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Glutathione –linking cell proliferation to oxidative stress

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Running head: Nuclear glutathione

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Abstract

Significance:

The multifaceted functions of reduced glutathione (gamma-glutamyl-cysteinyl-glycine; GSH) continue to fascinate plants and animal scientists, not least because of the dynamic relationships between GSH and reactive oxygen species (ROS) that underpin reduction/oxidation (redox) regulation and signalling. Here we consider the respective roles of ROS and GSH in the regulation of plant growth, with a particular focus on regulation of the plant cell cycle. Glutathione is discussed not only as a crucial low molecular weight redox buffer that shields nuclear processes against oxidative challenge but also a flexible regulator of genetic and epigenetic functions.

Recent Advances:

The intracellular compartmentalization of GSH during the cell cycle is remarkably consistent in plants and animals. Moreover, measurements of *in vivo* glutathione redox potentials reveal that the cellular environment is much more reducing than predicted from GSH/GSSG ratios measured in tissue extracts. The redox potential of the cytosol and nuclei of non-dividing plant cells is about -300 mV. This relatively low redox potential is maintained even in cells experiencing oxidative stress by a number of mechanisms including vacuolar sequestration of GSSG. We propose that regulated ROS production linked to glutathione-mediated signalling events are the hallmark of viable cells within a changing and challenging environment.

Critical Issues: The concept that the cell cycle in animals is subject to redox controls is well established but little is known about how ROS and GSH regulate this process in plants. However, it is increasingly likely that similar redox controls exist in plants,

although possibly through different pathways. Moreover, redox-regulated proteins that function in cell cycle checkpoints remain to be identified in plants. While GSH-responsive genes have now been identified, the mechanisms that mediate and regulate protein glutathionylation in plants remain poorly defined.

Future Directions: The nuclear GSH pool provides an appropriate redox environment for essential nuclear functions. Future work will function on how this essential thiol interacts with the nuclear thioredoxin system and nitric oxide to regulate genetic and epigenetic mechanisms. The characterization of redox-regulated cell cycle proteins in plants, and the elucidation of mechanisms that facilitate GSH accumulation in the nucleus are keep steps to unravelling the complexities of nuclear redox controls.

Introduction

Glutathione (GSH) is a ubiquitous low molecular weight thiol in eukaryotes. The 2GSH/glutathione disulphide (GSSG) redox couple is crucial in the regulation of cellular redox homeostasis. High GSH/GSSG ratios are maintained by the activity of glutathione reductase (GR), which ensures that the 2GSH/ GSSG and NADP/NADPH redox couples are in thermodynamic equilibrium and hence at the same redox potential.

Considerations of antioxidant functions in the prevention of oxidative stress still overshadow much of our current philosophy and understanding of the importance of GSH in animals and plants. However, accumulating evidence demonstrates that GSH is required for the operation of a diverse range of processes that include growth, stress tolerance and cell suicide programs (1,2). Within this context, the requirement for GSH is undoubtedly linked to signalling function, particularly interactions with nitric oxide (NO) and participation in thiol-dependent post-translational protein modifications, which modulate activities, sub-cellular localization, stability or their interactions with partner proteins in plants and animals.

Plant growth is driven initially by cell proliferation and primary morphogenesis, followed by cell expansion, secondary morphogenesis and endoreduplication (3).While the requirement for GSH biosynthesis for mitosis and root formation is well established in plants, the broader functions of GSH-regulation in the orchestration of plant organ formation is poorly understood, not least because of the diverse range of potential target genes and proteins that are involved in the promotion or inhibition of component pathways or processes.

GSH is abundant in the plant cell cytosol, chloroplasts, mitochondria and nucleus (Figure 1). Like other small molecules, GSH diffuses freely between the cytosol and nucleus through the nuclear pore complex (4). It is rather surprising therefore that the nuclear GSH pool is much more resistant to depletion than the cytosolic pool, a property that is particularly important during cell proliferation (5-8). Although relatively little is known about the nuclear thioredoxins (TRX) and glutaredoxins (GRXs) or their functions in plants (9) it is probable that these redox proteins participate in the plethora of thiol-dependent redox regulation mechanisms and post-translational modifications that are required for plant growth and development, particularly through functions in the nuclei.The many important roles of glutathione in plants have been well documented in recent reviews (1,2, 10) and hence the following discussion will focus on how GSH functions as a regulator of plant development, with a particular focus on the nuclear GSH and the regulation of mitosis.

GSH and redox signalling

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) act as signalling molecules to transfer extracellular or intracellular information to the nucleus to elicit specific and appropriate responses. In its classic function as an antioxidant, GSH serves to remove ROS and hence limit the lifetime of the oxidative signal. However, accumulating molecular genetic evidence suggests that GSH is also important in potentiating ROS signals in plants, particularly through interactions with plant stress hormones such as salicylic acid (SA) and jamonic acid (11-13).

Redox-sensitive cysteines, which can undergo a diverse spectrum of thiol modifications, play a central role in coupling changes in intracellular redox state to metabolic and molecular responses through the ROS- and RNS-dependent signalling pathways. The reactivity of any protein thiol group is largely determined by the structural environment of the cysteine, together with its p*Ka* value. Most protein thiols have p*Ka* values greater than 8.0, which means that the thiol group is predominantly protonated and largely nonreactive at cellular pH values. However, the thiol groups of redox-sensitive cysteines have much lower p*Ka* values ranging from 3 to 6. Acidic thiols therefore exist as highly reactive thiolate anions ($-S^-$) under physiological pH conditions. In contrast to their protonated forms, acidic thiols are highly susceptible to oxidation by ROS and RNS. Reactive cysteines can be oxidized by ROS into sulfenic (-SOH), sulfinic (-SO2H) or sulfonic (-SO3H) acids. Whereas cysteine oxidation to sulfenic acid is reversible, oxidation to sulfinic and sulfonic acids is generally considered to be irreversible. However, this is not the case for 2-cys peroxiredoxins, in which the sulfinic form of the catalytic cysteine can be reduced to the sulfenic acid form by proteins such as sulfiredoxin or sestrin (14-16).

The oxidation of the cysteine residues is catalyzed by cysteine oxidases that confer specificity to the reaction. These enzymes introduce two oxygen groups into an N-terminal thiol to produce cysteine sulfinic acid groups, a reaction that promotes protein degradation in the oxygen-dependent branch of the N-end rule pathway of targeted proteolysis (17) In the N-end rule pathway, the removal of the relatively stabilizing N-terminal methionine in certain transcription factors reveals a cysteine residue that is sensitive to *S*-nitrosylation as well as oxidation to sulfenic and sulfonic acid groups (18).

This process enhances the susceptibility of the transcription factor to proteolytic degradation, keeping levels low and preventing activation of gene expression. At the low oxygen concentrations that occur for example in seeds this continuous stimulation of degradation is prevented and appropriate gene expression is triggered (18). However, it is not as yet clear what determines whether a given cysteine in these circumstances will react with NO, oxygen, hydrogen peroxide or GSH, although each post-translational modification is likely to have a different effect on protein function.

Oxidative modifications of protein cysteines can involve formation of mixed disulphides with other protein thiol groups (intra- orintermolecular protein disulfide bonds) or with low molecular weight thiols such as GSH (S-glutathionylation). cysteine (S-cysteinylation) or cysteamine (S-cysteaminylation). In the presence of RNS, cysteines can be modified by either S-nitrosylation or S-glutathionylation. Most, if not all, of these processes provide routes of redox-regulated post-translational protein modification that can facilitate regulation of function, stability or interaction. Moreover, NO readily reacts with GSH to form S-nitrosoglutathione (GSNO), which functions as a natural NO donor for protein nitrosylation. The addition of the NO moiety to form an S- nitrosothiol (SNO) is a key mechanism for the transfer of NO bioactivity. This NO adduct has been proposed to be a significant player in NO regulatory mechanisms, particularly in S-nitrosylation of proteins. The role of GSNO in transferring NO to protein thiols has impacts on many regulatory processes in plants and other organisms, with increasing numbers of proteins reported to be reversibly nitrosylated The size of the GSNO pool in plants is regulated in plants by GSNO reductase 1, which reduces GSNO to oxidized glutathione (GSSG) and ammonium (19). Most redox-regulated post-translational protein modifications are

reversible, in a manner that is controlled by TRXs and GRXs, both of which play important roles in redox signalling.

Protein glutathionylation, in which a mixed disulfide bridge is formed between an accessible free protein thiol and the cysteine thiol of GSH, provides a potential mechanism for metabolic regulation and cell signalling (20). This process is relatively well characterised in relation to function in animals but remains poorly characterised in plants, often because of technological challenges (21). Since GSH is the most abundant low molecular weight thiol in plant cells (22, 23), glutathionylation has the potential to be a major form of *S*-thiolation-dependent regulation (21).

Glutathionylation is also possible through enzyme-independent thiol-disulphide exchange reactions between GSSG and cysteine thiolates on proteins, a process that is considered to be favoured under conditions of oxidative or nitrosative stress. In such situations, glutathionylation is considered to play a protective role against irreversible oxidation of sensitive cysteine residues. The proteins that promote protein glutathionylation *in vivo* in plant cells are unknown but the reverse reaction (de-glutathionylation) is thought to be catalysed by GRXs, which are reduced by GSH. Many important enzymes of photosynthetic carbon assimilationin the Benson-Calvincycle in chloroplasts and associated pathways are regulated by TRX in a redox-dependent manner (24). Chloroplast TRX and the enzymes of the Benson-Calvin cycle in chloroplasts are major targets of glutathionylation (25-27). To date however, this process has only been relatively well documented for only one enzyme NADP-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is also a key enzyme in glycolysis in both plastids and the cytosol. The activities of the chloroplast GAPDH form and its activating f-type TRX

are inhibited by glutathionylation (25-27). Glutathionylation of cytosolic GAPDH form is reversed by both GRX and TRX (28). Few studies have attempted to measure glutathionylated proteins in other cellular compartments. For example, while the presence of the cell cycle inhibitor ophiobolin A altered the profile of glutathionylated proteins, these changes were not characterized in detail (29).

The pK_a of GSH (about 9) in cells limits facile oxidation to sulfenic acid (GSOH). The pK_a therefore has to be decreased to allow de-protonation at physiological pH values and so allow nucleophilic attack. This is achieved by the action of glutathione *S*-transferases (GSTs), which decrease the $pK_a$of GSH to about 6 and so catalyze the conjugation of GSH with a range of reactive electrophilic compounds, particularly xenobiotics (30, 31). Interestingly, the rate of GST-catalyzedconjugation reactions of thiols with electrophiles is only slightly faster than the non-enzymatic reaction under physiological conditions. Although the reaction is not kinetically favorable, GSTs can also catalyze the glutathionylation of protein cysteine residues.

GSH synthesis and its role in plant development

The pathway of GSH synthesis from its component amino acids (glutamate, cysteine, and glycine) in plants is catalyzed by two ATP-dependent enzymes, as in animals. The first enzyme of this pathway, gamma-glutamylcysteine synthetase (γ-ECS), which is now often called glutamate-cysteine ligase, catalyzes the formation of glutamylcysteine in chloroplasts and other types of plastid (32-35). In contrast to γ-ECS, which is localized only in plastids, the second enzymes of the pathway GSH synthetase is found in both the plastids and cytosol (36).

Gamma-glutamylcysteine and GSH are transported across the bounding envelope membranes of the chloroplasts by a small family of transporters called CHLOROQUINERESISTANCE TRANSPORTER-LIKE TRANSPORTER (CLT:37). Thereafter, GSH is transported to all of the intracellular compartments including the nucleus (Figure 1). GSH synthesis in plants is regulated at the level of gene transcription. However, in stress situations flux through the pathway is largely regulated at level of regulation of γ-ECS activity, which is subject to thiol-based oxidative activation as well as feedback inhibition by GSH (38). The modifier (γ-ECS M) subunitof γ-ECS that is found in animals (39) has never been identified in plants.

Molecular genetic studies provided unequivocal evidence that GSH is an essential metabolite in plants. Null mutants in the *GSH1* gene, which encodes γ-ECS, fail to survive beyond the embryo stage (40,41). While null mutants in the *GSH2* gene, which encodes GSH synthetase, are able to grow to the seedling stage, they are unable to develop further. Hence, it appears that gamma-glutamylcysteine can compensate for GSH at the earliest stages of seedling development but not thereafter (41).

The specific requirement for GSH in root development has been revealed through the characterization of a number of *A. thaliana GSH1* mutants that have partially impaired γ-ECS activity but are still able to support a low level of GSH synthesis and accumulation. The *rootmeristemless1 (rml1*) mutants were originally identified on the basis of their inability to maintain cell division following germination because the cell cycle arrests in the primary root at an early stage (42,43). The factor required to activate and maintain the cell division in the root apical cells was shown to be GSH (43). The *rml1* mutants have only between 2-5% of the wild type GSH levels (44) and the cytosolic

redox potential is decreased by about 40 mV to about -260mV (45). Similar decreases in GSH caused by the presence of the GSH synthesis inhibitor, buthiomine sulfoxamine (BSO) caused the arrest of root, but not shoot, development in wild type seedlings (44, 46).

Other *A. thaliana GSH1* mutants that have less severe effects on GSH synthesis than *rml1* are able to support primary root development but they show altered root architecture (44; 47). For example, the cadmium-sensitive 2 (*cad2-1*) mutants, which have about 20-30% of the wild type GSH contents and exhibit hypersensitivity to cadmium are largely aphenotypic in terms of shoot growth in the absence of stress (48,49). However, *cad2-1,* like other similar *GSH1* mutants, such as *pad2-1 and rax1-1,* produces fewer lateral roots and hence have lower root densities than the wild type (44). Such studies have shown that GSH participates in the interactions between auxin and strigolactones, which are two of the major plant hormones that control of root architecture (44, 47).

In contrast to roots, TRX able to replace GSH functions in the control shoot development (50,51). Studies on *Arabidopsis cad2-1 ntra ntrb* triple mutants that lack both functional cytosolic NADPH-THIOREDOXIN REDUCTASES (*ntra,ntrb*mutants) and have a decreased capacity for GSH synthesis (*cad2-1*) were found to grow in a similar manner to the wild type throughout vegetative development(51). However, they were unable to sustain normal floral meristem development, producing instead a PIN-FORMED (PIN) structure that is characteristic of impaired auxin transport (51). Auxin transport is regulated by efflux (PIN) and influx (AUX1, LAX1–LAX3) transporters, as well as by B type ATP binding cassette subfamily G (ABCG) transporters (51). The PIN-

proteins are asymmetrically localized such that their polarity determines the directional flow of auxin. The regulated distribution of PIN activity is a crucial control point for auxin movement and for the regulation of plant development. The *cad2-1 ntra* ntrb triple mutants showed decreased polar auxin transport, together with lower auxin levels (51). Moreover, the BSO-triggered inhibition of root growth is accompanied by a decreased expression of PIN proteins (46). These results demonstrate that thiol-mediated redox pathways are linked to the signalling of auxin, which is the key regulator plant growth and development. (52-54). While there is interplay between the TRX and GSH/GRX systems in the regulation of auxin transport, the GSH/GRX system has specific effects. Further evidence in support of this conclusion comes from the characterisation of knockout mutants in the gene encoding GRXS17. The *grxs17* mutants exhibit decreased polar auxin transport and they have a weaker auxin response, as well as showing a temperature-dependent inhibition of root growth and agravitropism (55).

The differential distribution of auxin within and between tissues and organs governs a wide spectrum of plant growth and developmental responses (54). Indole-3-acetic acid (IAA) is the main form of auxin in many plant species including Arabidopsis. IAA is synthesized largely in meristems and nodes and is transported throughout the plant to activate cell-specific effects in response to developmental or environmental triggers. Auxin gradients control the growth and architecture of plant organs such as roots (52-54). Auxin accumulates in stem cells and at sites of cell division and is important in the production of lateral roots, adventitious roots and root hairs, as well as in the control of apical dominance and stem elongation (52-54). IAA is removed by

conjugation or catabolism when downstream responses have reached their optima (56) Oxidation of IAA to 2-oxindole-3-acid acid (oxIAA) attenuates the auxin signal.

Auxin-mediated responses are linked to localized ROS production byNAD(P)H oxidases/respiratory burst oxidase homologs (Rboh).This process requires the activation of phosphatidylinositol 3-kinase (PtdIns 3-kinase), which produces PtdIns(3)P, a metabolite that regulates endocytosis and vesicle trafficking (57). In this way, phospholipid signalling and redox pathways cooperate in the regulation of auxin transport (57).*Arabidopsis* mutants that are deficient in various Rboh forms show developmental changes that are similar to defects in auxin-responses (58). The *Arabidopsis* genome has ten *AtRboh* genes (*AtRbohA-AtRbohJ*) that encode NADPH oxidases (59). The activity of NADPH oxidases is required for root growth and for root hair development (60).Auxin-induced ROS production is mediated at least in part by the activation of RbohD (58, 60-63) but RbohC is involved in the control of root hair tip growth(64), a process that also involves the transcriptional Mediator subunits, PFT1/MED25 by activating a subset of hydrogen peroxide-producing class III peroxidases (65). As discussed below, the regulated activities of both NADPH oxidases and peroxidases are required for the orchestration of root development.

The interaction between auxin and redox signalling pathways facilitates a flexible regulatory hub that is highly responsive of cell metabolism. There appears to be a highly regulated zonal distribution of apoplastic superoxide and hydrogen peroxide accumulation that governs root growth and differentiation. This spatial distribution of different ROS forms in the apoplast appears to delineate the zone of cell proliferation from the zone of cell elongation and differentiation. Superoxide is localized

predominantly in the apoplast of cells in the elongation zone of the *A. thaliana*root, while hydrogen peroxide is found predominantly in the differentiation zone and in root hairs (66). This spatial distribution is perturbed in the *A. thaliana upbeat 1.* Loss of UPBEAT 1 functions led to meristem enlargement, increased cell elongation and generally increased root growth (67). UPBEAT 1 functions as a negative regulator of peroxidase gene expression in the elongation zone. The UPBEAT 1 transcription factor was shown to modulate the balance between cellular proliferation and differentiation in root growth by regulating the expression of a sub-set of peroxidases (67). The expression of these peroxidases exerted a strong influence on the balance between the zones of superoxide and hydrogen peroxide accumulation in the apoplast.

It appears therefore that opposing apoplastic gradients of superoxide and hydrogen peroxide in the root apoplastic environment are linked to the onset of root cell differentiation. UPBEAT 1is controlled independently of auxin or cytokinin, which is another hormonal regulator of root growth (67).. This zonal control of hydrogen peroxide and superoxide accumulation appears to occur largely in the apoplast, and as yet there is no information concerning the functions of intra-cellular antioxidants or thiol signalling that distinguish the zone of cell proliferation from the zone of cell elongation and differentiation. While the levels of ascorbate and glutathione are high in both zones, these antioxidants are more abundant in the cell proliferation zone than other zones in the root (Figure 2; Table 1). Similarly, the abundance of γ-ECS and GR proteins is high in the zone of cell division (Figure 2).

It is important to note that the levels of ascorbate and glutathione are negligible in the cells of quiescent centre of the primary root meristem. The cells in the quiescent

centre, which sits at auxin maxima, have an arrested (G0) or an extended G1 phase of the cycle (68). These cells are considered to be equivalent to stem cells and a sink for IAA because the highly oxidized state in the stem cell niche will favour auxin oxidation (68-70) . While quiescent centre cells are physiologically indistinguishable from the adjacent, actively dividing cells, they have altered mitochondrial function (71). The addition of GSH or ascorbate stimulates the cells in the quiescent centre to progress from the G1 to the S phase of the cell cycle (68). Conversely, the addition of GSSG or oxidized ascorbate (dehydroascorbate, DHA) to proliferating cells causes an arrest the cell cycle in G1 (72,73)

The importance of high GSH/GSSG ratios for plant development

The above discussion has emphasized the importance of the abundance of GSH in controlling plant growth and development. Other studies using Arabidopsis mutants that are defective in GR have demonstrated that the ability to maintain high cellular GSH/GSSG ratios is an essential determinant of plant organ growth and vigour. Most plants contain two GR genes (*GR1* and *GR2*). *GR1* encodes the cytosolic/peroxisomal localized form of the enzyme, while *GR2* encodes a dual-targeted chloroplast and mitochondrial GR form. Although the knockout mutants in GR1 give rise to a lower GSH: GSSG ratio they are able to undergo normal plant development (74). However, *GR2* knockout mutants produce a lethal phenotype and show growth arrest at the stage of embryo development (74). Hence, the activity of the cytosolic GR form is insufficient to compensate for loss of the chloroplast and mitochondrial enzymes. These findings

demonstrate that the GSH/GSSG ratios in the chloroplasts and mitochondria are crucial determinants of plant cell viability.

Further studies have shown that GR2 fulfils a key role in the maintenance of the root apical meristem (75). A GR2 mutant called *miao* (a weak mutation in *GR2*) displays major defects in the root apical meristem, together with an inhibition of root growth. The *miao* mutants have only about half the wild type GR activity and hence accumulate GSSG and have low GSH/GSSG ratios (75). Hence high GSH/GSSG ratios in root plastids are essential for the maintenance of the root apical meristem. It is not clear how GSH/GSSG ratios alter the viability of the cells in the root meristem but GSH is required for downstream effectors of PLETHORA (PLT), which is an auxin-inducible master regulator of root development. However, the altered plastid GSH/GSSG ratios also appear to have effects on root development through other auxin/PLT-independent redox signalling pathways (75).

In contrast to *GR2*, the cytosolic/peroxisomal GR1 form is not crucial for plant development (74).*GR1* accounts for over 60% of the total cellular GR activity. While the knockout *gr1* mutants do not show a growth or development phenotype even under stress conditions, and the glutathione redox potential of the cytosol is significantly shifted towards enhanced oxidation in the absence of cytosolic/peroxisomal GR activity. The NADPH-dependent TRX system can reduce GSSG in the cytosol and so act as a backup system for GR1 activity (74). Such a functional redundancy might have additional physiological important because the cytosolic glutathione pool is direct contact with the nuclear glutathione pool.

The *gr1* mutants show enhanced susceptibility to pathogens suggesting that *GR1* is essential for redox signalling through the plant hormone-mediated defence pathways (11-13, 76). Characterization of mutants such as the *clt* mutants that are deficient in the cytosolic GSH (37) and *gr1cat2* double mutants that lack both GR1 and the major form of leaf catalase (*cat2;* 76) have shown that the cytosolic GSH pool *per se* plays a crucial role in linking ROS signalling to downstream pathogen responses and associated hormone-dependent pathways (11-13, 37).

As mentioned above GSH is required flower development (50, 51). GSH is important in pollen germination and pollen tube growth (77). The *Arabidopsis ntra ntrb* mutants have decreased pollen fertility (50). Moreover, the pollen in *gr1ntra ntrb* triple mutants are sterile suggesting that TRX and GSH are required for optimal pollen production (74)

GSH is required for the cell cycle in roots

The mitotic cell cycle is the main driving force for plant growth (78). The basic underlying mechanisms of cell proliferation are conserved among all eukaryotes, with heterodimeric cyclin-dependent kinase (CDK)-cyclin complexes at the G1-to-S boundary in Arabidopsis cells that activate the E2F–DP pathway by phosphorylation of the retinoblastoma-related (RBR) repressor, a process that induces genes involved in nucleotide synthesis, DNA replication, and DNA repair (79). Interactome analysis revealed that all E2Fs, including E2Fc, and DP proteins interact with RBR (79) with E2F–DP–RBR network activity associated with both the G1-to-S and G2-to-M transitions, as in mammalian cells (80).

Cell cycle progression in animals is considered to be driven by an intrinsic redox cycle consisting of reductive and oxidative phases (81-85). The binding of growth factors, such as epidermal growth factor (EGF) to their receptors (such as EGFR) is facilitated by ROS generation leading to oxidation and activation of signaling pathways that trigger cell proliferation (85). While the concept that ROS promote proliferation in various animal cell types S (83), little is known about the mechanisms that govern cell cycle entry in plants. The G0-to-G1 transition, which is not governed by cyclin-dependent kinases, is activated by a redox-dependent signal transduction pathway that results in cyclin D1 activation. D-type cyclins are also important regulators of the G0/G1-to-S transition in plants, preceding the activation of the core cell cycle machinery (78).The redox-dependent expression of D-type cyclin genes has not been established in plants, although NO was found to activate the cell cycle though effects on CycD3;1 transcription and effects on cyclin-dependent CDKA1 protein kinase (86). The auxin-dependent activation of cell proliferation in the root meristem is associated with oxidation which is maximal in the quiescent centre cells, suggesting that cell proliferation is influenced by cellular redox state (69, 70). Redox controls also play a key part in the regulation of cell cycle progression (87, 88). In particular, the G1 and G2 checkpoints that regulate the cell cycle are highly responsive to oxidation (89). While the mechanisms than underpin these responses and the pathways of oxidative activation of the cell cycle are not as clearly defined in plants as they are in animals, antioxidants play a crucial role in providing an appropriate redox environment for cell cycle progression. Low GSH-dependent cell cycle arrest in *rml1* roots is accompanied by a decreased abundance of transcripts encoding many core cell cycle components such as cyclins and CDKs that are

necessary for the G2 to M transition (44), confirming that in the absence of adequate GSH the cell cycle arrests at an early stage (G1/S, 43).

The redox environment of the cytosol and nuclei

Changes in intracellular GSH compartmentation are a key component of many stress responses (90, 91). The distribution of GSH between different cellular compartments is important because it establishes and supports the redox environment in which metabolism and signalling events occur. The GSH redox potential of the cytosol of plant cells *in vivo* has been extensively monitored using redox-sensitive green fluorescent protein (roGFP) probes (92-94). Parallel analyses of the redox state of nucleus and cytosol using a yellow fluorescent protein-based redox sensor (rxYFP) in the yeast *S. cerevisiae* showed that nuclear GSH redox environment is highly reducing and similar to that of the cytosol (95), a situation that is also observed in plant cells (Figure 3; 44; 96). The average glutathione redox potential of the cytosol was about -288 mV while that of the nuclei was -294 mV in the cell division zone of *A. thaliana* root mersiem (Figure3; 96). However, cell division is asynchronous in the root meristem and so these data reveal nothing with regard to variations in the glutathione redox potential that might occur during the different phases of the cell cycle.

These values confirm that the GSH:GSSG and NADPH:$NADP^+$ redox couples are close to redox equilibrium in the cytosol and the nuclei, conferring a high sensitivity to the signaling functions of the glutathione redox potential, which is mediated through relevant pathways such as GRX-dependent changes in protein thiol-disulfide status. For example, a change in cytosolic redox potential of about 50 mV is sufficient to

significantly alter the balance between oxidized and reduced forms of TRX-regulated proteins (97). As discussed previously (1), an increase in redox potential from -350 to -300 mV converts the TRX-regulated chloroplast glucose-6-phosphate dehydrogenase 1 from almost completely inactive to active (98).Small changes in cellular NADP:NADPH ratiosmay be sufficient to allow significant changes in the glutathione redox potential, and hence facilitate signal amplification *in vivo*. Given the low K_M of GR for NADPH (99, 100) compared to likely cytosolic NADPH concentrations (around 150 μM; 101), this could occur through adjustment of relative concentrations in the NADP-glutathione equilibrium rather than kinetic limitation of GR activity by NADPH.

Assuming that the 2GSH/ GSSG and NADP/NADPH redox couples are in thermodynamic equilibrium, then at $NADP^+/NADPH = 1$ (representing a redox potential of -320 mV), there should be very little GSSG in the cytosol or the nuclei. Such negative redox potentials are achieved through continuous GSSG reduction by GR and also by GSSG transport into the vacuole (102). In this regard, it is worth noting that the tissue GSH/GSSG ratios of many organisms are often cited as being in the range of 10^2 to 10^3 (1, 2). However, given the values of the glutathione redox potentials obtained *in vivo* by roGFP measurements, it is clear that the measurements of GSH and GSSG levels in tissue extracts, while a useful indicator of redox perturbation, do not precisely reflect the GSH/GSSG ratios in intracellular compartments. The GSH/GSSG ratios measured in tissue extracts, such as those shown in Table 1, generally suggest that in the absence of stress between 90- 95% of the glutathione pool is present as GSH with about 5-10% present as GSSG. These GSH/GSSG ratios give much higher redox potentials than those obtained in compartments such as the cytosol using roGFP. It is likely therefore that the

GSSG measured in tissue extracts is either generated as an artefact during extraction or that a large proportion of the detected GSSG is not located in the cytosol. While it is inevitable that some oxidation of GSH will occur during extraction, the other possibility is that GSSG is continuously removed from the cytosol in order to stabilize the cytosolic redox potential, particularly in situations of oxidative stress (102). Evidence in support of this hypothesis was obtained from studies on the *A. thaliana cat2* mutants that lack the major form of leaf catalase. These mutants do not show significant increases in leaf H_2O_2 levels but they accumulate both GSH and GSSG (76; 102). These mutants accumulate GSSG to such high levels (55) that in theory, the glutathione redox potential of the leaf cells would collapse if this metabolite in the cytosol. However, while the *cat2* mutants grow more slowly than the wild type under ambient atmospheric conditions, they remain viable and only show lesions in a day-length and salicylic acid-dependent manner (103). This apparent anomaly was resolved by evidence concerning where the GSSG was localised in *cat2* mutant leaves (104). In this case of severe oxidative stress, GSSG was largely sequestered in the vacuole (104). Vacuolar sequestration therefore serves to limit the accumulation of GSSG in the cytosol, as illustrated in Figure 1.

GSH/GSSG ratios measured in tissue extracts are often used as a marker of oxidative stress. Removal of GSSG from the cytosol may reconcile the large discrepancies in redox potentials calculated from measured GSH/GSSG ratios to the modest adjustments in redox potential measured using roGFP probes in plants exposed to stress (92-94; 105). In this regard, it may interest to re-investigate the concept of a reductive limit that defines the relationship between redox regulation and the cell cycle in cancerous cells (106). While animal cells do not have vacuoles, it could be the other

mechanisms of GSSG sequestration exist that mean that the GSH/GSSG ratios measured in animal tissue extracts might not provide an accurate estimate of *in vivo* glutathione redox potentials.

The nuclear glutathione pool

As in animals (34), glutathione is abundant in the nucleus of plant cells (Figures 3 and 4). Data such as that shown in Figure 4 demonstrates that the nuclear GSH pool is in equilibrium with that of the cytosol. However, studies on animal and plant cells have demonstrated GSH is compartmentalized in the nucleus during the cell cycle (5-8, 107). Data showing that the nuclear GSH pool is more resistant to depletion than the cytosolic pool (108) suggest that mechanisms exist that facilitate GSH sequestration in the nucleus. For example, the addition of BSO to 3T3 fibroblasts significantly decreased the total cellular GSH pool (108). However, only the cytosolic GSH pool was rapidly depleted in the presence of BSO and the nuclear GSH pool was less depleted (108). Similarly, the addition of the sesterpenoid inhibitor Ophiobolin A to tobacco cells blocked the cell cycle at the S/G2 phases, trapping GSH in the nucleus (29).

GSH co-localizes with nuclear DNA during cell proliferation in animals (8) and plants (5, 6, 96, 107). In such studies, the nuclear GSH pool has been monitored largely by cconfocal microscopy using a double staining procedure involving Hoechst 33342 (Hoechst; blue stain) to localize nuclear DNA and CellTracker green 5-chloromethylfluorescein diacetate (CMFDA; green stain) to detect GSH (5, 6, 86, 107). Using such procedures, the nuclear localization of GSH can be visualized, for example, in synchronously dividing *A. thaliana* cells, as illustrated in Figure 4A-H (96). GSH was

also observed in the nuclei of the dividing pericycle cells following activation to form the lateral root meristem (5). These results suggest a dynamic regulation of the nuclear GSH pool during the cell cycle (6, 107). While *in vivo* staining procedures undoubtedly have their limitations, these results are sufficiently compelling to merit further investigation using other techniques. Moreover, the question of how GSH might be sequestered in the nucleus is less easily answered in plants than animals, where Bcl-2 proteins bind GSH to the Bcl-2 homology-3 domain groove and facilitate GSH transport and sequestration in the nucleus (110; 111). Plants do not have Bcl-2 proteins and GSH like any other molecule smaller than 40 KDa should move freely across the nuclear pore complex (4). It is tempting to speculate that auxin or auxin-mediated oxidation might regulate GSH transport and sequestration in the nucleus. The composition of the nuclear pore complex has an important influence auxin signalling because certain nuclear pore mutants show auxin hypersensitivity (4).

The functions of nuclear GSH pool including maintaining genome integrity were discussed in detail in a recent review (107). Nuclear GSH is likely to be involved in the redox modulation of genetic and epigenetic mechanisms that control gene expression (107, 112, 113). For example, the S-glutathionylation of Cys110 in histone H_3 was shown to alter the stability of the nucleosomes and chromatin structure in proliferating mammalian cells (107; 112). Moreover, genes encoding GSH-requiring enzymes such as GSTs are likely to be regulated by S-glutathionylation in plants in a similar manner to that observed in animals (114). In addition, similar GSH - and glutaredoxin-dependent mechanisms for the reductive activation of methionine sulfoxide reductases are found in animals and plants. These systems facilitate the reduction of methionine sulfoxide to

methionine, as well as preventing the oxidative generation of methyl radicals that can methylate cytosine residues (113). In addition, GSH fulfils essential functions in the regulation of mitosis in the root apical meristem. These effects could be mediated by GSH alone or through changes in the GSH/GSSG ratios in response to hormone-mediated oxidation. Since our recent review (107) little new information has become available concerning the specific interactions of GSH with nuclear proteins, although the scope for thiol-mediated post-translational modifications that alter nuclear protein functions is vast.

Studies on the effects of low GSH in *Δgsh1* yeast cells have shown that although cells lacking GSH grow like the wild type, they are very sensitive to oxidative stress (115). Moreover, proteins in the GSH-depleted *Δgsh1*yeast cells were found to be highly oxidized and translational activity was impaired in yeast, where the YAP1/GPx3-regulated system is responsible for augmentation of antioxidant potential (115). Moreover, oxidative stress generated a high level of genome instability in GSH-depleted yeast cells, despite the presence of Yap1 in the nuclei (111). Thus, the role of GSH in maintaining nuclear functions during exposure to a mild oxidative stress is crucial to the survival of yeast cells.

Glutathione depletion in the *rml1* mutants, where the cytosolic redox potential is decreased from -300 to -260mV (45) alters the expression of specific suites of genes, associated with the signaling of auxin and other plant hormones (44). A comparison of the transcript profiles of Arabidopsis cell at stages in the cell cycle where GSH was predominantly localised in the nuclei compared to when GSH was compartmentalised evenly between the cytosol and nucleus revealed that defence-related transcripts were less abundant at times when the nuclear GSH pool was larger than that of the cytosol (9,

10). This finding is in agreement with the results of other studies showing that cytosolic GSH pool is important in transducing oxidative signals linked to hormone-dependent defences against biotic and abiotic stresses (11-13; 37, 116).

Conclusions and perspectives

ROS are vital life signals in plants and animals that are required for the accurate orchestration of numerous processes in biology, many of which are mediated through thiol-dependent post-translational mechanisms. While the TRX and GRX systems can have overlapping functions in this type of regulation with numerous points of reciprocal control, each thiol modulator has unique and specialised roles, particularly in the regulation of cell proliferation and differentiation. The specific requirement for GSH and high GSH/GSSG ratios in the control of cell fate is manifest in the development of the root apical meristem, where a change in the glutathione redox potential of only 40 mV is sufficient to cause an arrest of the cell cycle. The GSH pool in the nucleus ensures that the nuclear machinery resides in a highly reducing environment (about -300 mV). An increase in the glutathione redox potential to -260mV, as measured for example in the roots of the *rml1* mutants is sufficient to prevent the G1/S transition, and causes large changes in the transcript profiles of roots and shoots (44). These findings show that there is a GSH-regulated cell cycle molecular master-switch in the root meristem and probably in other related meristems such as in flowers and pollen. However, redox regulation in these tissues in less well described than the root system, where gradients of superoxide and hydrogen peroxide in the apoplast are required for the transition from cell proliferation to differentiation. While the role of GSH in the control of these gradients

has not been explored to date, this elegant mechanism indicates how the redox regulation of the genetic regulatory network links environmental signals that are often perceived first in the apoplast and plasma membrane to developmental programs.

The identification of this GSH-regulated molecular master-switch that is the remains to be determined, but its characterization will lead to a step change in our understanding of cell cycle regulation Moreover, GSH-regulated steps are important in the regulators of the hormone-dependent control of cell proliferation and differentiation. This requirement has been partially characterised with regard to auxin and its functions in the elaboration of postembryonic root meristem development but few studies to date have explored the GSH-dependent post-translational mechanisms that regulate plant growth and development.

ROS and GSH-dependent thiol-dependent post-translational mechanisms are not only a key to monitoring metabolic flux by cells in order to regulate responses to biotic and abiotic stresses but are also key regulators of the cell cycle in plants, as they are in animals. The high abundance of reactive but relatively short-lived interacting molecules that can either directly or indirectly alter protein structure or function through thiol/NO intermediates *via* at least partially reversible mechanisms is a prerequisite to successful signalling cascades.

Plants have mastered the art of redox control using ROS and RNS as secondary messengers to regulate a diverse range of protein functions through redox-based, post-translational modifications that act as regulators of molecular master-switches. Much current focus concerns the impact of this regulation on local and systemic signaling pathways but the interpretation of data on *S*-modified proteins remains problematic. The

accurate identification of the modifications occurring *in vivo* is not trivial, nor is quantification in terms of the proportion of the protein cysteines that undergo any given modification.

GSH is recruited into the nucleus at the G1 phase of the cell cycle, an event that is linked to the oxidative activation of cell proliferation. Very little is known about the mechanisms that might enable GSH sequestration in the nucleus of plants that do not have Bcl-2 proteins that bind GSH. A key question that is still outstanding therefore concerns the mechanisms that facilitate this type of control in plants. Moreover, GSH depletion causes large changes in gene expression but the functional significance of of these GSH responsive genes remains poorly characterised. Thus, unravelling the biology of nuclear GSH and its functions in genetic and epigenetic controls that underpin plant growth and defence responses represents a new frontier in plant and animal science. Given the tremendous progress has been made over the last decade; it is likely that exciting new developments are on the horizon. Finally, it is worth remembering that in nature plants are often exposed to multiple stresses simultaneously. Regular exposures to changing mild stresses results molecular and physiological adjustments that include increased resistance to oxidative stress, which translate into plant growth and development that are optimised to prevailing environmental conditions. Without regular stress exposures that entrain genetic and epigenetic cross tolerance responses, it is likely that sustainability and vigour in natural environments. Understanding the mechanisms that create short and long-lasting molecular memories of stress, together with GSH functions in the central regulatory hub that controls cell proliferation and fate within an

environmental and developmental context will be key advances and help to resolve many of the outstanding issues discussed in this review.

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Legends to Figures

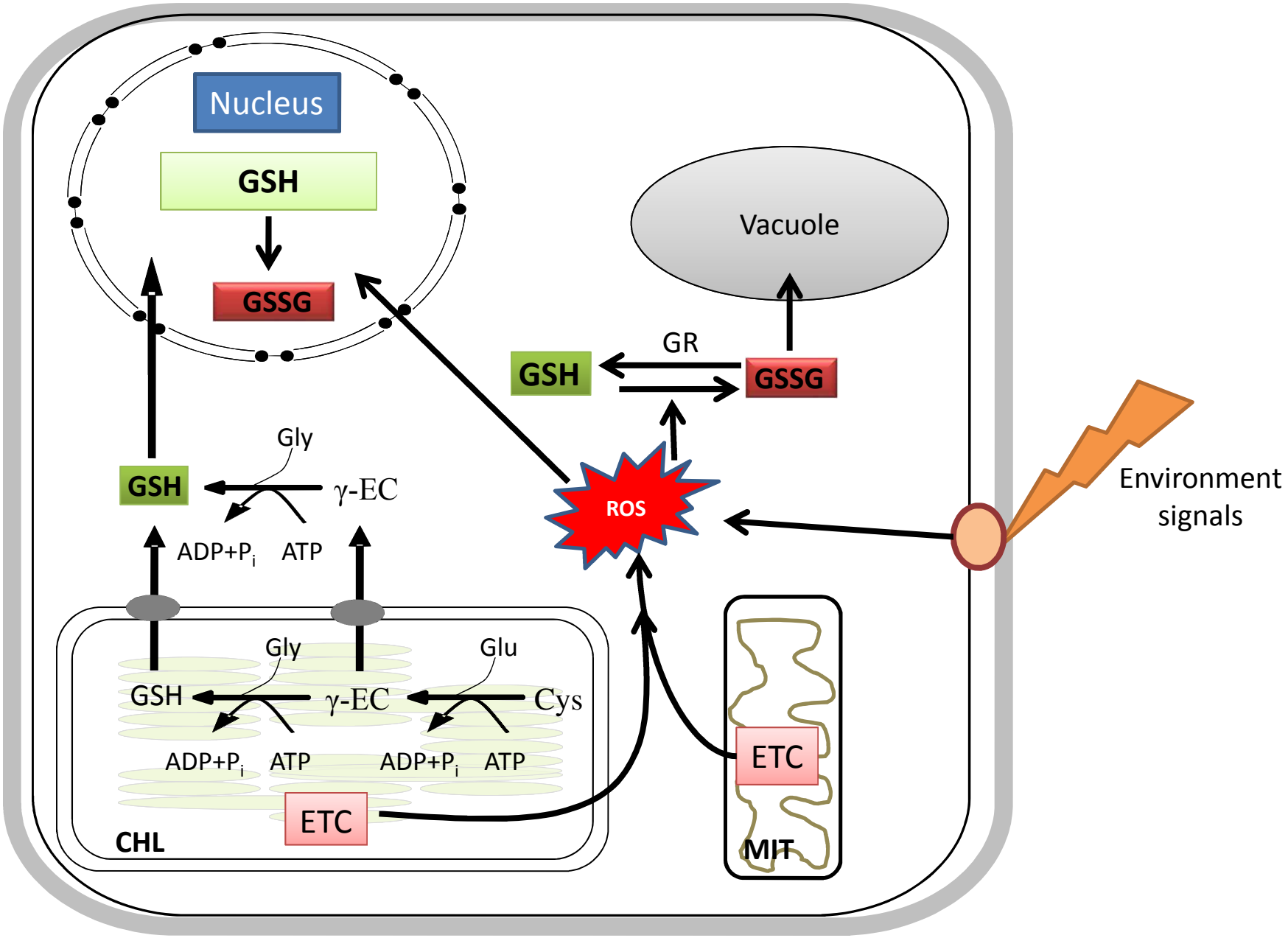
Figure 1. Intracellular compartmentation of glutathione in plants. CHl, chloroplast; MIT, mitochondria; ETC, electron transport chain.

Figure 2 Immunoblot analysis showing the relative abundance of glutathione reductase (A) and g-glutamylcysteine synthetase (B) proteins in whole roots (1) and in different regions that can be divided loosely into regions of cell proliferation (4) and expansion and differentiation (2/3) of 4-day old corn (*Zea mays*) seedlings (C). Zone 4 comprises of the root cap, the quiescent centre and meristematic cells, zone 3 is largely elongating cells and zone 2 contains mature cells. (see Ref 117 for experimental details).

Figure 3. Nuclear GSH localisation in plant cells. Confocal microscopy images showing GSH localization in the nuclei of Arabidopsis cells at the G1 (A, B, C, D) and G2 (E, F, G, H) phases of the cell cycle. Localization of GSH in the root tipsof *A. thaliana* wild type (I, K) and *rml1*mutant (J, L) plants. 5-chloromethylfluorescein diacetate(green) staining was used to detect GSH; Hoechst 33342 (blue) was used to stain chromatin and hence localize nuclei. Images B, D, F, H, J and L show the over-laid blue and green images. Synchronized proliferation in A-H was achieved by growing the cells in sucrose-free media supplemented with aphidicolin.

Figure 4. The glutathione redox potentials of nuclei and cytosol in the dividing cells of Arabidopsis roots measured using roGFP (A). The roGFP measurements used to produce

the data shown in (A) were performed in the zone indicated by the white square in the schematic model of root structure (B).



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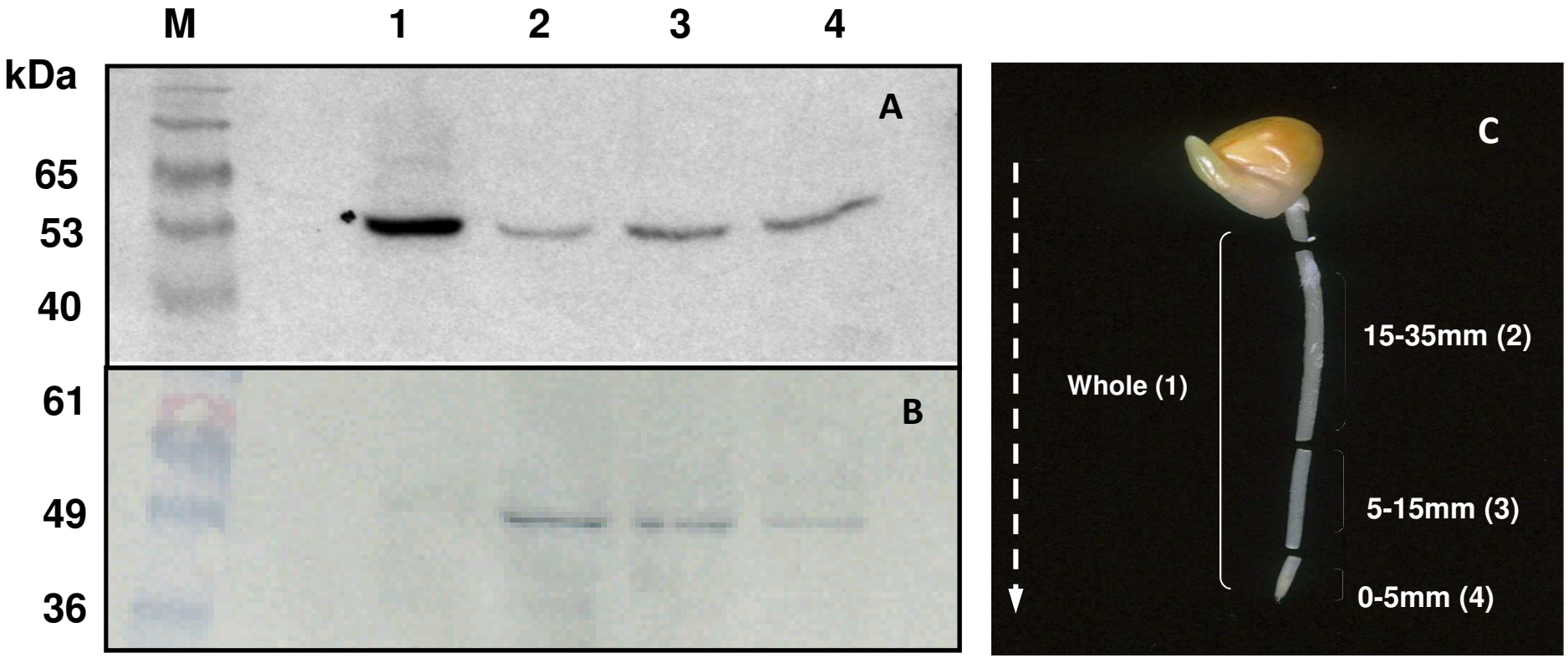

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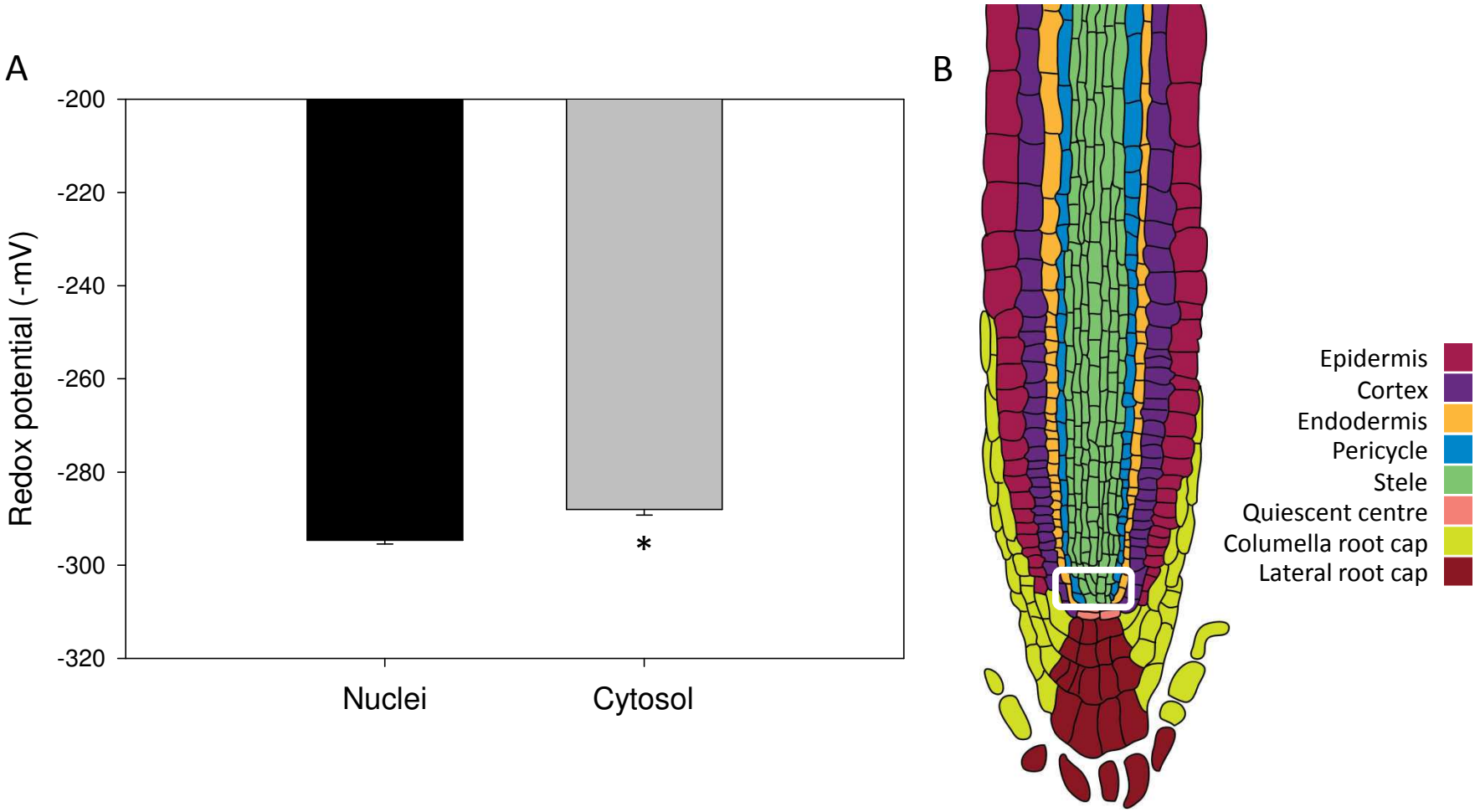

Figure 3. Redox potentials of the nuclei and cytosol of developing Arabidopsis radicles measured by roGFP (A). Asterisk indicate significant difference according to paired t-test (P<0,001). Redox potentials were measured as previously described by Schnaubelt et al. (2015) in the zone indicated by a white square in the root structure picture (B).

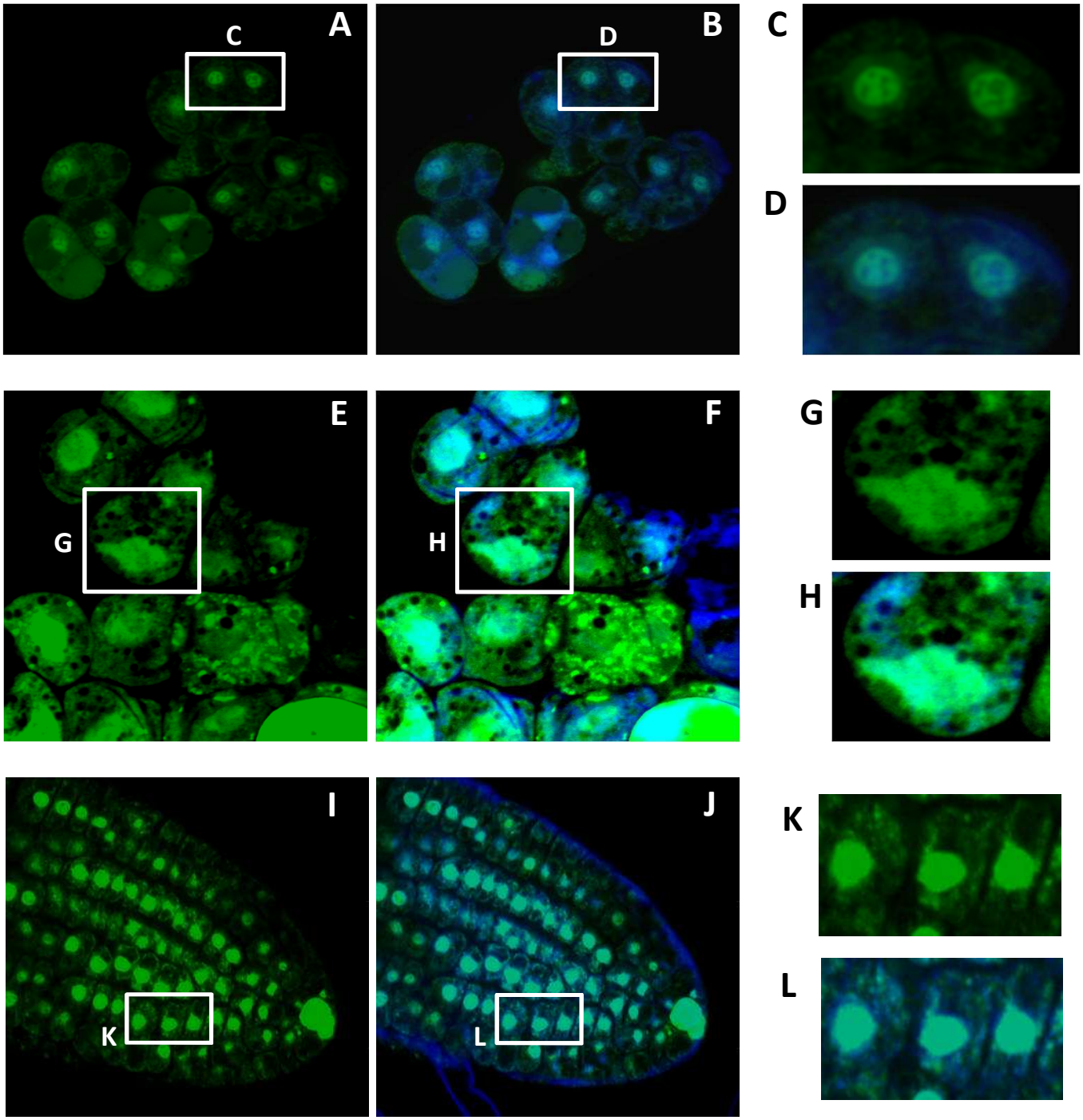

Figure 4 Confocal microscopy images showing GSH localization in *Arabidopsis* cells at the G1 (A, B, C, D) and G2 (E, F, G, H) phases of the cell cycle. Localization of GSH in *rml1* root tip (I, J, K, L). CellTracker green 5-chloromethylfluorescein diacetate (CMFDA, green) staining was used to detect GSH; Hoechst 33342 (blue) staining was used to localize nuclei. Images B, D, F, H, J and L show the over-laid blue and green images. Confocal microscopy approach was performed as previously described by Diaz-Vivancos et al. (2010a). Synchronized proliferation was achieved by growing the cells in sucrose-free media supplemented with aphidicolin.