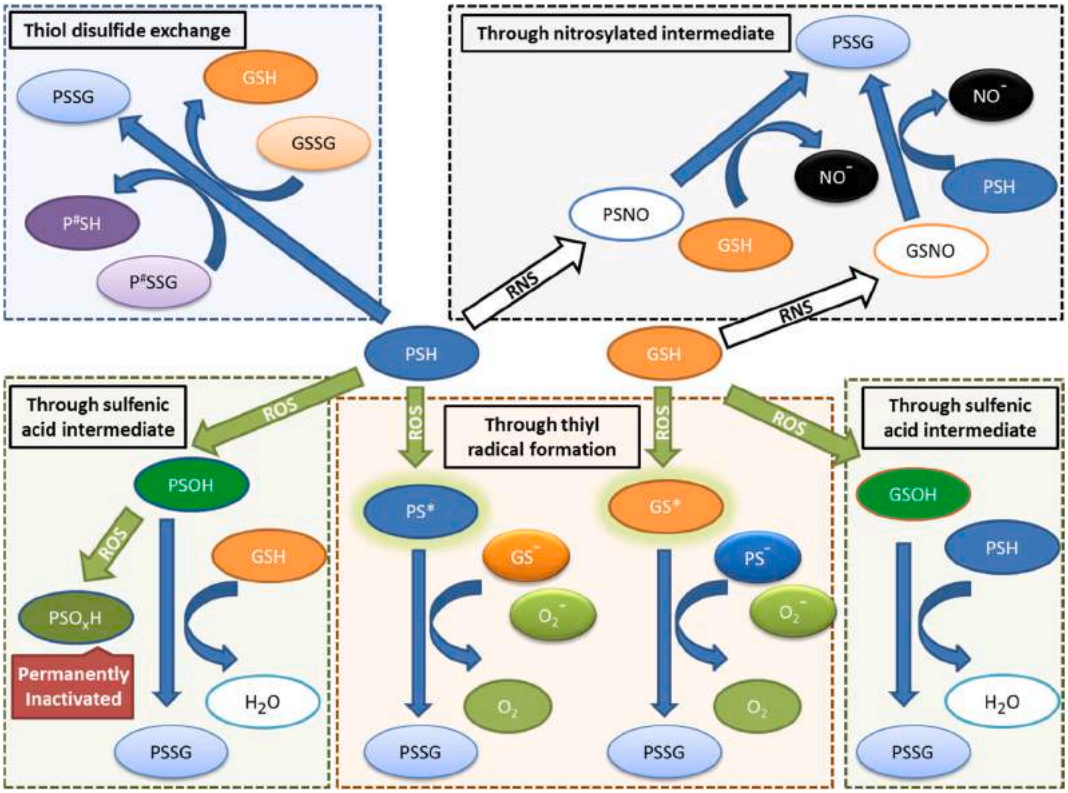

Fig. 3. Possible non-enzymatic pathways leading to S-glutathionylation at cysteine residues in proteins. PSH or PSH = Protein-SH, PSSG or PSSG = Glutathione bound protein, PS = thiyl radical on protein cysteine residue, GS$^{\bullet}$ = thiyl radical on cysteine of glutathione, PSOH/GSOG = sulfenic acid forms of protein and glutathione, ROS = Reactive oxygen species, RNS = Reactive nitrogen species, PSNO/GSNO = S-nitrosylated cysteine in protein or glutathione.

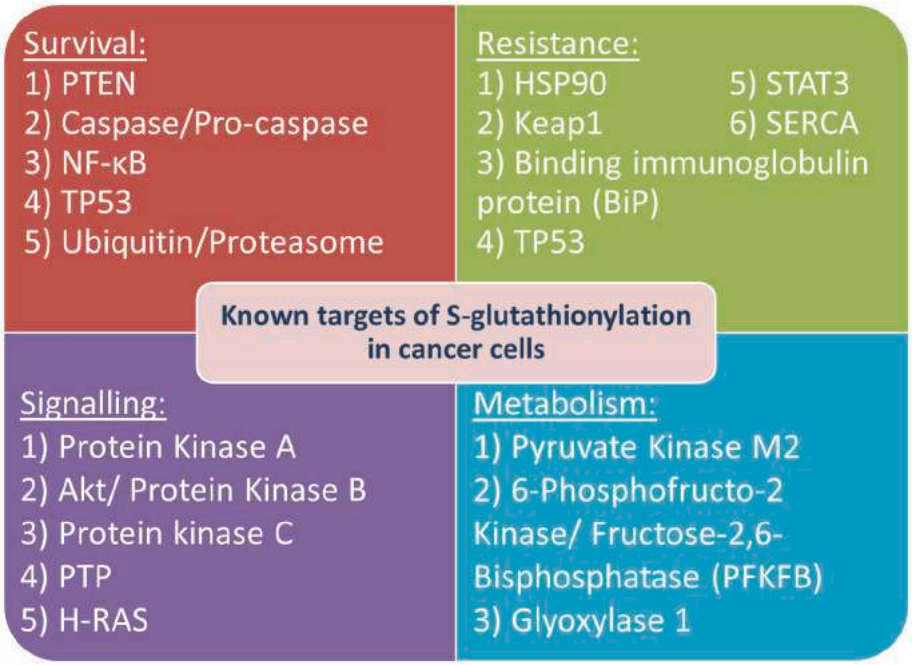

Fig. 4. Targets of S-glutathionylation which have functional implications in cancer cells. PTEN = Phosphatase and tensin homolog, PTP = Protein tyrosine phosphatases, TP53 = Tumour protein p53, STAT3 = Signal transducer and activator of transcription 3, SERCA = Sarco/endoplasmic reticulum Ca2+-ATPase, or SR Ca2+-ATPase.

Oxidative stress can cause oxidation of protein thiol groups to form sulfenic acid (PSOH) [37,38] or lead to the generation of thiyl radicals ($PS^{\bullet}$) [39]. Thiyl radicals are primarily generated from reactions with non-superoxide electron donors, as superoxide reacts 4–6 orders of magnitude faster with SOD than GSH/PSH. Although both these forms are known to undergo reaction with glutathione resulting in S-glutathionylation (reactions 2.1, 2.2, 3.1, 3.2, 3.3), recent calculations suggest that the formation of a sulfenic group from H_2O_2 in proteins might be a rare event. Most protein cysteine residues have second-order rate constants for reacting with H_2O_2 in the range of 10^2 M^{-1} s^{-1} as against 4.7×10^7 M^{-1} s^{-1} and 10^8 M^{-1} s^{-1} for detoxification of H_2O_2 by Glutathione Peroxidase-1 and Peroxiredoxin-2, respectively. This makes it highly unlikely that H_2O_2 would react with protein thiols/GSH to give rise to sulfenic forms of proteins/GSH [40,41]. Nevertheless, localized inactivation of hydroperoxidases leading to high levels of H_2O_2 may drive thiol sulfenylation. On the other hand, reaction through thiyl radicals can be one plausible mechanism through which ROS can drive S-glutathionylation of proteins.

$PSOH + GSH \rightarrow PSSG + H_2O$ …. (second order rate constant (k) = 2.9 M^{-1} s^{-1}; reaction with Human Serum Albumin [42]) (2.1)

$PSH + GSOH \rightarrow PSSG + H_2O$ ….. (2.2)

$PS^{\bullet} + GS^{-} + O_2 \rightarrow PSSG + O_2^{-\bullet}$ …. (3.1)

$GS^{\bullet} + PS^{-} + O_2 \rightarrow PSSG + O_2^{-\bullet}$ …. (3.2)

$GS^{\bullet} + PS^{\bullet} \rightarrow PSSG$ …. (3.3)

Nitrosative stress caused by reactive nitrogen species (RNS) can lead to the formation of S-nitrosylated form of glutathione (GSNO) [43]. Nitrosative stress leads to the generation of $NO^{\bullet}$ that can further react with oxygen to form N_2O_3, which subsequently reacts with GSH to form GSNO. Alternatively, GSNO can be formed directly from a reaction between $GS^{\bullet}$ and $NO^{\bullet}$, but this is dependent on the relative concentration of $GS^{\bullet}$ and superoxide radical in the milieu. The reaction rate between superoxide and $NO^{\bullet}$ is 10-fold higher than the reaction between $GS^{\bullet}$ and $NO^{\bullet}$, and hence localized non-availability of superoxide radicals is necessary for the formation of GSNO [44]. GSNO is only semi-stable (aqueous half-life in hours) and can potentially lead to subsequent S-glutathionylation [45,46] or S-nitrosylation, with the microenvironment playing an important role in determining whether the target cysteine undergoes glutathionylation (reactions 4.1) or nitrosylation (4.2) [47]. Certain calculations indicate a slight polar nature for the S–N bond in GSNO, with the N atom being slightly positively charged [48]. This should ideally lead to a nucleophilic attack on the N by the protein thiolate anion to favour S-nitrosylation, but certain studies also suggest a nucleophilic attack on the S atom of GSNO [45,49] to form PSSG. The reaction between GSNO and PSH has been demonstrated to be substantially faster compared to the reaction between GSSG and PSH [47]. The choice between S-nitrosylation and S-glutathionylation depends on the immediate microenvironment and adjoining residues of the target protein thiol. Studies have shown that proteins like papain and GAPDH have a propensity towards GSNO mediated S-glutathionylation while other target proteins like actin and BSA undergo preferential S-nitrosylation [47]. The accessibility and steric flexibility of the RSNO group also determines the bias between S-nitrosylation and S-glutathionylation reaction [48]. Nitrosative stress can also lead to S-nitrosylation of target cysteine residue through the formation of N_2O_3. N_2O_3 can directly interact with protein thiol to give rise to S-nitrosylation (4.3) [43]. S-nitrosylation is usually a transient modification, although stable forms having signalling roles have been reported [50,51]. S-nitrosylated residues are denitrosylated by reducing agents, such as GSH, to form PSSG(4.4) or give rise to PSH(4.5) through transnitrosylation reaction [47,48,52].

$GSNO + PSH \rightarrow PSSG + NO^{-}$ …. (second order rate constant (k) = 760 M^{-1} s^{-1}; *E coli* reduced Trx- [53];) (4.1)

$GSNO + PSH \rightarrow PSNO + GSSG$ …. (4.2)

$PSH + N_2O_3 \rightarrow PSNO + NO_2^{-} + H^{+}$ … (4.3)

$PSNO + GSH \rightarrow PSSG + NO^{-}$ …. (second order rate constant (k) = 1-300 M^{-1} s^{-1}) [54]. (4.4) (

$PSNO + GSH \rightarrow PSH + GSNO$ …. (4.5)

There is also evidence of sulfenylamide moiety being formed in protein tyrosine phosphatase 1B (PTP1B), which acts as an intermediate and can further react with GSH to form glutathionylated structures [55]. It is a possibility that such pathways are functioning in other proteins too. In addition to this, free cysteine or free homocysteine may bind to protein thiol groups to give rise to S-cysteinylation or S-homocysteinylation [56]. The second-order rate constant for the reaction between Cys with Cys-SOH is high ($>10^5$ M^{-1} s^{-1}) [57] but might be of limited significance due to the higher reactivity of Cys-SOH with Gpx1 and Prx2. Enzymatic pathways mediating cysteinylation have not been discovered till now. It is likely that enzyme-mediated S-glutathionylation events are much more plausible due to their faster reaction rates.

In summary, oxidative stress can result in enhanced synthesis of glutathione and promotes S-glutathionylation of target proteins in cancer cells.

3. Mediators of glutathionylation and how glutathionylation affects protein structure and function

Glutathionylation can either occur spontaneously or be assisted by specific enzymes. Kinetic data suggest that the majority of S-glutathionylation events using GSSG might be driven by enzymatic pathways. For example, second-order rate constant for the reaction of Grx with glutathione moieties like GSSG (10^5–10^6 M^{-1} s^{-1}) [58,59] is multiple orders higher than that between GSSG and protein thiols [35]. As such, high GSSG (under oxidative stress) is more likely to give rise to Grx-SG as intermediate than directly interacting with protein thiols. Grx-SG may further interact with protein thiols, but such reactions face competition from millimolar GSH concentrations, which has been shown to have a high rate constant in the range of 6×10^3 to 5×10^5 M^{-1} s^{-1} for reacting with Grx-SG. A relatively localized low GSH level might be essential for glutathionylation through a Grx-SG intermediate.

Another obstacle towards protein glutathionylation is the enzyme glutathione reductase. Glutathione reductase has a high $K_{cat(app)}/K_{m(app)}$ range of 3.2–4.85 $\times$ 10^6 M^{-1} s^{-1} [60] and can reconvert GSSG to GSH before it is utilized by Grx or takes part in spontaneous glutathionylation. Regulatory mechanisms on glutathione reductase (GR) may be in place to fine-tune the distribution of GSSG. Another hypothesis could be that a temporary decrease in NADPH levels as a result of oxidative stress can render GR temporarily inactive and thereby facilitate protein glutathionylation. Such a regulatory mechanism is expected, as otherwise, the emergence of a group of proteins like glutaredoxins would make no sense in evolutionary terms. Spontaneous protein glutathionylation through GSSG necessitates much more stringent conditions to be met. It is only possible under (a) high local concentration of protein relative to Grx (b) high GSSG (c) low GSH (d) kinetic uncoupling of Grx catalysis from GSH by an altered reductive half-reaction. Spontaneous protein glutathionylation generally occurs under conditions of oxidative stress inside the cell [61]. Although it can take place through thiol disulphide exchange, driven by a change in GSH/GSSG ratio under unique conditions as mentioned above, but more frequently, it is the ROS-induced thiyl radicals that drive the spontaneous reactions.

Experimental data indicate that most S-glutathionylation events take an enzymatic route (Figure: 2) due to kinetic constraints. Information regarding the site-specificity of enzyme-directed glutathionylation is unclear, although bioinformatic analysis suggests that certain sequences might have a higher propensity to undergo glutathionylation. Glutathionylating enzymes might provide the specificity of target proteins by virtue of their physical complex formation with certain known target proteins [62]. In recent years, we have started to have a better understanding of the intricacies of glutathionylating enzymes.

Glutathione S-transferases (GSTs) were originally thought to be phase II detoxification enzymes that were principally involved in the detoxification of electrophilic compounds in the liver [63] but their role in protein S-glutathionylation is gradually becoming more evident [64]. GSTs have multiple isoforms [65] among which glutathione -S-transferase Pi (GST-Π) is the most well-studied one for its effects on the glutathionylation process. Studies have shown that inhibiting a glutathionylation-favouring enzyme like GST-Π does drastically reduce the abundance of protein S-glutathionylation [64]. GST-Π is known to interact with 1-Cys Peroxiredoxin and bring about glutathionylation of its catalytic cysteine [62]. The absence of GST-Π leads to diminished S-glutathionylation levels [64,66,67], highlighting its profound role in the glutathionylation process. Like most enzymes, GST-Π is subject to negative feedback regulation through glutathionylation at its Cys47 and Cys101 residues [62].

As indicated by kinetic considerations, an important group of enzymes involved in the glutathionylation cycle is glutaredoxins (Grx). The predominant function of glutaredoxins is to “deglutathionylate” the already glutathionylated proteins at a basal GSH/GSSG ratio, but they are also known to catalyse the forward reaction in specific instances. Meiyal et al. hypothesized a mechanism involving stabilization of the $GS^{\bullet}$ thiyl radical as an enzyme disulphide anion radical intermediate ($Grx1\text{-}SSG^{\bullet}-$), which can then recombine with a protein thiyl radical [11]. This is in agreement with the high second-order rate constant value (10^5 and 10^6 M^{-1} s^{-1}) of reaction between GSSG and Grx1. The low pKa of the active site of Grx1 is proposed to drive the anion radical stabilization process. Often, its target residues lack a basic environment and low pKa value which may be a prerequisite for glutathionylation. Glutaredoxins can play a role in the glutathionylation process by making such reactions energetically favourable. Ukewela et al. have suggested that Grx has dual functions, as it can act as oxidase at low GSH/GSSG ratio and as a reductase at high GSH/GSSG ratios [68].

Glutaredoxins and sulfiredoxins (Srx) are also involved in catalysis of the deglutathionylation process, i.e., the removal of glutathione from the bound cysteine residue. Deglutathionylation by Grx occurs through a thiol-disulphide exchange reaction [59,69,70] which leads to the formation of oxidized Grx. Reduced Grx is regenerated by using GSH as the reducing equivalent. Peltoniemi et al. have suggested that the deglutathionylation activity of Grx is only functional when oxidative stress is removed [71]. Oxidative stress results in low GSH and high GSSG, both of which have an inhibitory effect on the deglutathionylating activity of Grx. This might be a possible adaptation to ensure that S-glutathionylation events do not get reversed as long as the oxidative stress persists.

Sulfiredoxin (Srx) is known to play a preferential role in the deglutathionylation of certain proteins like Prx1, actin, and PTP1B [72,73], possibly due to its higher affinity to such proteins compared to Grx. Unlike Grx, Srx is probably not inactivated by oxidative stress as evident from its ability to diminish overall S-glutathionylation under oxidative stress [73]. Recently, Hughes et al. have reported that GST isoform GSTω 1-1 can specifically deglutathionylate Cys253 in NIMA-related kinase 7 (NEK7) to promote NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) activation [74,75]. Protein disulphide isomerase may also play some role in the deglutathionylation process [76].

In terms of structural implications, glutathionylation involves the addition of a 305 Da moiety to the target cysteine of a protein. The resultant effect depends on the original function of the target cysteine. For catalytically active cysteine, S-glutathionylation precludes its catalytic activity. The inhibition of activity may just be a secondary effect with the primary purpose being protective in nature. S-glutathionylation can prevent certain labile Cys-SH/Cys-SOH groups from irreversible over-oxidation and permanent inactivation. As mentioned before, GST-Π can form a complex with 1-Cys Prx. 1-Cys Prx has a catalytic cysteine that is prone to oxidation to $–SO_XH$ forms, leading to loss of activity. GST-Π binds to 1-Cys Prx and brings about glutathionylation of the oxidized cysteine, thus preventing it from irreversible oxidation. Subsequently, the Cys-SSG bond is reduced to Cys-SH, leading to the resumption of its enzymatic activity [62].

Such glutathionylation mediated inactivation of catalytic residues

may also have associated functional significance. Under oxidative stress, PTP1B undergoes inactivation through glutathionylation at catalytically active Cys215. The inactivation of phosphatase activity has functional implications as it allows stress-activated kinases to function properly [77]. Glutathionylation can also cause alteration of structure that may have functional consequences. Glutathionylation of catalytic Cys152 of GAPDH alters its conformation and prevents it from binding to ET-1 mRNA, and forms an elegant circuit to allow prompt expression of ET-1 under conditions of oxidative stress [78]. In GST-Π, Cys47 and Cys101 play an important role in its heterodimerizing ability with JNK. S-Glutathionylation of Cys47 and Cys101 causes structural changes leading to its dissociation from JNK and subsequent activation of JNK [64]. Thus, alteration of structure and blocking of functional sites are the two mechanisms behind the ability of glutathionylation in exerting protective and functional roles.

4. Advances in measurement and identification of S-glutathionylation

Recent advances in methodologies for the detection and quantification of S-glutathionylation have made it possible to identify and quantify a large number of glutathionylated proteins. The earliest approaches were based on the use of radioactive ^{35}S labelled cysteine to selectively label glutathione along with concomitant inhibition of protein synthesis. Incorporation of radiolabelled glutathione into proteins as S-glutathionylated moiety would serve as a signal for quantification of S-glutathionylation and target proteins could be identified by mass spectrometry [79,80]. Although the radioactive method is sensitive, it requires careful handling and disposal, and it is not specific for S-glutathionylation. Moreover, in this method, protein synthesis has to be inhibited leading to disruption of the physiological behaviour of the cell. Further, a biotin-labelled method (BioGEE) with enhanced specificity and sensitivity was developed and this technique enabled enrichment of glutathionylated proteins [81] through interaction with streptavidin beads. A shortcoming of this method is that one can identify only those glutathionylation events which take place post-labelling of cells with BioGEE. Also, the bulky nature of BioGEE can interfere with enzyme-mediated glutathionylation events [82]. On the other hand, antibody-based methods do not need any prior labelling and would be specific to existing glutathionylated proteins. But antibody-based methods suffer from certain drawbacks, such as lack of specificity to all glutathionylated proteins. As S-glutathionylation is flexible in nature [83], minute structural variations can prevent the detection of certain glutathionylated variants by epitope-structure specific monoclonal *anti*-GSH antibodies, thus limiting its application.

Among high-throughput techniques, resin-assisted enrichment techniques have allowed the detection of S-glutathionylated proteins. This method can identify the exact site of modification along with quantification of the extent of glutathionylation at that site [84]. The technique revolves around an initial blocking of the thiol groups of non-glutathionylated cysteines, followed by a specific reduction of glutathionylated cysteines to –SH using a mutant form of glutaredoxin. A thiol-reactive (-SH reactive) resin is used to trap the glutathionylated proteins, followed by tryptic digestion, iTRAQ (Isobaric tag for relative and absolute quantitation)/TMT (tandem mass tag) labelling, and mass spectrometric detection. Although this method can help identify a large number of glutathionylated proteins, it is an indirect detection method. False positives can arise due to incomplete blocking of initial steps. Inaccessibility of certain S-glutathionylated sites to the mutant glutaredoxin enzyme may also compromise the sensitivity of the method.

Another prevalent method involves the use of azide-containing glutathione derivative, γGlu-Cys-azido-Ala [82]. This moiety is synthesized inside cells by transfecting them with a mutant form of glutathione synthetase (F152A/S151G), which incorporates externally supplied azido-Ala to γGlu-Cys to form γGlu-Cys-azido-Ala (clickable glutathione). The γGlu-Cys-azido-Ala modified proteins can react with externally supplied biotin-alkyne/rhodamine-alkyne, which provides a practical handle for detecting and enriching target proteins of glutathionylation [82]. This approach allows direct detection of S-glutathionylated proteins. Recently, isotopically-labelled heavy and light derivatives of azido-Ala have been used to allow direct quantification of these S-glutathionylated proteins at the level of individual glutathionylation sites [85].

5. Critical role played by proteins in redox regulation in cancer

Cancer growth, progression, and their development of resistance to chemotherapeutic drugs involve an intricate interplay of proteins, often in their aberrant forms [86–88]. The altered redox status of a cancer cell makes the situation even more complex in terms of redox regulation of such proteins. Many of the proteins involved in cancer are known to harbour redox-sensitive critical cysteine residues [89] within active sites or at locations important for maintaining structural integrity [90]. Such cysteines are often amenable to modifications that may have functional consequences, especially in the realm of redox regulation. S-glutathionylation of proteins is one of the crucial mechanisms used by cancer cells to adjust and respond to the redox imbalances. How cancer cells adapt to oxidative stress and exploit S-glutathionylation to shift the balance in their favour is a question to ponder upon.

It is certain that S-glutathionylation of proteins would have a major impact on the progression of cancer as a disease and how it handles the response to chemotherapeutic drugs/radiation. In the last couple of decades, numerous studies have shown the link between S-glutathionylation and cancer (Fig. 4). The following sections describe such findings in detail.

6. Role of glutathionylation in cancer progression

6.1. Regulation of cellular metabolism and detoxification

Cancer cells have altered metabolism to support their high rate of growth and they produce toxic by-products as a result of their enhanced growth rate. Glutathionylation is known to affect certain proteins involved in cellular metabolism and detoxification. One such example is Pyruvate Kinase M2 (PKM2), which is an important enzyme for cancer cells due to their high dependency on the glycolytic pathway, as well as for its possible role in modulating antioxidant responses [91,92]. PKM2 has been shown to confer a selective advantage to cancer cells in vivo [93]. PKM2 catalyses phosphoenol pyruvate's irreversible conversion to pyruvate in the glycolytic pathway and acts as the rate-limiting enzyme. Anastasiou et al. have demonstrated that S-glutathionylation of Cys358 of PKM2 is crucial for responding to oxidative stress by allowing inactivation of PKM2. Velden et al. highlighted the role of GST-Π 1 in mediating glutathionylation of PKM2 in non-small cell lung cancer model [94]. Interestingly, they have found an increase in glycolytic enzymes and lactate accumulation in response to the GST-Π mediated glutathionylation of PKM2. This is contrary to earlier reports of a decrease in glycolytic activity [95]. This difference may be attributed to different cell types being used in the respective studies. Velden et al. have reported that GST-Π1 inhibition with TLK117 (selective inhibitor of GST-Π) decreased PKM2 glutathionylation as well as glycolysis, which suggests that specificity imparted by GST-Π1 might have a role to play in the differential response.

Another protein affected by S-glutathionylation is 6-Phosphofructo-2 Kinase/Fructose-2,6-Bisphosphatase (PFKFB), which is a bifunctional enzyme that catalyses two opposing reactions involving synthesis and hydrolysis of Fru-2,6-P_2. Fru-2,6-P_2 is one of the most significant allosteric modulators of glycolysis, and thus exerts control over glycolytic flux [96–98]. PFKFB has four tissue-specific isoforms (PFKFB1-4) and constitutive expression of PFKFB3 is seen in rapidly proliferating cancer cells in vitro as well as in aggressive neoplasms like thyroid, ovarian, colon, and breast carcinomas. Its inhibition can significantly hamper the

relatively high glycolytic activity of cancer cells [99]. Excessive ROS like H_2O_2 causes S-glutathionylation of PFKFB3 that renders it inactive due to a massive decrease in its kinase activity. The critical Cys206 is located in the loop meant for entry of Fructose-6-Phosphate. The neighbouring basic residues are Arg131, His135, Arg208, and Lys205, and they attract glutathione to the site leading to deprotonation of Cys206. The S-glutathionylation at Cys206 of PFKFB3 restricts the conversion of Fru-6-P to Fru-1,6-P2, which is the very first committed step towards glycolysis. This glutathionylation mediated inhibition of PFKFB3 and resultant decrease in Fru-2,6-P_2 levels leads to increased flux towards Pentose phosphate pathway (PPP). Cancer cells being susceptible to autonomous metabolism, must have intermittent activation of PPP so as to facilitate sufficient NADPH production. The reducing power of glutathione, which protects cancer cells from the elevated ROS, is recovered from glutathione reductase which is a NADPH dependent enzyme. This highlights an intricate redox-dependent mechanism to ensure enhanced production of NADPH in response to conditions of oxidative stress. More importantly, this mechanism of S-glutathionylation based inactivation of PFKFB3, reinforces the therapeutic goal of redox-profile based targeting of cancers [100].

Methylglyoxal (MGO), a 13-dicarbonyl compound, is a product of catalytic degradation of triose phosphates and amino acid metabolism. The glyoxalase system is made of Glyoxalase 1 and Glyoxalase 2, and is a detoxification system comprising of two consecutive reactions. Detoxification of these molecules proceeds through a hemithioacetal intermediate wherein GSH is conjugated to MGO leading to the formation of D-Lactate and free GSH [101]. Human Glyoxalase 1 (Glo1) or S-D-lactoylglutathione lyase, is a Zn-dependent homodimeric enzyme [102]. When cells are exposed to extracellular nitric oxide, Glo1 gets reversibly inactivated in a GSH dependent manner [103,104]. Cys139 of Glo1 is the key residue responsible for its NO-based modulation [105]. Out of its four Cysteine residues, only Cys139 is amenable to glutathionylation. Incubation of Glo1 with GSSG renders it completely inactive, although, reversibly. GST-Π and Grx1 regulate the glutathionylation of Glo1 [106]. Upon S-nitrosylation of Cys139, and at relevant physiological levels of GSH, the S-glutathionylation of Glo1 prevents MGO detoxification. This indicates that the cellular redox state can have a direct impact on enzyme activity. Inactivation of Glo1 has implications in several pathological events like diabetes and cancer. MGO production is unavoidable at the cellular level and in an altered redox state, the inactivated detoxification system may have altered intermediary metabolism [107].

6.2. Regulation of cellular signalling pathways

Cellular signalling pathways are intimately linked with all cellular processes and are subject to regulation at multiple levels. Glutathionylation can have an inhibitory/stimulatory effect on cell signalling proteins and such modulations might have important repercussions in cancer cell physiology and adaptation. Reversible phosphorylation is probably the most important cell signalling mechanism inside the cell. This post-translational modification of proteins serves to control and regulate a wide spectrum of functions including cell division, cell growth, immune responses, apoptosis, signal transduction, ageing, and more [108–110]. There are over 568 kinases and 156 phosphatases, which act predominantly on serine, threonine, or tyrosine residues to modulate the activity of target proteins [111]. Phosphorylation events and their aberrant functions have profound implications in cancer development and its progression [112]. cAMP-dependent protein kinases/Protein kinase A (PKA) are ubiquitously present inside the cell and an immense volume of data converges on the notion that the cAMP/PKA pathway is altered in many cancers [113]. The cAMP/PKA pathway can be implicated in many events like differentiation of glioma and neuroblastoma cells, deregulation of AKAPs leading to genome instability and tumour formation, as well as modulation of RAS/ERK and Hedgehog signalling, both of which are involved in cell cycle progression [114–119].

The diverse involvement of the cAMP/PKA pathway in cellular proliferation and growth pathways has been exploited as a therapeutic as well as a diagnostic target in cancers. Several drugs that act as cAMP analogues have been identified as anti-cancer therapeutics [120–122]. Glutathionylation has also been reported to inactivate PKA activity. PKA catalytic domain has two cysteine residues at positions 199 and 343. Under an oxidative environment, there is an intramolecular disulphide bond formation between these two residues that results in loss of enzyme activity. Cys199 is highly susceptible to oxidation [123] and reports suggest that oxidants can also lead to the formation of disulphide bonds with Cys199 of the catalytic subunit and Cys97 of the regulatory subunit of the PKA dimer [124–126]. Glutathionylation of Cys199 renders the kinase inactive owing to steric hindrance that hampers its affinity for the substrate [127]. Given the multitude of PKA targets and their eventual role in various physiological cellular processes, its inactivation in the oxidative environment corresponds to a potential decline in proliferative signals and thus an important watch-point for anti-cancer signalling.

Protein Kinase C (PKC) is another kinase with abundant free cysteine residues in its catalytic domain, making it prone to glutathione mediated regulation [128]. Ward et al. have demonstrated that PKCα can be inactivated by using thiol-specific oxidant diamide in combination with GSH. Such inactivation occurs due to the binding of GSH to free thiol groups of PKCα [129]. Humphries et al. has suggested that glutathionylation of Cys499 of PKCα can induce changes that lead to dephosphorylation of adjacent threonine. The dephosphorylation event may be responsible for the inactivation of PKCα [130,131]. Studies have shown that almost all isozymes belonging to PKC subfamilies are prone to glutathionylation and subsequent inactivation. Only PKCδ shows a biphasic response, i.e. it gets activated during low levels of oxidative stress while higher levels deactivate it [132]. This activation might be because of glutathionylation/oxidation of the auto-inhibitory zinc fingers in the regulatory domain of PKC that in turn releases the catalytic domain for activity [133]. Chu et al. has also reported a differential role of S-cysteinylation on PKC family enzymes, with PKCδ showing activation while PKCγ and PKCε undergo deactivation. Choice of stimulation vs inhibition seems to be concentration-dependent [134]. It is possible that differential glutathionylation is dependent on ROS concentration and can elicit distinct responses from PKC isozymes.

Akt, also known as Protein Kinase B, is also subject to regulation by glutathionylation at multiple levels. Usually, glutathionylation of Akt leads to its deactivation and inhibition of its downstream pathways. Glutaredoxin-1 can prevent H_2O_2 induced glutathionylation of Akt in retinal pigment cells and keep it in its activated form [135]. GSTω1-1 has been implicated in preventing activation of Akt in SH-SY5Y cell line. The enzymatic activity of GSTω1-1 is indispensable in this regard, which suggests a possible role of S-glutathionylation [136]. Carver et al. has reported that PTEN inactivation through S-nitrosylation can lead to sustained activation of the Akt pathway [137]. Ser/Thr protein phosphatase 2A (PP2A) can dephosphorylate Akt and exert an inhibitory role. In Caco cells, oxidized glutathione could inhibit the activity of PP2A that would, in turn, allow unhindered Akt activity [138].

Protein tyrosine phosphatases (PTP) are essential for maintaining the balance of the phosphorylation based signalling pathways. These enzymes cause dephosphorylation at the tyrosine residues and thereby exert a negative regulation on the system. Townsend et al. have reported that PTP1B undergoes reversible glutathionylation under para-aminobenzoic acid/nitric Oxide (PABA/NO) stress, leading to its inactivation. Interestingly, this happens parallel to the activation of stress kinases and seems to be an adaptation to allow the unimpeded activity of the stress kinases [77]. Lou et al. have further demonstrated (in HepG2 and A431 cells) that PTP1B glutathionylation occurs specifically at the active site Cys215 while the other cysteine residues remain in a reduced state [139,140]. This is an excellent example of how cellular machinery has evolved to make certain cysteine residues prone to glutathione based

regulation. Salsman et al. have shown that the reactivity of PTPB1 is much higher with H_2O_2 in comparison to glutathione [141] but under cellular conditions, reaction with glutathione is expected to play a more important role.

Low molecular weight protein tyrosine phosphatase (LMW-PTP) is also known to undergo glutathionylation mediated inhibition in response to VEGF or oxidative stress. This occurs in concert with focal adhesion kinase (FAK) activation, which facilitates endothelial cell migration [142]. Such pathways may also be active in cancer cells. c-Jun N-terminal kinase (JNK) is a stress-activated protein kinase with a major role in cancer cell signalling. It phosphorylates and activates c-Jun (a component of activator protein 1 (AP1)) that initiates transcription of a large number of downstream genes. This pathway is under redox control through glutathionylation at different stages. At the bottom of the cascade, c-Jun can be glutathionylated at Cys269, preventing it from binding to its target DNA sequence [143]. At the level of JNK, regulatory control is exerted through its physical association with GST-Π. Such complexation with GST-Π keeps JNK in an inactive form. It has been shown that GST-Π undergoes glutathionylation at Cys47 and Cys101, which are critical for its interaction with JNK [64]. This possibly releases JNK from the complex and allows it to regain its functionality.

Further upstream, MAPK/ERK kinase kinase 1 (MEKK1) and ASK1 are responsible for activating JNK through the intermediate mitogen-activated protein kinase kinase 4. MEKK1 provides a pro-survival signal while ASK1 is known to be pro-apoptotic in nature. Cross et al. have reported that under conditions of oxidative stress, glutathionylation at Cys1238 of MEKK1 leads to its inactivation while ASK1 gets activated through a Trx1 dependent mechanism [144,145]. Thus, glutathionylation acts as the redox sensor, allowing ASK1 to carry out apoptotic function while shutting down the antagonistic function of MEKK1. Checker et al. have demonstrated that under conditions of oxidative stress, dual specific phosphatase- 1 and 4 (MKP-1 and MKP-2) undergo glutathionylation dependent inactivation along with persistent JNK activation, leading to apoptosis in T-cell lymphoma [146].

Apart from kinases, G-proteins constitute another important group of signalling proteins. Ras has been one of the most widely studied GTP-dependent signalling proteins. There are three main members of the Ras superfamily namely H-Ras, K-Ras and N-Ras. Ras and its downstream effectors like Raf, MEK1/2, PI3K, AKT etc., have been in focus for cancer therapy [147]. Ras functions as an oncoprotein and approximately 30% of human cancers display activating mutation in Ras [148], leading to hyper-activation of downstream pathways of survival and proliferation contributing to the tumour growth [149,150]. H-Ras functions both as an initiating factor of signalling in response to oxidative stress as well as a target for oxidative regulatory mechanisms. H-Ras has six cysteine residues and out of which Cys118, Cys181, Cys184 and Cys186 are susceptible to oxidation as these are surface-exposed residues [151]. S-glutathionylation of Cys118 of H-ras serves as a redox-sensitive switch resulting in its activation. The activation due to Cys118 oxidation happens through the formation of a Ras-thiyl radical intermediate. This radical accentuates Ras activation through nucleotide exchange and reacts with $NO^{\bullet}$ and $GS^{\bullet}$ radicals to form Ras-SNO and Ras-SSG respectively. The glutathionylation mediated activation of H-Ras can therefore stimulate downstream signalling and thus encompasses a wide range of proliferative, survival and apoptotic pathways as mentioned earlier [152].

The role of H-Ras concerning cellular redox balance was analysed in a study based on OIS (Oncogene Induced Senescence) in human fibroblasts. A relatively depleted state of antioxidant potential was observed in OIS fibroblast expressing H-RasV12. A remarkably lower level of GSH and GSSG was noted in these cells indicating probable employment of GSSG towards glutathionylation of proteins since the H-Ras expressing cells also reported lower activity of glutathione reductase. There was a significant difference in the S-glutathionylation patterns of H-Ras expressing cells. Vimentin, ATP synthase β-subunit and α-enolase were also more glutathionylated in these cells compared to control cells [77,79]. The functional analysis of these proteins indicated their involvement in senescence and in other phenomena contributing to cancer progression [153–155]. Thus, during OIS, cells have higher H-Ras levels, lower GSH levels and altered pattern of glutathionylation [156].

Glutathionylation of H-Ras at Cys118 is also known to enhance phosphorylation indirectly by activating downstream kinases. Glutathionylation of H-Ras modulates its GTPase activity leading to activation of downstream p38 and Akt [157].

6.3. Regulation of proteins involved in cell survival and homeostasis

The ubiquitin-proteasome system plays an important role in the regulation of activity of certain critical proteins like NF-κB, p53, c-Jun and also plays the broader function of ensuring the survival of cell by recycling of damaged/unfolded protein. Oxidative stress, as prevalent in cancer cells, can cause transient glutathionylation of E1 (ubiquitin-activating enzyme) and E2 (ubiquitin-conjugating enzyme) proteins of ubiquitinylation machinery leading to their inactivation. This stalls this process of specific degradation of target proteins, thereby leaves them in an activated state [158]. Similarly, Rpn2, the regulatory subunit of the 19S subunit of proteasome, is also known to undergo glutathionylation and cause subsequent inhibition of proteasomal function [159]. Such glutathionylation events may drive a cancer cell towards enhanced or suppressed growth depending on the context.

NF-κB is a major transcription factor whose activity is tightly regulated by IκB protein, which keeps NF-κB in an inactive form via physical binding. Activation of IκB Kinase α (IKKα) and IκB Kinase β (IKKβ) causes phosphorylation of IκB, leading to its ubiquitin-mediated degradation, thereby releasing NF-κB [160]. Glutathionylation can act as a signal to modulate the activity of NF-κB at various levels. Cys62 of p50 subunit of NF-κB is a well-studied target for various oxidative modifications including glutathionylation, which leads to inhibition of NF-κB DNA binding activity [161,162]. NF-κB undergoes glutathionylation leading to a decrease in its binding ability on the promoter region of the iNOS gene in BCR-ABL positive cells [163]. Liao et al. have demonstrated that glutathionylation of p65 subunit of NF-κB causes suppression of TNF-α induced p65 translocation and downstream activities. This could be reversed by using a Grx1 inhibitor, suggesting an important role of Grx1 in mediating this glutathionylation event [164]. A similar effect of glutathionylation of the p65 subunit has been shown by Qanungo et al. in pancreatic cancer cells [165].

Seidel et al. have shown that the IκB alpha subunit can undergo glutathionylation in airway smooth muscle cells in a manner that prevents its degradation and thereby keeps NF-κB in an inactive state [166]. Reynaert et al. have demonstrated that Cys179 of IKKβ undergoes glutathionylation under oxidative stress in alveolar epithelial cells, leading to its inactivation [167]. This specific glutathionylation is reversed by Grx1 [167]. GST-Π is the causal agent of glutathionylation of IKKβ in lung epithelial cells, which consequently decreases its ability to phosphorylate IκB, and in turn, prevents NF-κB activation [168]. NF-κB activation could be achieved by using TLK117, a specific inhibitor of GST-Π, emphasizing the role of glutathionylation in the process.

Cancer cells undergo uncontrolled cell proliferation by evading apoptosis, a crucial mode of cell death required for the maintenance of homeostasis. Evasion of apoptosis is a major hallmark of various cancer types and is manifested by the up-regulation of anti-apoptotic genes and down regulation of pro-apoptotic genes [169]. Caspase-3 is an important mediator of apoptosis and is produced from its precursor zymogen procaspase-3 by proteolytic cleavage. Both procaspase-3 and caspase-3 undergo glutathionylation at redox-sensitive cysteine residues. Glutathionylation of procaspase-3 inhibits its ability to undergo proteolytic activation. Caspase-3 undergoes glutathionylation at Cys135 of p17 and Cys45 of p12 protein subunits. Cys135 is present at the active site and its glutathionylation affects the substrate access and thereby its activity. It is not well understood how glutathionylation at Cys45 of p12 affects the activity, although a most likely explanation is that glutathionylation at

Cys45 of p12 may induce conformational change which may cause dissociation of the subunits or may prevent substrate access [170].

6.4. Modulation of tumour suppressor proteins

Dysregulation of tumour suppressor proteins is a characteristic feature of cancerous cells. Apart from genetic mutations, post-translational modifications like glutathionylation are known to play a role in the modulation of the activity of certain tumour suppressor proteins.

p53 is a tumour suppressor which plays diverse roles including cell cycle arrest, genome repair, regulatory roles and apoptosis. Mutation in the p53 gene is often associated with a cancer phenotype and glutathionylation of p53 serves to modulate its effects.

Velu et al. have reported that oxidative stress can induce glutathionylation at Cys124, Cys141 and Cys182 of p53, with the modification at the former two sites rendering p53 unable to dimerize and bind to its target DNA [171]. Yusuf et al. have shown that glutathionylation of Cys141 of p53 is prevalent in different types of cancer cells. Melanomas seem to have the highest abundance of glutathionylated p53 followed by colon cancer and prostrate adenocarcinomas, while glutathionylated p53 was negligible in gastric cancers [172]. Cys277 of p53 has also been identified as a site undergoing glutathionylation in p53 under physiological conditions (reduction potentials of −163 mV and more) [173]. Cys277 has a possible role as a redox regulatory switch because it allows p53 to differentiate between its consensuses sequences and thereby allows it to respond differently under conditions of oxidative stress. Redox state of Cys 277 of tumour suppressor protein p53 regulates its sequence-specific DNA binding following DNA damage. The addition of glutathione to Cys277 of p53 is expected to inhibit its binding to certain subset of consensus sequences which are under redox control.

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a tumor suppressor having phosphatase-dependent and independent roles. Loss of function of PTEN is one of the most significant mutations found in human cancers [174,175]. The most well-established mechanism of tumour suppressive activity of PTEN is through dephosphorylation of PIP_3 and subsequent inhibition of PI3K signalling. The PI3K signalling is central to cancer survival, growth, and metabolism [176,177]. PTEN is a very prominent target for redox regulation and the phosphatase activity of PTEN is reversibly lost when S-nitrosothiols cause its oxidative modification [178]. In the presence of oxidative phosphorylation induced ROS, S-glutathionylation causes inactivation of PTEN leading to activation of PI3K/Akt pathway of cell survival [179]. However, since this is a reversible process that occurs in response to elevated ROS, the Cys at the active site is prevented from oxidation beyond sulfenic acid [180]. Phosphatase family proteins are as such susceptible to S-glutathionylation and the process is critical to their activity [138].

6.5. Protein glutathionylation serving as cancer biomarkers

Certain glutathionylation events are of unknown functional significance but are nevertheless consistent with cancerous phenotype. Such modifications can be indicative of cancer initiation and its prognosis.

Prostate cancer is the most common cancer in men and radiotherapy is the mainstay treatment mode [181]. Generally, prostate-specific antigen (PSA) is used for disease screening and prognosis in prostate cancer patients [182], but since PSA is only released subsequent to the death of cancer cells, it can take a longer time to appear after radiation exposure. Hence PSA levels may not suffice as a reliable biomarker for radiation exposure or delivered dose [183].

Serine protease inhibitors (serpins) are abundantly present in human blood and various tissues. There are 37 serpins that have been identified in the human genome so far and they have been divided into 9 clades (A to I) [184]. Serpins contain sensitive cysteine residues which undergo reversible glutathionylation under low ROS levels. Under oxidative stress conditions, serpin A1 and A3 undergo S-glutathionylation at Cys256 and Cys263, respectively [183]. Glutathionylated serpins may serve as a molecular biomarker of radiation exposure in prostate cancer patients.

Haemoglobin (Hb) is another abundant moiety that shows cancer-specific changes in glutathionylation levels. H/DX (hydrogen-deuterium exchange) mass spectrometry reveals that the quaternary structure of haemoglobin (Hb) changes upon glutathionylation [185]. Glutathionylated Hb is a marker of oxidative stress in many pathological states like hyperlipidaemia, uraemia and diabetes mellitus [186,187].

Out of the three Cys residues found in Hb (α-Cys104, β-Cys93, and β-Cys112), β-Cys93 has the highest affinity for electrophiles. However, incubation of Hb with GSSG results in glutathionylation of all three residues. With an increasing dose of GSSG, the extent of modification of all glutathionylated peptides increases and is significant enough to detect and grade oxidation status on a relative quantitative scale [188]. Glutathionylation of α-Cys104 and β-Cys93 of Hb is significantly enhanced in gastric cancer patients as compared to healthy persons. The extent of glutathionylation at α-Cys104 was 5.3 times higher in gastric cancer patients as compared to healthy individuals, whereas β-Cys93 glutathionylation was 13.9 times higher [189]. Thus, glutathionylation status β-Cys93 of Hb has been proposed as a potential marker of gastric cancer.

7. Role of glutathionylation in resistance to anti-cancer drugs

Anti-cancer drugs work through a wide spectrum of pathways and the efficacy of a drug is dependent on the level of expression/activity of certain proteins inside the cell. Glutathionylation, through its effect on target proteins, exerts influence over the effectiveness of anti-cancer drugs. The effect of glutathionylation on resistance to anti-cancer drugs is context-specific. The following section describes the recent findings in the field of glutathionylation and cancer chemoresistance.

7.1. Proteins involved in protein stabilization, unfolded protein response and protein turnover

HSP90 is a major molecular chaperone having an important role in stabilizing structurally labile proteins, including ones which are crucial for cell growth, proliferation and differentiation [190,191]. HSP90 has a vital role in supporting the adaptability and heterogeneity of tumours [192]. Being highly active in cancerous cells, HSP90 has also been implicated in conferring chemo-resistance in various cancer cell types [193,194].

Cellular degradation of HSP90 is dependent on the ubiquitin proteasomal pathway and Shih et al. have recently demonstrated that glutathionylation might be playing a crucial role in the process [195]. They have reported site-specific glutathionylation of HSP90, occurring both in vivo and in vitro. Oxidative stress was found to induce glutathionylation and subsequent structural changes in HSP90. These structural changes are thought to be important in priming HSP90 for proteasome-mediated degradation. Thus, oxidative stress might prove to be useful complimentary interventions to circumvent HSP90 based resistance to chemotherapeutic drugs.

Further, Hamamoto et al. have shown that the histone methyltransferase SMYD2 can methylate HSP90AB1 at Lys531 and Lys574, thereby bringing about its dimerization and activation [196]. Interestingly, SMYD2 can itself undergo glutathionylation at Cys13, preventing its association with HSP90 and blocking its subsequent activation [197].

An important aspect of cancer cell physiology is their necessity to handle an increased rate of protein synthesis to support their rapid proliferation. Such a high protein synthesis rate leads to the accumulation of unfolded or misfolded proteins inside the cells, leading to endoplasmic reticulum (ER) stress [198]. Unfolded protein response (UPR) is the cellular stress response cascade that allows such cells to either cope with the ER stress and restores homeostasis [199], or get

overwhelmed and undergo apoptosis [200]. The UPR has been shown to be a possible target for anti-cancer drugs to kill cancer cells [201].

Ye et al. have demonstrated that GST-Π can bring about glutathionylation of several ER proteins including PDI, calnexin, endoplasmin and calreticulin [67,202]. These proteins are responsible for the proper folding of newly synthesized proteins in the ER, thus alleviating ER stress. Tunicamycin, which brings about ER stress (and subsequent UPR dependent apoptosis) by inhibiting N-linked glycosylation of proteins, shows a higher efficacy in GST-Π knockout cells, although the exact role of glutathionylation in this process is unclear. Protein disulphide isomerase (PDI) is a key protein involved in protein folding. Townsend et al. have shown that PDI undergoes glutathionylation at the active sites (inside fragments 43–57 and 387–401) in response to nitrosative stress (PABA/NO treatment). This modification alters its tertiary structure and diminishes its isomerase activity [203]. Such glutathionylation of PDI is consistent with the induction of unfolded protein response, presumably due to accumulation of unfolded proteins as a result of PDI inactivation. Indeed, the cytotoxic potential of PABA/NO in SKOV3 cells could be attributed to overwhelmed UPR leading to apoptosis, brought about by functional inactivation of PDI through S-glutathionylation. It is worth mentioning that Erp5, which plays an important role in immune evasion on the surface of cancerous cells [204], shares a WCGHC motif with PDI, and can be a possible target for glutathionylation.

Another example of chemoresistance through glutathionylation can be found in the case of multiple myeloma. Multiple myeloma (MM), which are malignant monoclonal plasma cells, are characterized by the secretion of proteins containing disulphide bonds at a very high rate [205]. Such activity requires a high rate of proteasomal flux and tight regulation of redox balance in MM cells [206]. The dependency on high proteasomal activity is exploited using bortezomib, a proteasome inhibitor, which causes impairment of the proteasomal pathway and subsequent apoptosis of the MM cells due to overloading of unfolded and undegraded proteins. Unfortunately, bortezomib-induced cytotoxicity is prone to be nullified due to rapid development of resistance. Starheim et al. have shown that elevated intracellular GSH level is one of the factors which can reverse resistance to bortezomib [207], implying a crucial role of glutathione in bortezomib cytotoxicity. Multiple myeloma cells maintain a high level of oxidative stress to counter bortezomib-induced cytotoxicity [208]. Basal levels of S-glutathionylated proteins are elevated in such resistant cells. In particular, GST-Π mediated glutathionylation of binding immunoglobulin protein (BiP) is a pivotal process for bortezomib resistance. BiP is an HSP70 molecular chaperone having foldase and ATPase activities. These activities get modulated by glutathionylation at Cys420 and Cys41 respectively. While ATPase activity is dampened as a result of glutathionylation, the foldase activity is enhanced, which results in refolding of unfolded proteins at a higher rate, ultimately nullifying the proteasome inhibitory effect of bortezomib. Use of GST-Π inhibitors like TLK199 has been shown to reverse bortezomib resistance by preventing glutathionylation of BiP.

7.2. Proteins involved in survival signal

Signal Transducer and Activator of Transcription (STAT) proteins are a pool of latent transcription factors, that exhibit rapid and transient activation upon binding of cytokines/growth factors to cell surface receptors. In turn, they regulate multiple physiological processes including inflammation, apoptosis, differentiation and metastasis [209]. STAT3, one of the members of this family, is activated after phosphorylation of Tyr705 residue by receptor-associated Janus kinases (JAK) [210]. Persistent activation of STAT3 has oncogenic potential leading to uncontrolled growth, promotion of angiogenesis, suppression of anti-tumour immunity followed by invasion and metastasis which is associated with various human cancers including head & neck, prostate, breast and haematological malignancies [211,212]. STAT3 is subject to several post-translational modifications such as phosphorylation, methylation, acetylation, ubiquitylation, sumoylation and glutathionylation, and these modifications can regulate its activity [213,214]. Its activation is sensitive to redox regulation. Butturini et al. has observed that mild oxidative stress due to a decrease in GSH content induced by natural terpenes inhibited STAT3 activation via S-glutathionylation in THP-1 cells [215]. It was observed that oxidative stress induced S-glutathionylation of STAT3 exhibited concomitant inhibition of Tyr705 phosphorylation indicating a possible cross-talk between these two post-translational modifications [216]. Thus, the S-glutathionylation of STAT3 impairs JAK2 mediated Tyr705 phosphorylation, a pre-requisite for STAT3 DNA binding and transcriptional activity. This might be attributed to the addition of the GSH group to STAT3 leading to limiting the accessibility and recognition of Tyr705 by JAK2. Out of twelve 12 Cys residues present in STAT3, Cys328 and Cys542 are subject to redox regulation by S-glutathionylation.

Recently, it was also observed that sesquiterpene lactone (SL) induced mild oxidative stress could bring about STAT3 S-glutathionylation and significantly enhance the sensitivity of DU145 cells to cisplatin and docetaxel [216]. Cynaropicrin, costunolide, dehydrocostuslactone and alantolactone scavenge the cellular pool of GSH and disrupt GSH/GSSG homeostasis, leading to oxidative stress induced S-glutathionylation of STAT3, thereby switching off its downstream signalling cascade [216–218]. Their promising anti-STAT3 activity with little or no non-specificity provides an effective alternative for overcoming issues related with poor therapeutic success using conventional drugs and enhancing their anti-cancer efficacy.

7.3. Proteins involved in membrane transport

Another drug resistance mechanism employed by cancer cells is the increased expression of uncoupling protein 2 (UCP2), a protein that regulates proton leakage and diminishes mitochondrial ROS production [219]. Over-expression of UCP2 has been reported to result in increased proton leak from the mitochondria that helps the cells to control ROS upon exposure to ROS inducing chemotherapeutic agents [220,221]. Pfefferle et al. have hypothesized that since UCP2 is modulated by glutathionylation, pharmacological inhibition of UCP2 may sensitize drug-resistant leukaemia cells (MX2) to chemotherapeutic drugs. They showed that glutathionylation deactivates proton leakage through UCP2 in drug-resistant MX2 cells. Further, diamide treatment significantly increased cell death in MX2 cells and sensitized them to menadione and doxorubicin. They concluded that glutathionylation of UCP2 inhibits proton leakage, which makes the cells sensitive to chemotherapeutic drugs [222]. These results indicate that glutathionylation of UCP2 and its subsequent inhibition can be employed as a novel therapeutic strategy for drug-resistant cancers.

Ye et al. have also demonstrated the effect of GST-Π in bringing about glutathionylation of Sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) [67,202]. Increased sensitivity to thapsigargin (inhibitor of SERCA) in GST-Π deficient cells (knockout MEF cells and BMDD cells) highlights the importance of the protective role being played by GST-Π. It is likely that GST-Π causes reversible glutathionylation and preserves the function of SERCA, and thus hinders SERCA dependent apoptosis. SERCA is known to induce apoptosis in cancer cells through calmodulin/calcineurin-dependent apoptotic cascade [223].

7.4. Structural proteins

Microtubules are polymers of α and β tubulin dimers which constitute linear protofilaments. In turn, protofilaments are associated laterally to form long, hollow cylinders consisting of thirteen parallel protofilaments [224]. Microtubules are critical for several vital cellular processes including the formation of cytoskeletal structure, intracellular trafficking of vesicles, organelles, macromolecules, maintenance of cell shape, structure and major component of mitotic spindle responsible for cell division via mitosis/meiosis [225–227]. Targeting microtubule

leads to disruption of chromosome segregation forcing cells to aberrantly exit mitosis, exhibit G2/M arrest and undergo cell death [228]. Hence, several anti-mitotic agents such as taxanes and alkaloids are used extensively in clinics with decent therapeutic success for various cancer types [229]. However, cancer cells develop resistance to antimitotic agents. Such agents also exhibit undesirable effects on rapidly proliferating normal cells (neutrophils, skin, hair) [228]. There is a need for identifying alternative antimitotic agents with novel mechanism of action.

Interestingly, α-tubulins contain a total of 12 free thiol groups and β-tubulins contain 8 free thiol groups that are critical for tubulin polymerization, and their oxidative modification hampers microtubule formation [230]. Recently, it was observed that tubulins can be glutathionylated under oxidative stress and extent of glutathionylation directly correlated with their depolymerization and subsequent lack of microtubule formation leading to mitotic arrest and apoptosis in cancer cells [228]. Agents that can glutathionylate tubulin and thereby exhibit anti-cancer activity are being explored as putative novel anti-mitotic drugs.

7.5. Tumour suppressor proteins

Mutation in p53 results in the loss of wild-type tumour suppressive functions and aids in carcinogenesis and cancer progression. The majority of p53 mutations are missense due to which cells cannot activate the canonical p53 pathway. However, occasional reactivation of mutant p53 into its functional form has been employed as a strategy for the development of anti-cancer therapies [231]. Interestingly, reactive cysteines (Cys124, Cys141 and Cys182) in the DNA-binding domain of p53 are targets for oxidation and thiolation, and human p53 has been reported to undergo glutathionylation leading to changes in p53 functions [171]. A recent report showed that piperlongumine induced glutathionylation of mutant p53 restores some functionality via induction of structural changes in the defective DNA-binding domain. Induction of oxidative stress in Human colon carcinoma cell lines (HT29 and SW620) led to increased levels of WT-like p53 with a concomitant decrease in levels of mutant p53 protein. A subsequent up-regulation of p53 target genes (MDM2 and Bax) and an increase in the levels of apoptotic markers were also observed in both cell lines [232]. This functional restoration of the p53 also potentiated the cytotoxic effects of anti-cancer drugs like doxorubicin, indicating that oxidative stress mediated functional restoration of p53 may prevent cancer progression and highlights a novel strategy for cancer treatment.

7.6. Proteins serving as redox response regulators

The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) is the major regulator of the cytoprotective response of cells to oxidative stress [233]. Keap1, a negative regulator of Nrf2, is a cysteine-rich protein with 25 cysteine residues in mouse Keap1 and 27 cysteine residues in human Keap1 [234]. Genetic mutations and copy-number alterations to the Nrf2 signalling pathway have been reported in several cancers such as lung squamous cell carcinoma (19%), uterine (20.6%), head and neck (17.4%), oesophageal (19.8%), and bladder carcinomas (14.8%) [235]. The reactive thiol groups in Keap1 are known to get modified leading to Keap1-Nrf2 dissociation and subsequent Nrf2 nuclear translocation [236]. Zhang et al. reported that depletion of intracellular glutathione by electrophilic agent 2-(pro-1-ynyl)-5-(5,6-dihydroxypenta-1,3-diynyl) thiophene (PYDDT) could modify Keap1 by S-glutathionylation leading to activation of Keap1-Nrf2 pathway. They concluded that S-glutathionylation of Keap1 is an important post-translational modification and may have a critical role in regulating oxidative stress and signal transduction [237]. Another report showed that induction of oxidative stress in lymphocytes led to glutathionylation of KEAP-1 [238]. Nrf2 overexpression leads to chemoresistance in many solid tumours such as gastric cancer, osteosarcoma, non-small cell lung cancer [239–241]. An earlier report by Wang et al. showed that Nrf2 overexpression makes cancer cells resistant to chemotherapeutic agents, whereas Nrf2-downregulation increased drug susceptibility [242]. A recent report showed that P53-induced protein with a death domain (PIDD) interacts with Keap1 and promotes Nrf2 stabilization which is responsible for increased chemo-resistance in lung cancer cells in vitro and in vivo [243]. In conclusion, studies indicate that oxidative stress induced S-glutathionylation of Keap1 can result in Nrf2 activation leading to chemoresistance in cancer cells. Thus, inhibitors of Keap1 glutathionylation can be exploited as adjuvants to chemotherapeutic agents to sensitize cancer cells to these drugs and maximize cancer cell death.

8. Inhibition of proteins involved in downstream glutathionylation and detoxification

Activation of drug-detoxification system can cause chemo-resistance in cancer cells. GSTs, being one of the most crucial detoxification enzymes, catalyses the nucleophilic addition of glutathione to the electrophilic molecules and makes them hydrophilic for subsequent elimination [244]. GSTs also act as negative regulators of apoptotic pathways and related signalling cascades.

Reportedly, many cancers have overexpression of GSTs and they render the administered drug ineffective either via detoxification mechanisms or through signalling cascades, thus accentuating the problem of drug resistance resulting in poor clinical outcomes of therapy in cancers [245]. GST-α and GST-π have been thoroughly analysed for their potential as diagnostic and prognostic markers. Irrespective of whether a given drug is a substrate of glutathionylation or not, GST-π overexpression is prevalent in many drug-resistant solid tumours [246, 247]. Combining cancer therapy with compounds that would target GSTs seems to be a rational approach to ensure the effective killing of the cancer cells. In this regard, two major groups of drugs: Tricyclic Antidepressants (TCAs) and Perylene derivatives have been analysed.

Amitriptyline belongs to the group of TCAs deployed for the treatment of depression symptoms [248,249]. Amitriptyline reversibly inhibits GST-π and GST-α by competing with the substrate GSH, suggesting that they vie for the same site. This inhibition can increase the efficacy of anti-cancer drugs by preventing their elimination [250]. However, more studies must be done to identify the overall redox status post administration of Amitriptyline because inhibition of the activity of GSTs would also result in increased exposure to electrophilic molecules.

Perylene derivatives are used in oxidation based applications and 1,3,4,6,8,13-hexahydroxy-10,11-dimethylphenanthro 1,10,9,8-*opqra* perylene-7,14-dione or Hypericin is one of the most popularly used perylene derivatives. It is a natural pro-oxidant and its application in the enhancement of cancer cells killing is well established [251]. The most prominent example of hypericin use is in photodynamic therapy where it acts as a photosensitizer by mediating its oxidative effects through enhanced generation of ROS [252]. The increased levels of ROS facilitate therapy by effectively killing cancer cells [253]. Ability of hypericin to enhance cytotoxic killing in photodynamic therapy could also be attributed to the inhibition of GST. Hypericin was able to combat the GST-dependent resistance along with increasing ROS levels [254–256]. Hypericin can also modulate the downstream signalling pathways like tyrosine kinases and MAP Kinases, which are otherwise activated in the presence of high levels of GSTs [257]. To conclude, glutathionylation mediated by GSTs is often a causal factor behind drug resistance, and inhibition of GSTs by TCAs or perylene derivatives like hypericin can make way for more efficient administration of chemotherapeutic drugs.

9. Conclusion and future prospects

Traditionally, glutathione has been thought to be primarily involved in buffering and detoxification processes, but that notion is changing quickly. At present, glutathione is known to play a crucial role as a redox

molecule to signal the changes in redox state and help the cell to adjust to oxidative stressors. Cancer cells need to adapt to the stresses arising out of the high burden of constant proliferation and threats from chemotherapeutic drugs. Protein S-glutathionylation provides an exciting route to fine-tune the adaptation process by regulating redox-sensitive proteins. In this review, we have discussed the current state of knowledge about the role of protein S-glutathionylation in cancer. The recent spike of interest in this topic has led to the discovery of several pathways that are under the regulation of glutathionylation, but still, plenty remain to be understood in the realm of protein glutathionylation. Further evidence and a deeper knowledge would allow us to better understand the process development of cancer and its acquisition of resistance to chemotherapeutic drugs. Such knowledge will be helpful in our quest to develop effective modalities to treat cancer and overcome chemo-resistance.

Author contributions

DJP, AR, RC, RSP, BS, DS, SSK contributed to writing the manuscript. DJP, RC contributed to making figures. DS and SSK contributed to editing and correcting the manuscript. SSK contributed to conceptualizing the idea.

Funding details

The work was supported by funding from the Department of Atomic Energy, Government of India.

Declaration of competing interest

All the authors declare that they have no conflicts of interest with the contents of this article.

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Functional & Integrative Genomics (2022) 22:1211–1227
https://doi.org/10.1007/s10142-022-00906-z

RESEARCH

Transcript profiling of Polycomb gene family in *Oryza sativa* indicates their abiotic stress-specific response

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Received: 6 April 2022 / Revised: 16 August 2022 / Accepted: 27 September 2022 / Published online: 5 October 2022

Abstract

The precise regulation of gene expression is required for the determination of cell fate, differentiation, and developmental programs in eukaryotes. The Polycomb Group (PcG) genes are the key transcriptional regulators that constitute the repressive system, with two major protein complexes, Polycomb Repressive Complex 1 (PRC1) and Polycomb Repressive Complex 2 (PRC2). Previous studies have demonstrated the significance of these proteins in regulation of normal growth and development processes. However, the role of PcG in adaptation of crops to abiotic stress is still not well understood. The present study aimed to a comprehensive genome-wide identification of the PcG gene family in one of the economically important staple crops, *Oryza sativa*. Here, a total of 14 PcG genes have been identified, which were distributed over eight chromosomes. Protein structure analysis revealed that both the complexes have distinct domain and motifs that are conserved within the complexes. In silico promoter analysis showed that PcG gene promoters have abundance of abiotic stress-responsive elements. RNA-seq based expression analysis revealed that PcG genes are differentially expressed in different tissues and responded variably in different environmental stress. Validation of gene expression by qRT-PCR showed that most of the genes were upregulated at 1-h time point in shoot tissue and at 24-h time point in root tissue under the drought and salinity stress conditions. These findings provide important and extensive information on the PcG family of *O. sativa*, which will pave the path for understanding their role in stress signaling in plants.

Keywords Polycomb gene · *Oryza sativa* · Abiotic stress · Genome-wide · Epigenetics

Introduction

In comparison to other diverse life forms, plants are subjected to various biotic and abiotic stresses, and their development is dependent on cues from the environment. The response for biological processes that arise from environmental cues like different kinds of stresses, defense response against pathogens, metabolic pathways, and photosynthesis are mostly raised by changes in the gene expression level and patterns which essentially indicate that transcription is one of the important regulatory factors. While transcription as a process involves elaborate machinery, therefore, this study is intended to determine the role of a group of proteins (i.e., Polycomb group) that regulate transcription via chromatin remodeling.

Polycomb group (PcG), a highly conserved epigenetic modifying protein complex, has been widely found in plants and animals, and could continuously and stably inhibit gene transcription (Mozgova and Hennig 2015). In higher organisms, epigenetics plays an important role in regulating gene expression and is involved in coordinating the gene activity by chromatin remodeling via histone modifications (acetylation, methylation, phosphorylation, and ubiquitination). The well-known post-translational modifications include methylation of lysine 4 on histone H3 (H3K4me) which is mainly associated with transcriptional activation, whereas di- and trimethylation of lysines 9 and 27 (H3K9me2 and H3K27me3, respectively) leads to transcriptional repression (Wu et al. 2009). In plants, several regulatory and transitions development processes such as cell differentiation, organ initiation, and flower morphogenesis are controlled by chromatin modifiers (Ojolo et al. 2018). In various studies, epigenetics had also been found to play an important

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role in stress signaling (Chang et al. 2020; Kim et al. 2015; Luo et al. 2012).

The repressive epigenetic regulatory processes of genes are usually controlled by PcG proteins that were first identified in *Drosophila* as regulators of homeotic genes during development (Lewis 1978). It dictates the transcriptional status of target genes and therefore decides the fate between alternative developmental programs. PcG proteins belong to diverse families that function as a multiprotein complex. The most well-known complexes are Polycomb Repressive Complex 1 (PRC1) and Polycomb Repressive Complex 2 (PRC2) (Derkacheva and Hennig 2014). In animals, PRC2 is involved in trimethylation of lysine 27 of histone H3 (H3K27me3) which further recruits PRC1 to the chromatin that mono-ubiquitylates it on lysine 119 of histone H2A (H2AK119ub) in mammals (lysine 118 in *Drosophila*) (Simon and Kingston 2013). This is the classic view of PcG complex recruitment, although PRC1 can be recruited to some targets without H3K27me3 (Tavares et al. 2012), and the presence of PRC2 and H3K27me3 at some genes does not result in PRC1 recruitment (Simon and Kingston 2013).

In *Drosophila*, PRC1 primarily contains four subunits: Polycomb (Pc), Polyhomeotic (Ph), Posterior sex combs (Psc), and Sex combs extra (Sce) (Francis et al. 2001; Mohd-Sarip et al. 2002). In plants, the PRC1 complex is yet to be explored in detail. In Arabidopsis, a single homolog of Pc has been found named LIKE HETEROCHROMATIN PROTEIN1 (LHP1) or TERMINAL FLOWER 2. The chromodomain of LHP1 binds to the H3K27me3 in vitro, and its genome-wide localization overlaps strongly with H3K27me3 rich regions (Turck et al. 2007; Zhang et al. 2007). Two Arabidopsis homologs of Sce have been identified as REALLY INTERESTING NEW GENE1 (RING1A and RING1B). Both of these proteins contain the RING domain as well as ubiquitin-like RAWUL domain, the key characteristics of RING1 of the animal PRC1 complex (Sanchez-Pulido et al. 2008). These proteins function mainly in the repression of Class 1 KNOTTED1 LIKE HOMEOBOX (KNOX) genes that are involved in meristem identity (Katz et al. 2004; Xu and Shen 2008). Three homologs of *Drosophila*'s Psc have been reported in Arabidopsis as B LYMPHOMA Mo-MLV INSERTION REGION 1 HOMOLOG (BMI1A, BMI1B, and BMI1C); moreover, these three proteins along with the RING1A and RING1B have E3 ligase activity (Baile et al. 2022). BMI1A/B are responsible for H2AK121 ubiquitinylation in seedlings, and also target DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN2A (DREB2A) for proteasome mediated degradation by ubiquitylating them in response to water stress, suggesting that they might also regulate some PcG genes by targeting them for degradation (Qin et al. 2008). To date, no plant homolog of Ph has been discovered (Bemer and Grossniklaus 2012; Shen et al. 2021).

PRC2 in *Drosophila* consists of four subunits, namely, Enhancer of Zeste [E(z)], Suppressor of Zeste [Su(z)12], Extra sex combs (Esc), and p55 (Czermin et al. 2002; Müller et al. 2002). Plants have multiple families of PRC2 homologs following the gene duplication event during the evolutionary processes. In Arabidopsis, FERTILIZATION INDEPENDENT ENDOSPERM (FIE) is the only Esc homolog; CURLY LEAF (CLF), SWINGER (SWN), and MEDEA (MEA) are three E(z) homologs (Chanvivattana et al. 2004; Goodrich et al. 1997). Three known homologs of Su(z)12 are EMBRYONIC FLOWER2 (EMF2), VERNALIZATION2 (VRN2), and FERTILIZATION INDEPENDENT SEED2 (FIS2), and there are five p55 homologs known as MULTICOPY SUPPRESSOR OF IRA 1–5 (MSI1,2,3,4,5) (Hennig et al. 2005; Kiyosue et al. 1999). At different developmental stages, different homologs of E(z) and Su(z) are found to be functional in Arabidopsis (Derkacheva and Hennig 2014). MEA plays an important role in early endosperm development (Köhler et al. 2003), whereas SWN and CLF are active during vegetative development and vernalization. In Arabidopsis, the *clf swn* double mutant completely loses H3K27me3, which indicates the possible inactivation of PRC2 (Lafos et al. 2011). The Su(z) homolog FIS participates in regulating female gametophyte and seed development (Köhler et al. 2003), and EMF regulates the vegetative development and transition to flowering (Yang et al. 1995).

Genome-wide identification of PcG gene family has been done earlier in species such as bread wheat (Strejčková et al. 2020) and *Medicago truncatula* (Zhao et al. 2021). Studies of these types are helpful in understanding the contribution of a gene/gene family to a particular process and also pave the way for future work. Several PcG genes have been identified from the *O. sativa* genome, although the investigation of these genes has not been extensive (Chen et al. 2016; Luo et al. 2009). Therefore, genome-wide identification and transcript profiling of PcG members was performed in order to better understand the gene structure, expression pattern, and probable significance of these genes in abiotic stress responses in *O. sativa*.

Materials and methods

In silico identification of PcG gene family members in O. sativa

To identify the PcG gene members in *O. sativa*, the known amino acid sequences of Arabidopsis PcG proteins were used as query to conduct a BLASTp search (http://www.ncbi.nlm.nih.gov/BLAST). Sequences of Arabidopsis were retrieved from TAIR database (https://www.arabidopsis.org/). Afterward, the Batch web CD search tool (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) was

used to further check for the conserved domains of candidate PcG members. The genes were named according to the sequence homology with Arabidopsis primarily, and if more than one homolog was found in *Oryza sativa*, they were named according to their chromosomal location. Chromosomal location of PcG genes was determined with respect to their position and information retrieved from rice genome sequences. The physical map information on chromosome number, length, and gene loci were obtained from the Rice Genome Annotation Project (RGAP) (http://rice.plantbiology.msu.edu/). Elementary physical map depicting the location and distribution of PcG gene family was drawn using Map Tool software from Oryza base (https://shigen.nig.ac.jp/rice/oryzabase/) with default parameters.

Prediction of physicochemical characteristics and subcellular localization of PcG proteins

ProtParam ExPASy server (http://www.expasy.org/tools/) was used to analyze the MW (molecular weight), p*I* (isoelectric point), and GRAVY (grand average of hydropathicity index) of all the identified members. The subcellular location of PcG proteins was predicted using Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/). In addition, secondary and tertiary structures were predicted using SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html) and Phyrev2.0 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index), respectively.

Sequence analysis of PcG genes

Multiple EM for Motif Elicitation (MEME) program (http://meme-suite.org/tools/meme) was used for the conserved motif detection in PcG proteins with the parameters of minimum width of 6 and maximum width of 105 amino acids. The maximum number of motifs to be searched were kept ten.

The conserved domain information of PcG genes was obtained using Pfam (http://pfam.xfam.org/). Genomic features (UTRs, CDS, etc.) of PcG genes were retrieved from RGAP and were represented on PcG genes using GSDS 2.0 (http://gsds.cbi.pku.edu.cn/).

Prediction of protein interaction network of PcG family

The protein interaction network of PcG proteins was predicted through the STRING v11.0 program (https://www.string-db.org/). The confidence parameter (combined score) was set at a 0.40 threshold, and the "Network type" was "full network (the edges indicate both functional and physical protein associations)".

Analysis of putative promoter of PcG genes

The 2-kb upstream sequences from the translation start site of all of the *O. sativa* PcG genes were obtained from RGAP database and scanned through PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to know about the presence and position of different *cis*-regulatory elements.

Expression profiling of PcG genes using microarray and RNA-seq data

RNA-seq data and microarray data available at EBI expression atlas (https://www.ebi.ac.uk/gxa/home) and GENEVESTIGATOR (https://genevestigator.com/gv/), respectively, were used for the expression analysis of PcG genes at different developmental stages, anatomical tissues, in abiotic stress, heavy metal stress, and hormone treatment. RNA-seq datasets available at EBI expression atlas were queried using the Bioconductor package Expression Atlas (https://bioconductor.org/packages/release/bioc/html/ExpressionAtlas.html). Gene level counts obtained from Expression Atlas (E-MTAB-1624 and E-GEOD-58603) were analyzed for differential expression using Wald's test in the DESeq 2 package (Love et al. 2014). The resulting *p* values from Wald's test were adjusted for multiple testing using the Benjamini and Hochberg method (Benjamini and Hochberg 1995). In the case of stress conditions, log2FC estimated by DESeq 2 is plotted as a heatmap. Data from Genevestigator was retrieved and heatmap was generated using the log2 signal values with the help of MeV software package (Saeed et al. 2003).

Plant material and qRT-PCR analysis

IR64 seeds were grown under standard growth condition in growth chamber with 28 ± 2 °C and photoperiod of 16 h at 70% humidity. Initially, seeds were treated with Bavistin for 15–20 min and allowed to germinate in water hydroponically. After germination, Yoshida Media was provided for 10 days, and on the 11th day, stress treatments were given (Yoshida et al. 1971). For salinity stress, 200 mM NaCl was added to Yoshida Media; for drought stress, the seedlings were removed from the media and kept on tissue paper (Aggarwal et al. 2020; Nagar et al. 2020). Seedlings grown in Yoshida Media without any stress were used as control. Samples were harvested at the 1-h and 24-h time points.

Total RNA was isolated from shoot and root tissue of control and stressed rice plants using IRIS kit (Bangalore, Genei) as per the manufacturer's protocol. RNA quality was checked by 260/280 and 260/230 absorbance ratios and further running the RNA on denaturing agarose gel. RNase-free DNase I (Fermentas Life Sciences, USA)

enzyme was used to get rid of the genomic DNA contamination in RNA samples. First-strand cDNA synthesis was carried out using Maxima first strand cDNA synthesis kit for qRT-PCR (Fermentas Life Sciences, USA). Primers for real-time PCR analysis of PcG genes in rice were designed using NCBI primer BLAST for a product length ranging between 70 and 120 bp. Rice *EIF4α* gene was used as reference gene for the normalization of real-time data (Kumar et al. 2018). The PCR mixture contained 2.5 µl first-strand cDNA (10 times diluted), 5 µl of 2 × SYBR green PCR master mix (Fermentas Life Sciences, USA), and 2 µM of each gene-specific primer in a final volume of 10 µl. No template controls were also performed for each of the primer pair. The real-time PCRs were performed employing ViiA7 real-time PCR machine (Applied Biosystems, USA). Three biological replicates were analyzed for each sample. The relative expression ratio was calculated using delta Ct value method (Livak and Schmittgen 2001). The melt curve was analyzed to ensure the presence of single PCR product and no primer dimer.

Results

Identification and annotation of PcG members in O. sativa

After a systematic BLASTp search with the query sequence of Arabidopsis PcG proteins (Fig. 1 showing an outline of methodology), a total of 14 proteins were obtained in *O. sativa*. PcG genes have been previously identified in various studies (Chen et al. 2016; Luo et al. 2009). In this study, previously published results from *O. sativa* were compared and 11 genes out of 14 genes were same as formerly known PcG genes, and three PcG genes found in this study are *Os_MSI1*, *Os_MSI3*, and *Os_MSI4*. Genes have been named according to their homology with Arabidopsis genes. Table 1 shows the components of PRC1 and PRC2 among various species.

In Arabidopsis, there are five p55-like genes, *MSI1,2,3,4,5*, but only three were found in *O. sativa*, namely, *Os_MSI1* (LOC_Os03g43890) since it was most similar to *At_MSI1* and, likewise, *Os_MSI3* (LOC_Os09g36900) and *Os_MSI4* (LOC_Os01g51300).

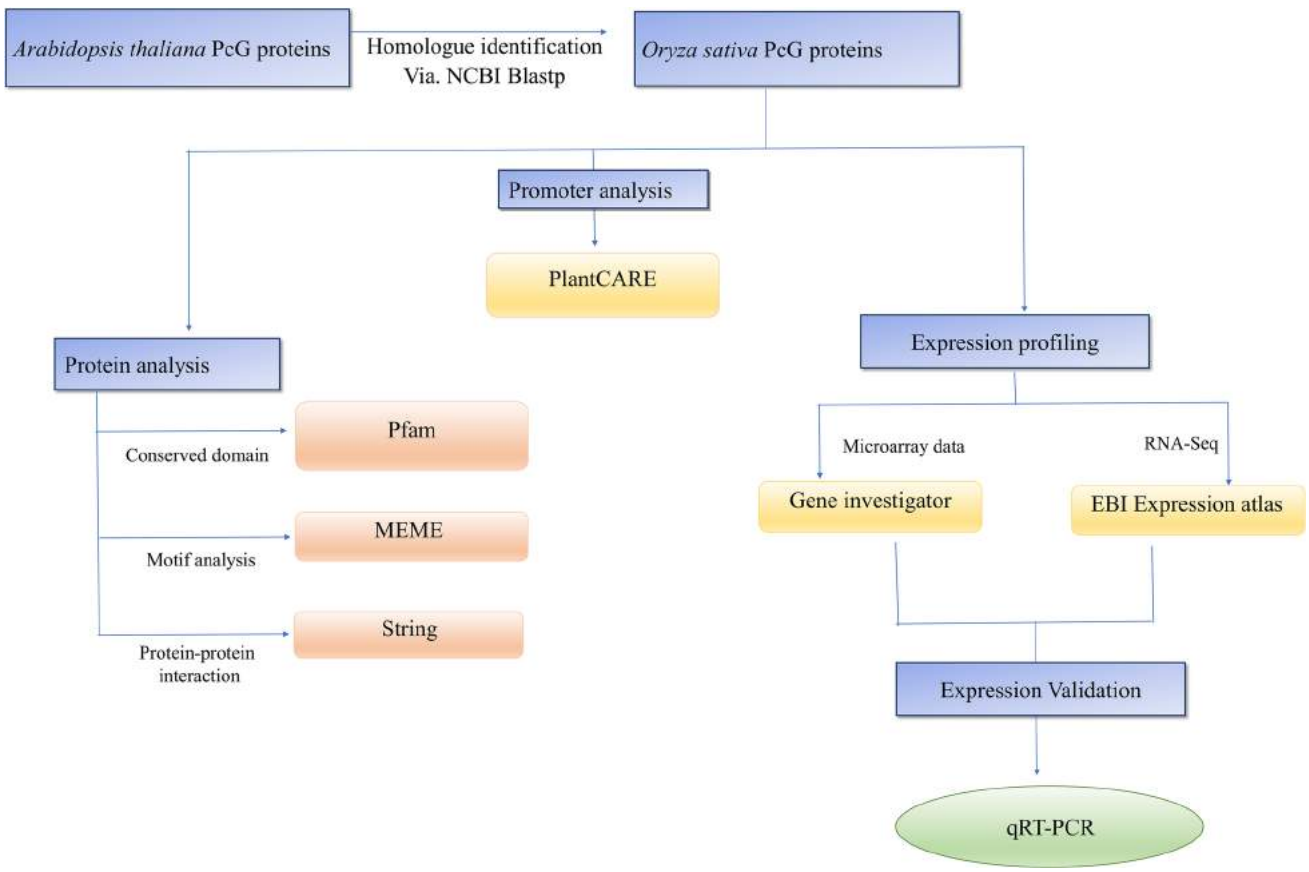

Fig. 1 Outline of methodology

Table 1 Components of PRC1 and PRC2 in *Drosophila melanogaster* and their homologs in *Arabidopsis thaliana* and *Oryza sativa*

	Drosophila melanogaster	*Arabidopsis thaliana*	*Oryza sativa*
PRC1	Pc (Polycomb)	At_LHP	Os_LHP
	Psc (Posterior sex combs)	At_BMI1A, At_BMI1B, At_BMI1C	Os_BMI1A, Os_BMI1B
	Sce (Sex combs extra)/RING	At_RING1A, At_RING1B	Os_RING1A.1, Os_RING1A.2
	Ph (Polyhomeotic)	-	-
PRC2	E(z) (Enhancer of zeste)	At_CLF, At_SWN, At_MEA	Os_CLF, Os_SWN
	Su(z) (Suppressor of zeste)	At_EMF2, At_VRN2, At_FIS2	Os_EMF2A, Os_EMF2B
	Esc (Extra sex combs)	At_FIE	Os_FIE1, Os_FIE2
	p55	At_MSI1,2,3,4,5	Os_MSI1, Os_MSI3, Os_MSI4

Table 2 provides the detailed information about all the 14 members of this family giving the basic information on all PcG genes (including gene name, locus ID, splice forms, nucleotide length, polypeptide length, CDS coordinates, chromosome number, MW, p*I*, and GRAVY). The results showed that the nucleotide length of the *O. sativa* PcG genes ranges from 1131 bp (*Os_FIE1*, encoding 376 amino acids) to 2691 bp (*Os_CLF*, encoding 896 amino acids), while the MW of PcG proteins varied from 42 kDa (Os_FIE) to 100.3 kDa (Os_CLF), the p*I* ranged from 4.77 (Os_MSI1) to 9.37 (Os_BMI1B), and all of the PcG proteins were hydrophilic.

The secondary structure analysis (Table S1) of *O. sativa* PcG proteins showed that the α-helices range from 11.7% (Os_FIE1) to 40.57% (Os_RING1A.2), the extended strand varies from 6.35% (Os_RING1A.2) to 34.57% (Os_FIE1), the β-turn spans from 1.82% (Os_BMI1B) to 10.37% (Os_FIE1), and the random coil ranges from 43.35% (Os_FIE1) to 61.05% (Os_BMI1B). Concurrently, the subcellular location analysis of PcG proteins indicated that all of them were located in the nucleus. Furthermore, the tertiary structures of PcG proteins were also built. Predicted models of PRC2 members were based on the reported templates c4xyhA, c7ktpC, c2cklA, c2rsnA, c3cfvA, c5ch2B, c3dm0A, c5wjcA, and c3h8hA, and those of PRC1 members were based on c3h8hA, c5ch2B, c2h0dB, c5a1vK, and c7ktpC to maximize the alignment coverage and confidence score (Fig. S1).

Gene structure analysis and chromosome location of PcG gene family

The exon–intron structure of PRC1 members indicated that the number of exons of all members ranged from 5 to 10. Both of the *Os_RING* members exhibited a similar intron–exon structure with 10 exons each, *Os_BMI* members contain 7 exons each, and *Os_LHP* carries the least number of exons (Fig. 2a). The motif prediction of PRC1 members showed that motif 7 had the most occurrence and is present in all the PRC1 members while motif 9 has the least occurrence present only in *At_BMI1A* (Figs. 2c, S2a). Motif 1 was present in all homologs of RING and Psc, and apart from

Table 2 Characteristics of identified PcG genes

Gene	Locus ID	Splice form	CDS (bp)	Protein (aa)	Coordinates 5′–3′	Chr	MW (kDa)	p*I*	GRAVY
Os_CLF	LOC_Os06g16390	1	2691	896	9353713–9362101	4	100.3	8.02	−0.747
Os_SWN	LOC_Os03g19480	1	2688	895	10959835–10952133	3	99.9	8	−0.704
Os_EMF2A	LOC_Os04g08034	6	1875	624	4274885–4290697	4	71.5	5.87	−0.502
Os_EMF2B	LOC_Os09g13630	1	1815	604	7938161–7925560	9	68.6	6.52	−0.434
Os_FIE1	LOC_Os08g04270	3	1131	376	2077234–2083327	8	42	5.68	−0.107
Os_FIE2	LOC_Os08g04290	1	1401	466	2095644–2100604	8	51.8	7.55	−0.295
Os_MSI1	LOC_Os03g43890	1	1287	428	24629504–24621292	3	48.4	4.77	−0.49
Os_MSI3	LOC_Os09g36900	1	1233	410	21276447–21280620	9	44.7	4.98	−0.363
Os_MSI4	LOC_Os01g51300	2	1389	462	29501122–29506955	1	51.1	5.79	−0.505
Os_RING1A.1	LOC_Os01g58400	2	1503	500	33745858–33740650	1	54.9	8.37	−0.719
Os_RING1A.2	LOC_Os05g41795	1	1467	488	24473136–24478871	5	53.6	5.5	−0.867
Os_BMI1A	LOC_Os03g43360	1	1533	510	24180460–24174317	3	56.2	8.86	−0.856
Os_BMI1B	LOC_Os03g53080	1	1320	439	30445269–30440115	3	48.6	9.37	−0.726
Os_LHP	LOC_Os10g17770	1	1248	415	8992294–8997104	10	45.7	4.89	−0.954

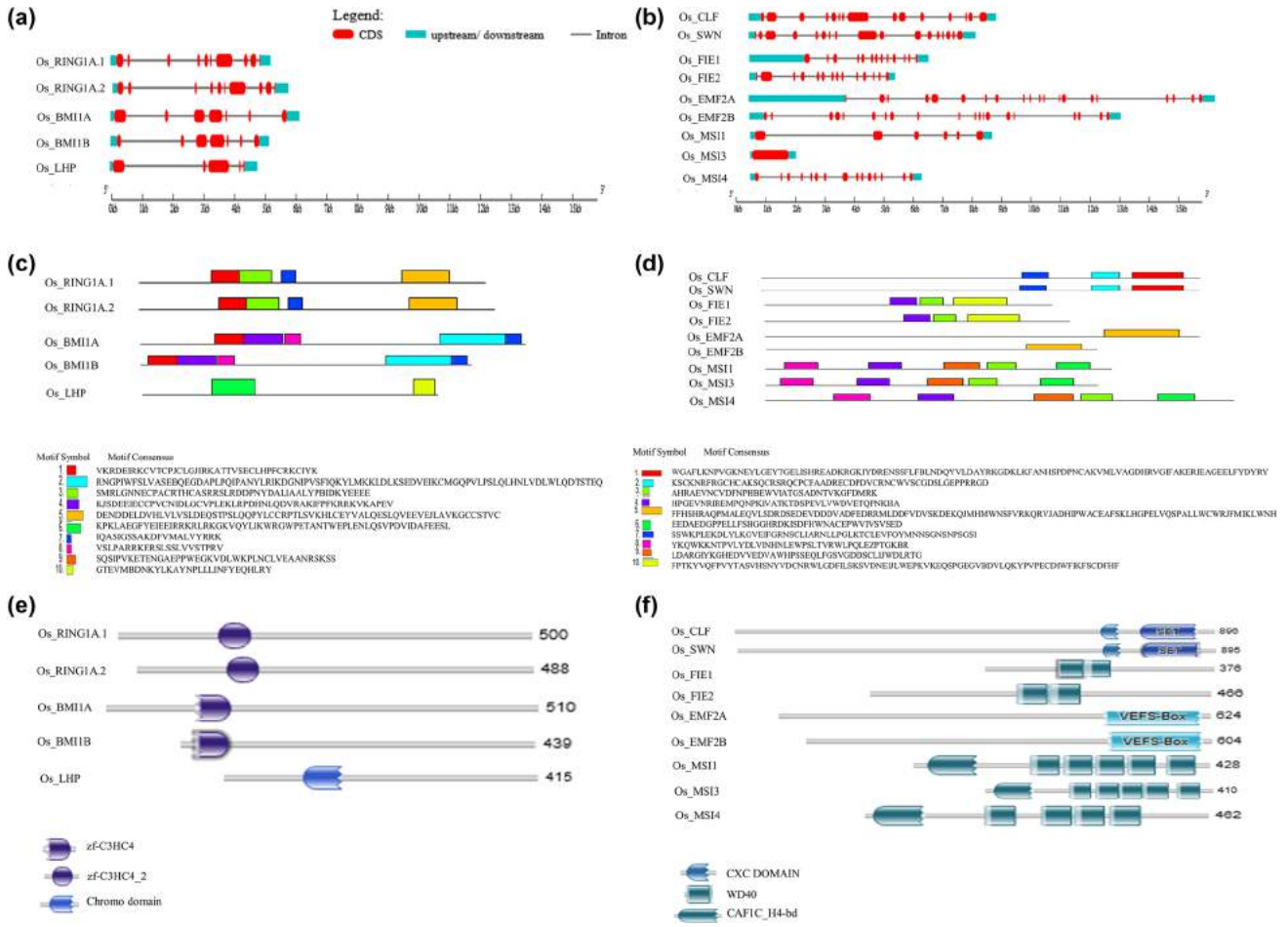

Fig. 2 Gene architecture and conserved domains of PRC1 and PRC2 complex members. Exon/intron structure of **a** PRC1 complex, **b** PRC2 complex. Distribution of conserved motif in **c** PRC1 complex, **d** PRC2 complex and conserved domain organization of **e** PRC1 and **f** PRC2 complex members in *Oryza sativa*

these motifs, RING homologs consist of motifs 3 and 5; motifs 2, 4, and 8 were present in homologs of Psc while Pc homologs contain 6 and 10. The conserved domain analysis of the PRC1 members revealed that the type of domains varied between different components. RING component contains zf-C3CH4_2 domain and BMI component contains zf-C3CH4 domain whereas LHP contains Chromo-domain (Fig. 2e).

The exon–intron structure of PRC2 members (Fig. 2b) indicated that the number of exons of all members ranged from 1 to 20. EMF members carried the highest number of exons and *Os_MSI3* being intron-less carries the least number of exons.

In PRC2 members, Su(z) homologs contain a single motif whereas all other components of PRC2 contained more than two motifs (Figs. 2d, S2b). Motifs 3 and 4 had the highest occurrence present in all p55 and Esc homologs, and the rest of all the motifs have similar occurrence rate—motifs 1, 2, and 7 were present only in homologs of E(z), motif 5 was present only in Su(z) homologs, motif 10 is unique to Esc homologs, and p55 homologs contain motifs 6, 8, and 9.

The conserved domain analysis of the PRC2 members revealed that the type of domains was more diverse than the PRC1 members and that the type of domains within each component was similar (Fig. 2f). Among them, the SWN/CLF contained SET and preSET-CXC functional domains, while the EMF2 component only possessed the VEFS-Box domain. The FIE component contained the WD40 domain whereas the MSI contained CAF1C_H4-bd along with WD40 domains.

PcG genes were mapped on the 12 different chromosomes of *O. sativa* and all of the PcG members were distributed unevenly over eight chromosomes (Fig. 3). Chromosome 3 contained the highest number of PcG members (4 genes), while chromosome 4, 5, 6, and 10 contained single gene each. Simultaneously, chromosomes 1, 8, and 9 harbor two genes each. No correlation was found between the length

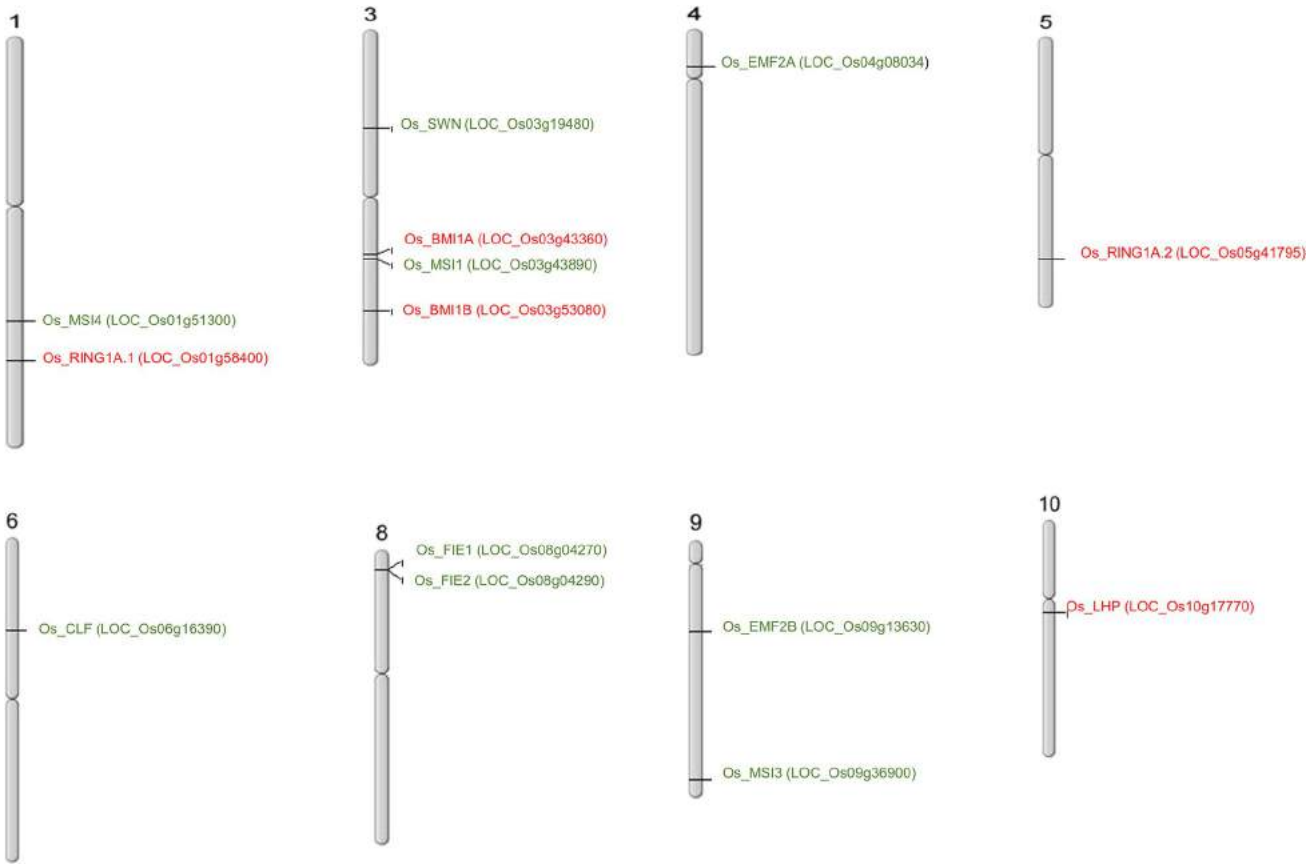

Fig. 3 Chromosomal distribution of PcG genes in *Oryza sativa*. Chromosome numbers are shown at the top of each chromosome. The names of each PcG gene are shown on the right side of each chromosome with positions in bracket. Red fonts represent PRC1 members and green fonts represent PRC2 members

of the chromosome and the number of PcG members it contained.

Protein–protein interaction analysis of PcG family

Protein–protein interaction data was analyzed for both PRC1 and PRC2 (Fig. 4) which showed interaction with other stress-responsive proteins. Os_RING1A.1 and \Os_RING1A.2 were found to be interacting with C3HC4 zinc finger proteins; similarly, Os_LHP showed interaction with the component of chromatin assembly factor complex (CAF-1). Os_MSI3 and Os_MSI4 were shown to interact with proliferating cell nuclear antigen (PCNA) and histone deacetylase, respectively. Majority of the PRC2 complex members were found to be interacting with C2H2 zinc finger proteins (Supplementary file 1). Apart from other proteins, PRC1 and PRC2 complex members showed interaction among themselves also. Compared to *O. sativa*, Arabidopsis PcG proteins show more intensive interaction within the complex. At_BMI1A and At_BMI1B were found to be interacting with DREB2A (Supplementary file 1).

Identification of putative cis-acting regulatory elements (CAREs) present in the upstream region of O. sativa PcG genes

The analysis of *cis*-acting regulatory elements revealed the presence of various plant hormone- and stress-responsive elements in the upstream region of PcG genes' promoters (Fig. 5). Fourteen hormone-responsive elements were found like ABA-responsive-ABRE (found in all the genes except *Os_SWN* and *Os_EMF2B*), ABRE2 (present only in *Os_CLF*), ABRE3a and ABRE4 (present in *Os_BMI1A*, *Os_RING1A.2*, *Os_CLF*, *Os_EMF2A*, *Os_FIE1*, and *Os_MSI1*), CARE (only in *Os_BMI1A* and *Os_MSI1*), auxin-responsive-AUXRR-core (found only in *Os_CLF* and *Os_FIE1*), and TGA-element (found in half of the genes).

Some other hormone-responsive elements are as follows: ethylene-responsive-ERE (present in *Os_RING1A.1*, *Os_RING1A.2*, *Os_LHP*, *Os_CLF*, *Os_FIE2*, and *Os_MSI1*), gibberellin-responsive-GARE (only in *Os_MSI1*, *Os_MSI3*, and *Os_MSI4*), P-box (present in *Os_BMI1B*, *Os_RING1A.1*, and *Os_RING1A.2*), TATC-box (found only in *Os_BMI1A* and *Os_EMF2B*), salicylic acid-responsive-TCA-element

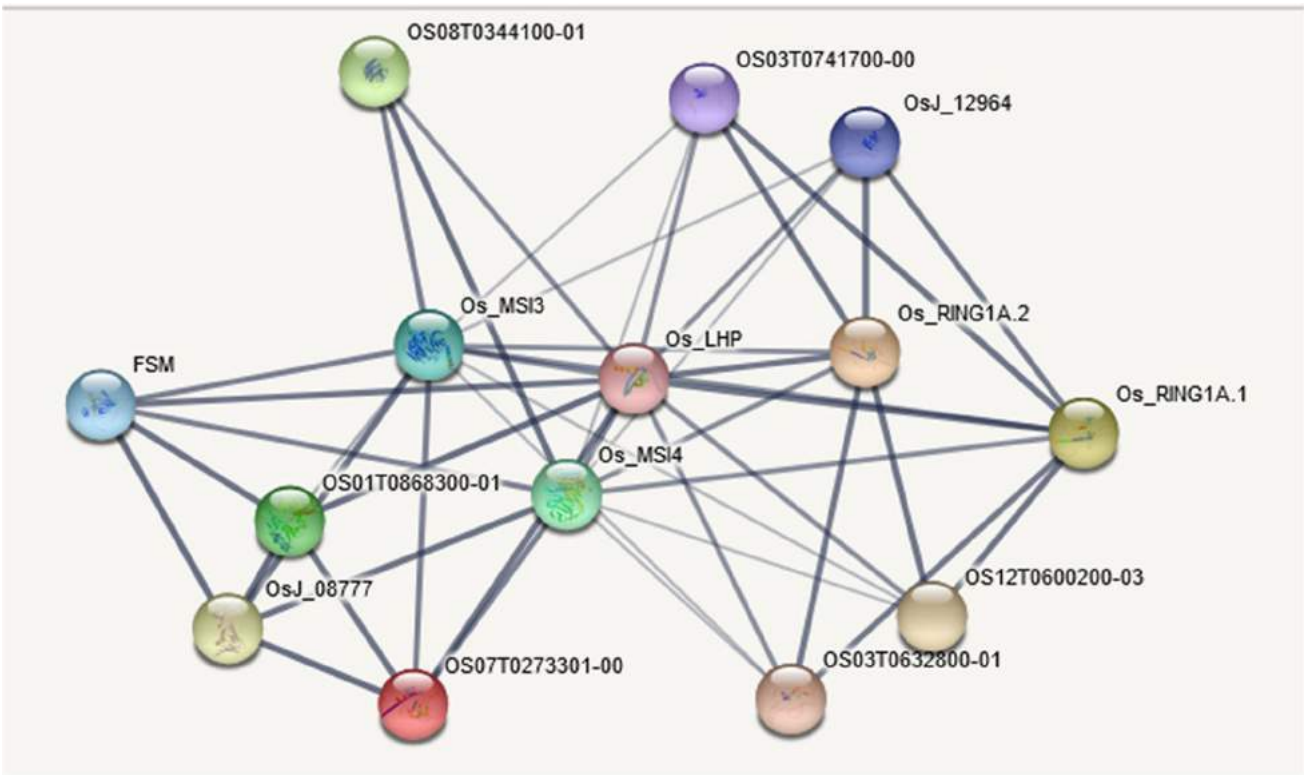

Fig. 4 Protein–protein interaction among PcG members in *Oryza sativa*. The interaction network was predicted through STRING and thickness of line indicates the strength of data support

(present in *Os_BMI1B*, *Os_EMF2A*, *Os_EMF2B*, and *Os_MSI1*) and methyl jasmonate-responsive element-CGTCA-motif (found in all the genes except *Os_BMI1B* and *Os_FIE2*), and TGACG-motif (found in all the genes except *Os_BMI1B*).

Regarding defense and stress-related elements, the following elements were found-water-responsive MYB (present in all the genes), drought-responsive-as-1 (found in all the genes except *Os_BMI1B*, *Os_RING1A.1*, and *Os_MSI4*), DRE core (in *Os_BMI1A*, *Os_CLF*, *Os_EMF2A*, *Os_EMF2B*, *Os_FIE1*, and *Os_MSI3*), DRE1 (present only in *Os_SWN*), and MBS (present in all PRC1 members, *Os_EMF2B*, *Os_FIE1*, *Os_FIE2*, and *Os_MSI1*).

Various other stress elements were also found like MYB recognition site (found in all except *Os_RING1A.1*, *Os_LHP*, *Os_SWN*, *Os_EMF2B*, *Os_MSI1*, and *Os_MSI4*), MYC (present in all except *Os_MSI4*), cold-responsive-LTR, heat, osmotic stress, low pH, and nutrient starvation stresses responsive-STRE (present in all except *Os_RING1A.2* and *Os_FIE2*).

Other elements are found related to anoxic responsive-ARE (present in all the genes), GC-motif (found in *Os_BMI1B*, *Os_RING1A.2*, *Os_CLF*, *Os_EMF2A*, *Os_FIE1*, and *Os_MSI3*), defense responsive TC-rich repeats (present only in *Os_FIE2*), wounding and pathogen responsive-W box (found in all except *Os_RING1A.1*, *Os_RING1A.2*, *Os_CLF*, *Os_SWN*, *Os_FIE2*, and *Os_MSI3*), WRE3 (found in all except *Os_BMI1B*, *Os_RING1A.1*, *Os_RING1A.2*, *Os_LHP*, *Os*_MSI1, and *Os_MSI3*), WUN-motif (present only in *Os_RING1A.2*) along with the presence of other core promoter elements, light-responsive elements, and circadian responsive elements. The most abundant element was the ABA-responsive ABRE element followed by water-responsive MYB and the least occurring elements were DRE1, WUN, and TC-rich repeats. The promoter of *Os_FIE1* contains the highest number of elements and the least number of elements were found in the promoter of *Os_FIE2*.

PcG gene expression in various developmental stages and anatomical tissues

To investigate about the role of *O. sativa* PcG genes, the expression pattern at different developmental stages and in different anatomical tissues (Fig. 6) was analyzed. Most of the genes had lower expression at the initial stages of development (Fig. 6a). *Os_CLF*, *Os_MSI3*, and *Os_MSI4* showed high expression compared with other genes at the germination stage indicating that these genes might have a role in seed dormancy. *Os_FIE2* had low expression at all stages except the milk stage. Most of the genes were showing a

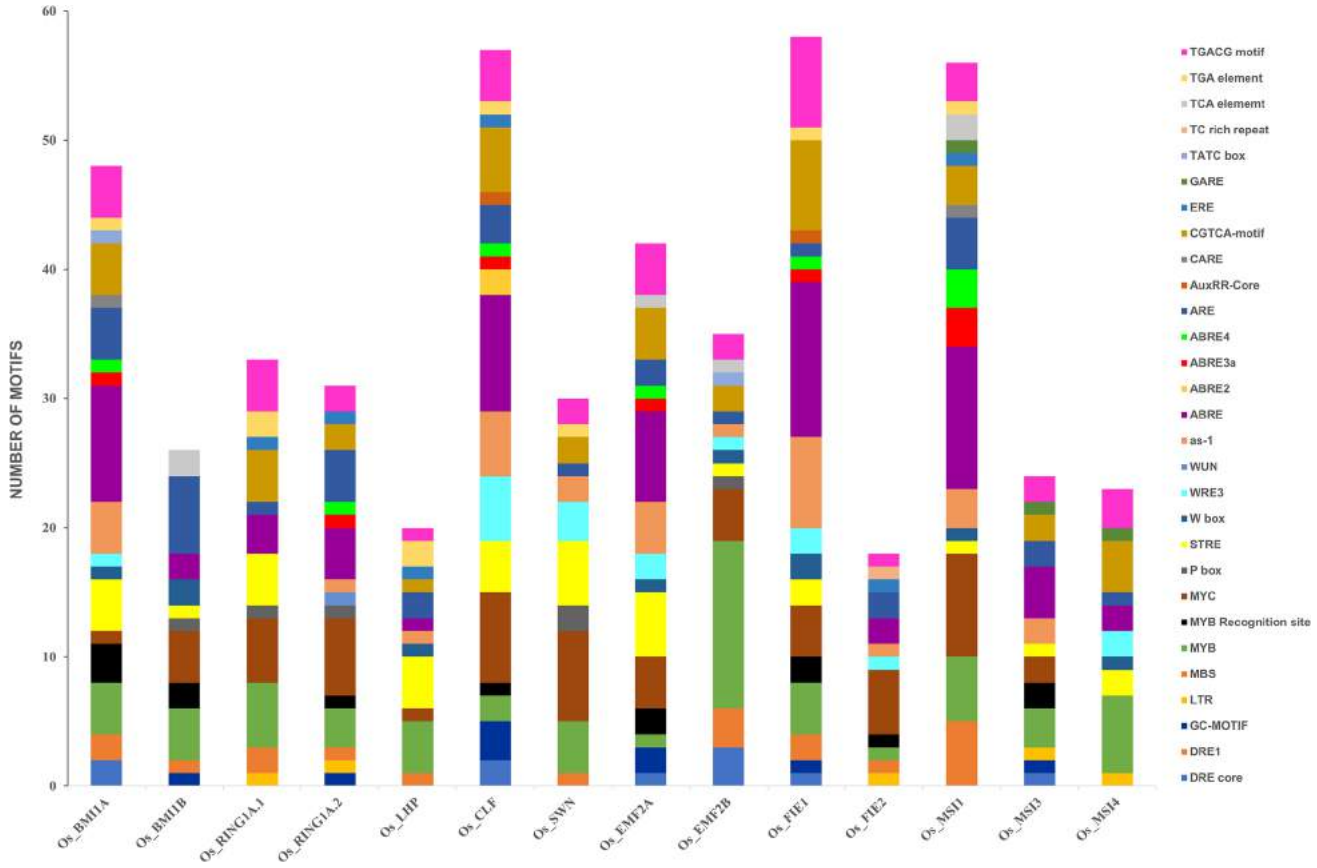

Fig. 5 Analysis of *cis*-acting regulatory elements in the putative promoter regions of PcG genes in *Oryza sativa*. The 2-kb 5′ upstream region of all the PcG genes were analyzed to check the presence of stress and hormone-responsive *cis*-acting regulatory elements. The identified elements were plotted against each gene. Different elements are represented with different colors

moderate level of expression at the stem elongation stage depicting the role of Polycomb complex in controlling plant height. *Os_BMI1B* showed a high level of expression at the reproductive phase than the vegetative phase. *Os_EMF2B* had the highest expression in the flowering stage contrary to other stages.

In case of anatomical tissues, PcG showed a great variation in their expression pattern (Fig. 6b). *Os_FIE2* had the highest expression level in endosperm out of all the tissues suggesting its role in seed development in rice. *Os_CLF* and *Os_FIE1* had the highest expression in male gamete cells. *Os_CLF*, *Os_MSI4*, and *Os_MSI3* also showed relatively high expression in the root tip, and compared to other genes, *Os_EMF2B* showed the highest expression in pollen.

Expression pattern analysis of PcG genes under different stress conditions

A number of stress-responsive CAREs were present in the promoter region of PcG genes; therefore, to gain more insight into its role in response to abiotic stress, the expression pattern of PcG genes under different abiotic stress conditions was analyzed.

Both microarray and RNA-seq data were analyzed to look upon PcG genes' role and the genes were found to be differentially regulated in response to different abiotic stresses. Along with *O. sativa* PcG genes, the expression pattern of Arabidopsis PcG was also analyzed using available microarray data.

Microarray data analysis of Arabidopsis PcG genes in root tissue (Fig. 7a) showed that expression of *At_MSI* and *At_FIE* genes was getting downregulated at the late time points such as 6 h, 12 h, and 24 h under salinity and osmotic and oxidative stress conditions while it was getting upregulated in cold condition and remains unchanged during drought conditions.

PRC1 complex members showed an increase in expression level at 1-h time point under drought and oxidative stress conditions; in cold conditions, 50% of them were getting downregulated at 0.5 h and expression of *At_RING1A* was getting decreased at 6-h, 12-h, and 24-h time points. Under salinity and osmotic stress conditions, they did not show much change.

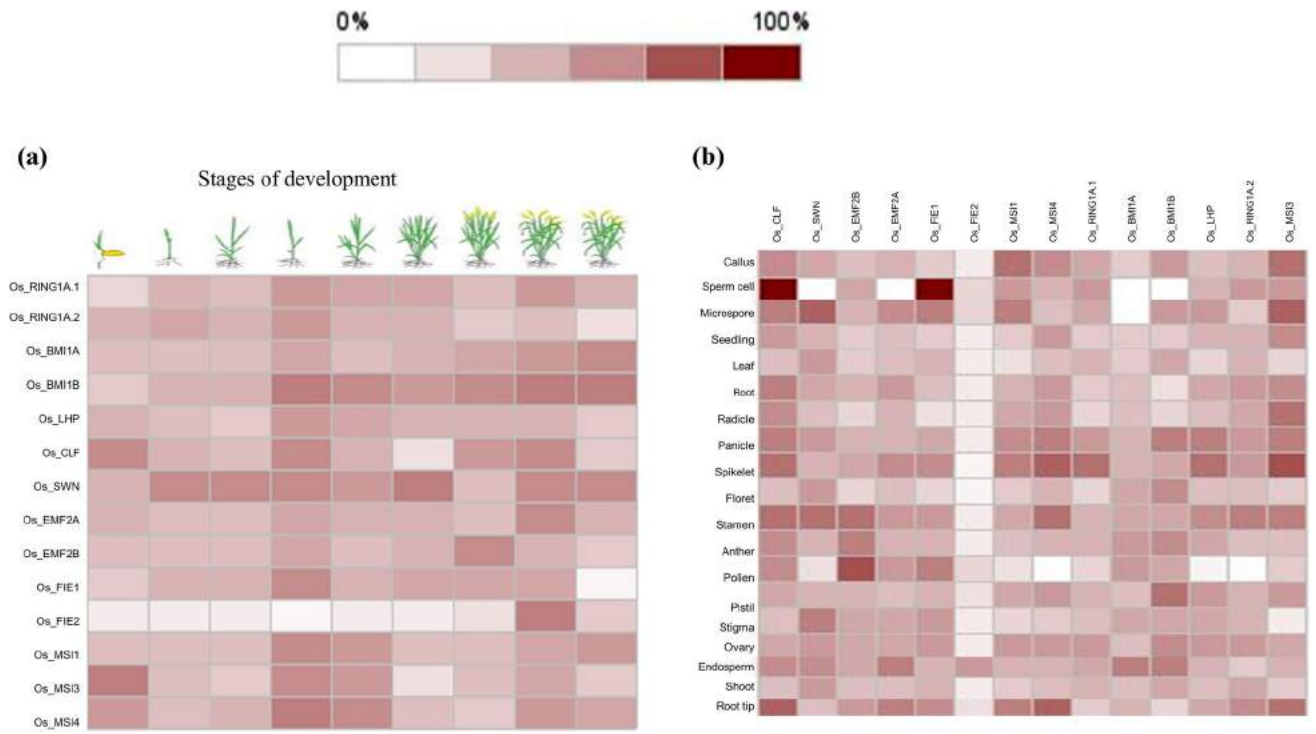

Fig. 6 Heatmap showing expression analysis of PcG genes in different developmental stages and tissues of *Oryza sativa*. Microarray data depicting expression of PcG genes in (a) different developmental stages. Stages of development shown in the picture are germination, seedling; tillering; stem elongation, booting, heading, flowering, milk stage, dough stage. (b) Different tissues. Expression analysis was carried out with RNA-seq datasets using Genevestigator

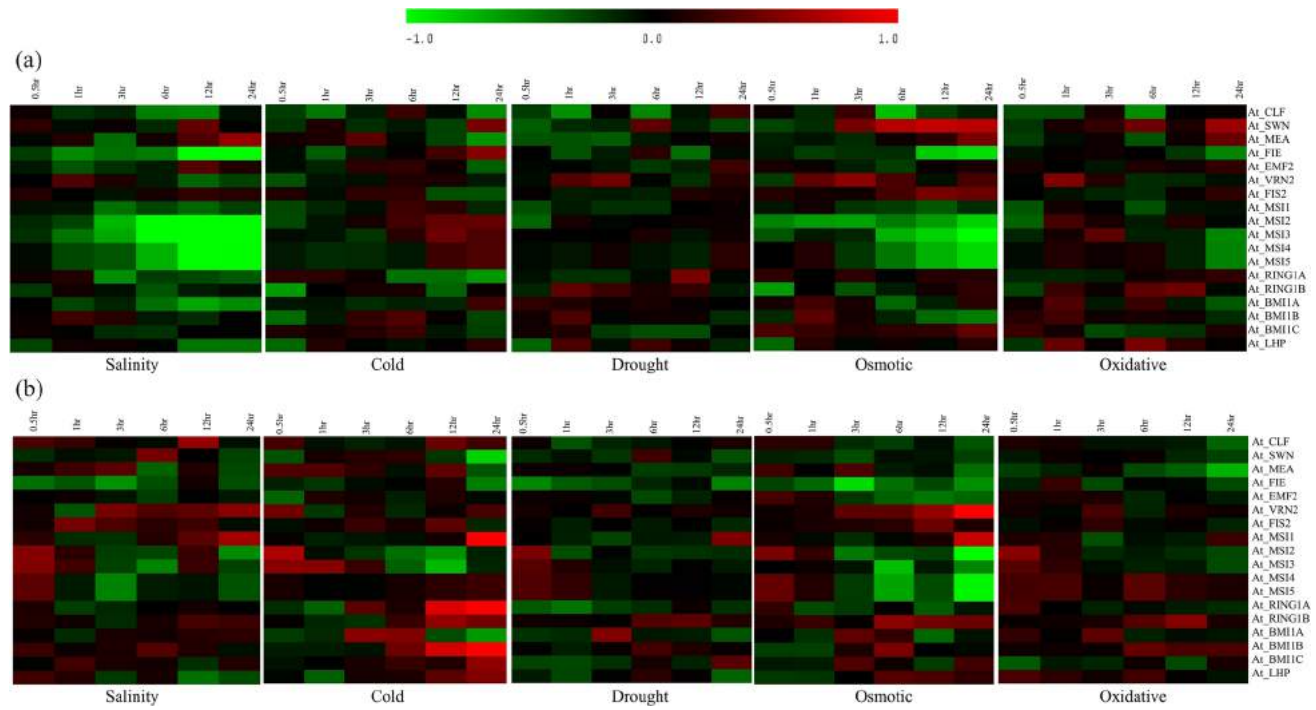

Fig. 7 Heatmap analysis of PcG genes from 16-day-old *Arabidopsis thaliana* seedlings. Microarray data depicting expression of PcG genes under various abiotic stress in (a) root and (b) shoot tissue at different time points such as 0.5, 1, 3, 6, 12, and 24 h with respect to control. The color scale represents Log2 of average signal values. Expression analysis was carried out with microarray datasets using Genevestigator

In shoot tissue (Fig. 7b), PcG genes were showing massive variation in their expression pattern under cold stress conditions. Expression of PRC1 complex members showed an increase at 12-h and 24-h time points except for *At_BMI1A* which showed downregulation. The PRC2 complex members had almost the same kind of expression throughout except for *At_MSI2*, *At_MSI3*, and *At_CLF* which were showing downregulation at 12-h and 24-h time points, respectively, whereas expression of *At_MSI2* and *At_MSI3* was showing an increase at 0.5-h and 1-h time point. The mRNA transcript level of *At_MSI3* and *At_MSI1* showed a rise at 24-h time point.

In oxidative stress condition (Fig. 7b), most of the PRC1 complex members were getting a slight increase in their expression pattern from 3 to 12 h. In osmotic stress conditions (Fig. 7b), most of the genes had varied expression patterns at later time points compared to early time points. Expression level of most of the PcG genes was decreasing under the drought stress conditions (Fig. 7b) throughout 0.5-h to 24-h time points except in a few cases such as *At_MSI2,3,4,* and *5* which showed upregulation at 0.5 h. *At_MSI1* expression levels showed increase at 24 h, and at 3 h, mRNA transcript levels of *At_BMI1A* were increased.

Microarray data analysis of rice under abiotic stress conditions (Fig. 8a) showed that four out of five PRC1 complex members upregulates under drought conditions. *Os_RING1A.2* and *Os_BMI1B* also showed upregulation in cold stress whereas the other three genes did not show much difference in their expression pattern during cold stress. In salinity stress, apart from *Os_LHP*, the other four PRC1 members were showing little increase in their expression pattern.

In PRC2 complex, *Os_MSI3*, *Os_MSI4*, and *Os_EMF2A* were getting downregulated during both salinity and drought stress conditions, and cold stress reduced the *Os_FIE1* expression levels. The expression pattern of PcG genes upon treatment with hormones and in the case of metal stress was also analyzed (Fig. 8b). *Os_MSI3* was downregulated more than twofold upon the external application of ABA and more than onefold upon application of JA and salicylic acid, and it also showed a decrease in the presence of heavy metal such as Cr (VI) with more than threefold. Expression of *Os_RING1A.1* and *Os_BMI1B* was increased by more than twofold in the presence of Cr (VI).

RNA-seq data analysis of IR-64 in salinity stress condition (Fig. 8c) revealed that levels of *Os_EMF2B*, *Os_RING1A.1*, and *Os_FIE2* were decreased both in root and leaf tissue whereas *Os_RING1A.2* and *Os_BMI1B* were getting reduced only in leaf tissue. *Os_BMI1A* was getting downregulated in the leaf, whereas in the root, it was getting upregulated. The expression of *Os_EMF2A*, *Os_EMF2B* in leaf was decreasing more after the application of ABA along with salinity stress condition compared to salinity stress alone.

In Nipponbare species, during salinity stress condition (Fig. 8d), expression of *Os_SWN* and *Os_EMF2B* showed a decrease at the time point of 1 h whereas *Os_FIE* was getting increased. *Os_MSI1*, *Os_MSI3* and *Os_MSI4*, *Os_CLF* levels were getting upregulated at the time point of 5 h. At the 24-h time point *Os_LHP*, *Os_BMI1A*, and *Os_BMI1B*, *Os_FIE1* showed upregulation whereas *Os_RING1A.2* and *Os_SWN* showed a decrease in expression level. Expression of most of the genes was changed at the time point of 5 h and 24 h making them late responsive genes.

Expression profiling of PcG gene family using qRT-PCR

PcG genes were screened for qRT-PCR analysis to validate the microarray and RNA-seq data under drought and salinity stress conditions in both root and shoot tissues.

In shoot tissue (Fig. 9a), most of the genes were getting upregulated in drought and salinity stress conditions at the time point of 1 h except a few genes such as *Os_FIE2*, *Os_MSI1* (downregulated in both the stress conditions) and *Os_CLF*, *Os_MSI4* (getting decreased in 1-h salinity stress condition). *Os_FIE1* showed the highest level of transcript with approximately fourfold increase in both salinity and drought stress conditions followed by *Os_RING1A.1* which showed around fourfold increase in its expression under salinity stress condition and *Os_RING1A.2* showed around fourfold increase in its expression under drought stress condition.

At 24-h time point, expression of *Os_BMI1A*, *Os_BMI1B*, and *Os_SWN* was not observed and therefore not included in the expression analysis. At 24 h, the expression level of most of the genes were either getting decreased or was same as the control under both the stress conditions except *Os_FIE2* (upregulated under drought stress condition). The mRNA transcript levels of *Os_FIE1* were decreased fivefold under salinity stress condition and about fourfold in drought condition.

In root tissue (Fig. 9b), *Os_BMI1A* and *Os_BMI1B* did not show any expression at a particular growth condition and hence not included in expression analysis. At 1-h time point, salinity induced the expression level of *Os_CLF*, *Os_SWN* by fourfold and 1.7-fold, respectively; however, expression level of all the other genes was decreased. *Os_FIE2* showed about sixfold downregulation under 1-h drought stress condition. At 24-h time point, under salinity stress condition, *Os_FIE2* showed the abundance in its transcript level (≈sevenfold) along with other PRC1 members. The expression data shows that transcript level of *Os_EMF2A* decreased fivefold, whereas the transcript levels of most of the other genes showed an increase in level under 24-h drought stress

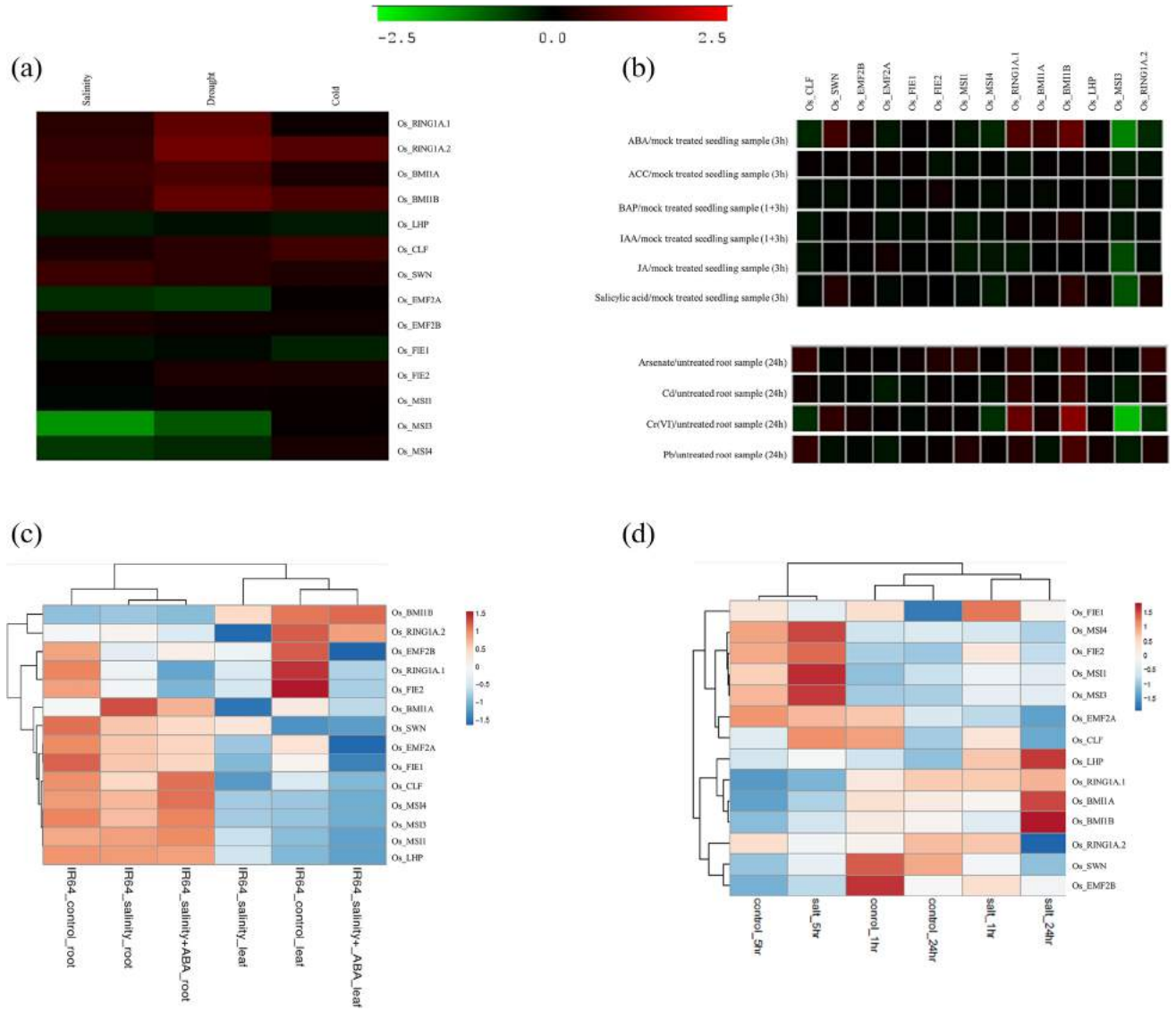

Fig. 8 Heatmap showing the stress-related expression of PcG genes in *Oryza sativa*. (a) Expression of PcG genes in IR64 seedling under various abiotic stresses such as salinity, drought, and cold. (b) Expression analysis in the presence of various hormones and heavy metals. Expression analysis was carried out with microarray datasets using Genevestigator. (c) Expression pattern of PcG genes in IR64 under salinity stress in leaf and root tissue. (d) Expression pattern of PcG genes in Nipponbare under salinity stress at different time points—1, 5, and 24 h. Expression analysis was carried out with RNA-seq datasets using Rice Expression Database. The color scale represents Log2 of average signal values

condition. *Os_EMF2B*, *Os_RING1A.1*, and *Os_FIE2* under 1-h salinity stress condition show the same expression pattern as seen in RNA-seq data.

Discussion

Abiotic stress is one of the major reasons for the yield loss in crop plants throughout the world. The continuously changing climate will impose more stress on the plant species. In eukaryotic cells, the gene activity is controlled by the epigenetic marks along with the DNA sequence (Luo et al. 2012) and thus provides a powerful means to regulate the plants' response against abiotic stress conditions. PcG, being one of the epigenetic regulatory mechanisms, could be an important player in the regulation of plant's behavior in such abiotic stress conditions, but very little information is known about its role regarding abiotic stress. In our study, 14 PcG genes in *O. sativa* have been identified based on the sequence similarity and conserved domains. We did not find any homolog of *At_MEA*, *At_VRN2*, and *At_FIS2* in *O. sativa* genome. The protein sequences of 14 PcG members

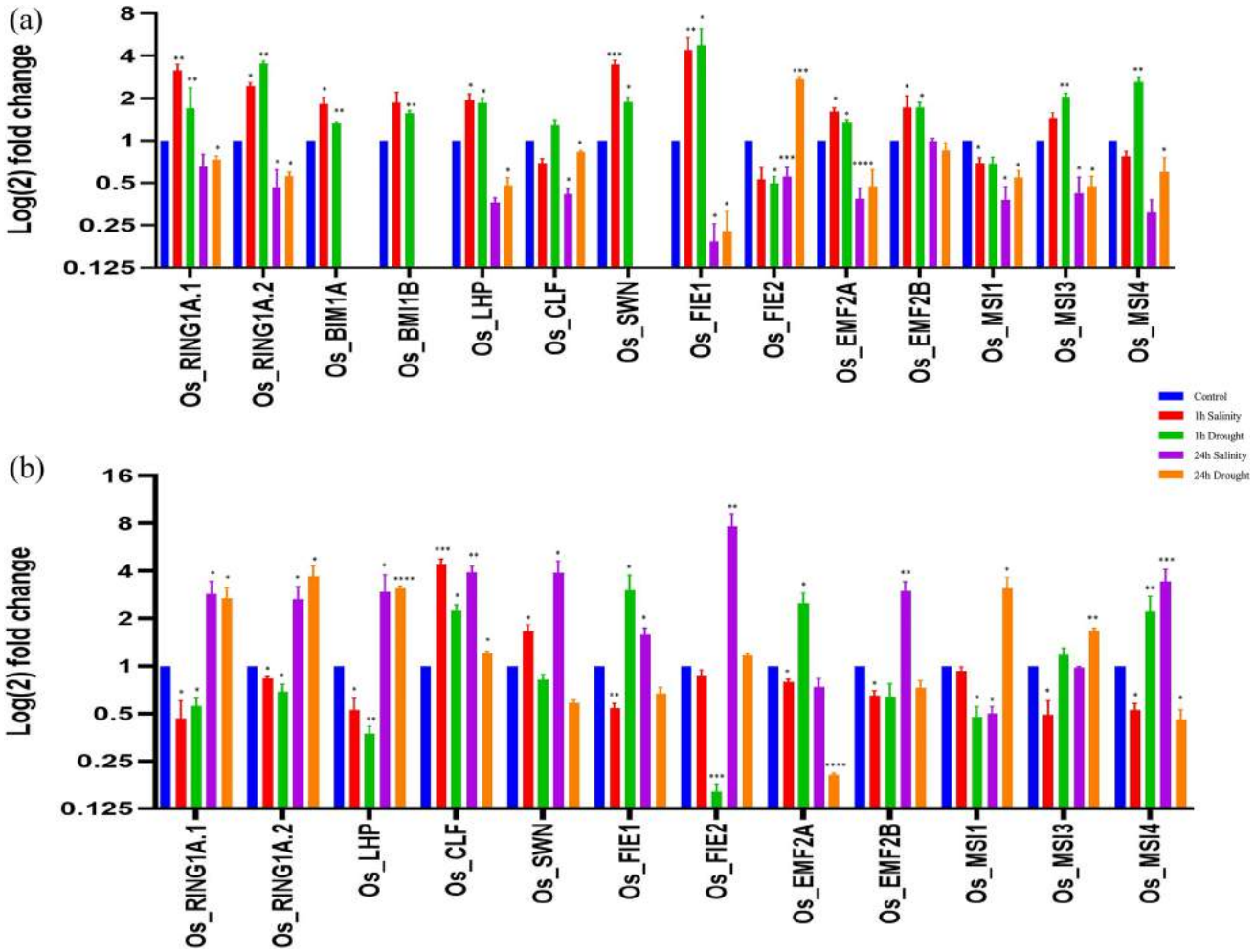

Fig. 9 Relative expression analysis of PcG genes under abiotic stress conditions. qRT-PCR was used to analyze the expression patterns of the Polycomb genes in (a) shoot and (b) root tissues of rice seedlings under high saline (200 mM NaCl) and drought conditions for 1 and 24 h. The data were normalized to the rice eIF4a expression level. Asterisks indicate the stress groups that showed a significant difference in mRNA abundance compared with the control group: $*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$

had an average length of 536 amino acids with an average molecular weight of 59 kDa. Furthermore, investigating about the localization of PcG genes, we discovered that they are all found in the nucleus, which is consistent with earlier research on Arabidopsis PcG genes (Calonje et al. 2008; Gendall et al. 2001; Li et al. 2011; Schatlowski et al. 2010; Xu and Shen 2008). Chromosomal location analysis showed that all of the PcG members were unevenly distributed and even the members of the same component were present on different chromosomes. A similar pattern of uneven PcG genes' distribution was reported in other species too (Aubert et al. 2001, p. 1; Luo et al. 1999; Strejčková et al. 2020; Zhao et al. 2021).

The protein motif and gene structures of PcG family showed highly conservative patterns. Ten conserved motifs were acquired for both the PRC1 and PRC2 complex each, and we found that in PRC1, nine motifs out of ten were present both in *O. sativa* and Arabidopsis sequences (Figs. 2, S2a). Only one motif (motif 9) was different which was present only in At_BMI1A. In PRC2, all ten motifs were present in protein sequences of both species. All of the conserved domains of PcG genes found in Arabidopsis earlier by various researchers (Ach et al. 1997; Chen et al. 2009; Goodrich et al. 1997; Huang et al. 2019) were also found to be present in *O. sativa* PcG protein sequences.

Being a complex, PcG proteins showed interaction among themselves apart from the other proteins. Majority of the PcG proteins were found to be interacting with C2H2, C3HC4 zinc finger proteins, and previously, these zinc finger proteins were shown to be regulating various abiotic stress and hormone responses by directly targeting the downstream ion balance–related genes, antioxidant genes associated with ROS scavenging, and genes involved in hormone signal transduction (Agarwal and Khurana 2020; Han et al. 2020, p.

2; Jung et al. 2013). They also showed interaction with other histone modification enzymes such as histone deacetylase (LOC_Os08g25570.1), and this post-translational modification helps plants survive the unfavorable conditions by regulating the expression of stress-responsive genes (Yuan et al. 2013). Simultaneously, in Arabidopsis, it generated a complex interaction network. PcG protein showed interaction with the proteins within the same complex such as FIE interacting with CLF (Katz et al. 2004), FIE interacting with MEA (Luo et al. 2000), and BMI interacting with either RING family members or LHP (Chen et al. 2010). Apart from interacting within the same complex, members from different complexes also reacts with each other such as CLF of PRC2 complex interacting with RING1A and RING1B of PRC1 complex (Xu and Shen 2008), and MSI1 from PRC2 complex interacting with LHP from PRC2 complex (Derkacheva et al. 2013). These hypothetical interactions indicate the probable mechanism of the PcG family proteins in various stress conditions. In the future, we might validate these interactions experimentally. These interactions may pave the way to characterize individual PcG member and understand their role in the plant system further.

In our study, we showed that all of the PcG genes' promoter contains various types of *cis*-regulatory elements that are either stress responsive or hormone responsive (Fig. 5), indicating that these genes might respond to various environmental factors such as drought and low temperature along with various plant hormones such as ABA and ethylene, and thus possibly regulate the abiotic and biotic stress responses in plants.

The RNA-seq dataset analysis of PcG genes in various developmental stages and tissues showed that PcG genes had a varied expression in all stages and tissues. *Os_FIE2* expression was found to be highest at milk stage of development—the stage at which the process of grain filling takes place; the same role of Os_FIE2 had been reported earlier by Nallamilli et al. (2013), and *Os_EMF2B* showed the highest expression during flowering stage which was also reported by Conrad et al. (2014) stating that EMF2B in rice is essential for maintenance of floral meristem determinacy. *Os_CLF*, *Os_EMF2B*, *Os_FIE*, and *Os_BMI1A* showed a moderate level of expression in endosperm and they were also found to have O2-SITE *cis*-regulatory element in the upstream region of their promoters. This element is involved in the metabolism of zein protein found in endosperm, comprehending their expression in endosperm.

Diverse stress conditions lead to rapid and coordinated changes at the transcript level of the entire gene network (Santos et al. 2011). Difference in the expression pattern may rise due to the difference in the type of stress, stage, as well as variety. In this study, the microarray, RNA-seq, and qRT-PCR data were compared to analyze the expression pattern of PcG genes in *O. sativa.* The real-time-based expression analysis presented *Os_RING1A.1*, *Os_RING1A.2*, *Os_FIE1*, and *Os_FIE2* as important candidate genes in response to abiotic stress. Consistent with the qRT-PCR data, *Os_RING1A.1*, *Os_RING1A.2* showed upregulation in their expression pattern in microarray data also (Fig. 8a) and their homolog in Arabidopsis also showed a change in expression under various stress conditions. *Os_FIE1* expression data was similar for both qRT-PCR and RNA-seq showing a decrease in expression level at 1-h time point under salinity stress condition in root tissue and then getting upregulated at later time points (Figs. 8, 9b). Moreover, the promoter of all these four genes showed the presence of CAREs such as MBS, MYB, MYC, ABRE, ARE, and TGACG motif which were earlier shown to regulate the expression of stress-responsive genes such as RD29B (Tuteja 2007). MYB transcription factors are involved in the regulation of phenylpropanoid pathway which produces various secondary metabolic compounds involved in abiotic stress response in plants (Roy 2016). Previously, RING1A and RING1B in Arabidopsis were shown to be involved in various ABA-dependent processes such as seed germination, seedling establishment, and root growth (Zhu et al. 2020). Thus, the presence of these and other ABA-responsive elements is indicative of the role of PcG genes in stress signaling via ABA-dependent or -independent pathways. These studies suggest that PcG genes perform crucial functions in the adaptation of plants to the external environment.

Conclusion

Despite tremendous advances in recent years, there are still major gaps in our understanding of the PcG repressive mechanism in plants. Furthermore, Arabidopsis is used in the majority of the investigations to understand the roles of PcG genes. Thus, it was critical to first locate and analyze the PcG gene family in crop plants like rice, to see further if the two systems function similarly. In this study, 14 PcG genes were identified in *O. sativa* genome. However, there have been some reports showing the involvement of PcG genes in regulating rice growth and development. The significance of the PcG gene family in abiotic stress signaling in rice is yet to be investigated. Promoter analysis shows the involvement of certain rice PcG family members in growth, development, and abiotic stress response (MBS, MYB, MYC, ABRE, ARE). Microarray- and RNA-seq-based expression profiling correlates with the promoter analysis and suggests the involvement of PcG genes in plant development and abiotic stress signaling. qRT-PCR-based expression analysis further validates the probable role of certain PcG genes in drought and salinity stress. These findings indicate that the PcG genes affect the expression of diverse stress-responsive genes and prove to be useful in transgenic research and also

as candidate genes for plant breeders. This preliminary study of PcG gene family in rice has set the stage for future product development activities. We have identified four probable stress-responsive PcG genes, namely, *Os_FIE1*, *Os_FIE2*, *Os_RING1A.1*, and *Os_RING1A.2*, on the basis of their expression pattern in stress conditions for further functional characterization.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10142-022-00906-z.

Author contribution The idea, concept, and design of experiments, analysis, interpretation of data, fund, and project management were done by AM. NY has done the bioinformatics analyses and wet laboratory experiments. NY drafted the original manuscript. NY, AM, PN, and RR critically analyzed the data and revised the manuscript. AK helped in retrieving the RNA-seq data. AR helped in designing and initiating few experiments.

Funding The study was supported by funding from South Asian University, New Delhi, to AM for consumables and the infrastructure. NY received financial assistance from the Council of Scientific and Industrial Research.

Data availability All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Competing interests The authors declare no competing interests.

Conflict of interest The authors declare no competing interests.

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