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Changes in low-molecular-weight thiol-disulphide redox couples are part of bread wheat seed germination and early seedling growth

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ABSTRACT

The tripeptide antioxidant glutathione (γ-L-glutamyl-L-cysteinyl-glycine; GSH) essentially contributes to thiol-disulphide conversions, which are involved in the control of seed development, germination, and seedling establishment. However, the relative contribution of GSH metabolism in different seed structures is not fully understood. We studied the GSH/glutathione disulphide (GSSG) redox couple and associated low-molecular-weight (LMW) thiols and disulphides related to GSH metabolism in bread wheat (Triticum aestivum L.) seeds, focusing on redox changes in the embryo and endosperm during germination. In dry seeds, GSH was the predominant LMW thiol and, 15 h after the onset of imbibition, embryos of non-germinated seeds contained 12 times more LMW thiols than the endosperm. In germinated seeds, the embryo contained 7 and 11 times more LMW thiols than the endosperm after 15 and 48 h, respectively. This resulted in the embryo having significantly more reducing half-cell reduction potentials of GSH/GSSG and cysteine (CyS)/cystine (CySS) redox couples (ECySS/GSH and ECySS/CySS, respectively) than the endosperm. Upon seed germination and early seedling growth, CyS and CySS concentrations significantly increased in both embryo and endosperm, progressively contributing to the cellular LMW thiol-disulphide redox environment (ECyS/CySS, ECySS/CySS, respectively). The changes in ECySS/CySS could be related to the mobilisation of storage proteins in the endosperm during early seedling growth. We suggest that ECySS/GSSG and ECySS/CySS can be used as markers of the physiological and developmental stage of embryo and endosperm. We also present a model of interaction between LMW thiols and disulphides with hydrogen peroxide (H2O2) in redox regulation of bread wheat germination and early seedling growth.

KEYWORDS

Cysteine; embryo; endosperm; glutathione; reactive oxygen species; Triticum aestivum L.
Introduction

There is a long-standing interest in the role of the low-molecular-weight (LMW) thiol tripeptide glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH) in the seeds of higher plants. Glutathione has a plethora of roles in eukaryote and prokaryote cells [1], and in seeds is crucial to many seed quality traits, including viability and longevity [2,3]. Glutathione synthesis involves two ATP-dependent reactions. First, γ-L-glutamyl-L-cysteine (γ-Glu-Cys) is formed, catalysed by γ-Glu-Cys ligase (EC 6.3.2.2), and then glycine (Gly) is added by glutathione synthase (EC 6.3.2.3). Glutathione degradation likely involves the formation of cysteine-glycine (Cys-Gly) and 5-oxoproline [4,5]. As a major water-soluble antioxidant and redox buffer, the nucelophile GSH is involved in the control of reactive oxygen species (ROS) through non-enzymatic and enzymatic reactions, including the glutathione–ascorbate cycle [6]. Redox control by GSH is crucial throughout the whole seed life cycle from seed development, maturation drying, imbibition, germination, to seed ageing, and death. Furthermore, GSH is the storage and long-distance transport form of reduced sulphur during early seedling growth [3,7]. The majority of crop plants produce orthodox (i.e. desiccation tolerant) seeds, which gradually desiccate upon maturation drying and, when shed from the mother plant, can survive in a desiccated state for many years before germination. During seed maturation drying, GSH is progressively oxidised to glutathione disulphide (GSSG), which can bind to cysteine (Cys) residues of proteins via S-glutathionylation, resulting in a temporary increase of protein-bound glutathione (PSSG). This redox conversion protects free protein thiol groups (PSH) from auto-oxidation and oxidation to sulphonic or sulphenic acids [3,7–10]. Once shed, a further conversion of thiols to disulphides can accompany after-ripening of dry seeds [11], and another oxidative shift accompanies seed ageing [2,12].

The seeds of bread wheat (Triticum aestivum L., Poaceae) have received attention regarding thiol-disulphide conversions since the discovery of GSH by Hopkins [13–15]. Furthermore, the LMW thiol dipeptides Cys-Gly and γ-Glu-Cys were first isolated from wheat seeds [16,17]. Species of the Poaceae produce caryopases (in which the fruit coat, or “pericarp”, and the seed coat, or “testa”, are fused), hereafter called “seeds” for simplicity. Wheat is among the three most important crops used for staple food, including bread [18]. Starch is the main energy reserve in wheat seeds which, on a dry weight (DW) basis, also contain 10–15% of proteins in the aleurone layer and the starchy endosperm (a seed tissue that provides nutrients to the emerging seedling), mostly prolamins [19]. In wheat, prolamins are major components of gluten, a continuous matrix within the endosperm whose structural properties are governed by disulphide bonds between Cys residues [19,20].

Germination of orthodox seeds starts with the uptake of water, which enables metabolism to resume and energy reserves to be mobilised [21–23]. By definition, germination sensu stricto is completed when the radicle protrudes through the structures surrounding the embryo [24]. PSSG and GSSG are rapidly reduced early upon germination [25,26], and protein redox state is enzymatically controlled by thioredoxins (TRXs) and glutaredoxins (GRXs), which use NADPH and GSH for de-glutathionylation [7,10]. Cellular redox changes during seed imbibition also involve the production of ROS, which promote key events in germination and seedling development. Particularly, the ROS hydrogen peroxide (H2O2) is thought to be a critical player in alleviating seed dormancy (i.e. the inability to germinate under optimal conditions), stimulating reserve mobilisation, and participating in the regulation of early seedling growth and development [27–32]. However, if the control of ROS levels by antioxidants fails, oxidative damage may result in seed ageing, and eventually death [33].

The half-cell reduction potential (Eh) is an accurate descriptor of the redox state of concentration-dependent redox couples, and changes in the glutathione half-cell reduction potential (E[GSSG/2GSH]) accompany cell development from mitoses to cell death in human cells [34]. In the orchard grass, Dactylis glomerata L., more negative (i.e. more reducing) values of E[GSSG/2GSH] accompany proliferation, and more positive (i.e. more oxidising) values accompany differentiation upon somatic embryogenesis [35]. Furthermore, E[GSSG/2GSH] has been proposed to be a useful marker of seed viability and ageing, as a pronounced shift towards strongly oxidising conditions accompanies programmed cell death (PCD) [2,11,36,37]. Besides the GSH/GSSG redox couple, the Cys and cystine (CySS) couple and the dipeptides γ-Glu-Cys and Cys-Gly, along with their corresponding disulphides (i.e. bis-γ-glutamyl-cystine (bis-γ-Glu-Cys), and cystinyl-bis-glycine (Cys-bis-Gly)) contribute to the cellular LMW thiol-disulphide redox environment (Ethiol-disulphide) in seeds [37]. Ethiol-disulphide is calculated by the sum of the products of the individual half-cell reduction potentials (Eh,s) of all LMW thiol-disulphide redox couples and the reducing capacity of each individual couple [34]. Changes in Ethiol-disulphide have been related to somatic embryogenesis and seed ageing [11,35,37].

In summary, thiol-disulphide conversions of the GSH/GSSG redox couple play important roles during seed
maturation and germination, but their relative contribution in different wheat seed structures has not been studied. Moreover, the intermediates of GSH metabolism, namely Cys, γ-Glu-Cys, and Cys-Gly, have not been assessed in different seed tissues. In the present study, we elucidate changes in the concentrations and redox state of the GSH/GSSG redox couple, alongside those of its metabolic intermediates, in the embryo and the endosperm of wheat seeds during germination and early seedling growth. To provide a more comprehensive view of the changes in LMW thiol-disulphide redox couples that accompany the establishment of the next plant generation, their concentrations, and redox state are considered in embryos and endosperms, separately.

Materials and methods

All chemicals were purchased from Sigma Aldrich, Co. (St. Louise, MO), unless specified otherwise.

Seed material, germination assays, and water content measurements

Bread wheat (*Triticum aestivum* L.) cultivar Rebelde (Aprsovsementi S.p.a., Voghera, Italy – CO.NA.SE. Consorzio Nazionale Sementi S.r.l., Italy) was grown in an open field in Sant’Angelo Lodigiano (Lodi, Italy) in 2013-2014. After harvest, seeds were immediately stored at 4°C and <8% water content (WC), on a fresh weight (FW) basis, and used within 1 year. Seed samples were cleaned (i.e. damaged seeds were discarded and residual glumes were removed) and germinated in 90 mm Petri dishes between two layers of filter paper (Whatman 1, GE Healthcare, Little Chalfont, United Kingdom) moistened with 3 mL of distilled water, each containing 35 seeds (*n* = 7), in darkness at 20°C. Completion of germination was defined as radicle protrusion by at least 1 mm through the coleorhiza, a protective sheath of the radicle, and the seed coat was and scored regularly until all viable seeds had germinated. After 15 h from the onset of imbibition, it was possible to cleanly excise the embryo, including the scutellum (a part of the single modified cotyledon), from the seed using a sterile scalpel. Two time points, 15 h and 48 h, were chosen for detailed studies into the redox state of different seed structures. The remaining seed parts with the endosperm, including the aleurone layer and the fused seed coat and pericarp are hereafter referred to as “endosperm”, for simplicity. All material was weighed to determine the FW, frozen in liquid nitrogen, freeze-dried for 5 days, and then weighed to record the DW. Freeze-dried seeds were finely ground in 5-mL liquid-nitrogen-cooled Teflon capsules with one 7 mm diameter agate ball, using a Mikro-Dismembrator S (B. Braun, Biotech International, Melsungen, Germany) at 3000 rpm for 4 min. Seed WC was calculated on a FW basis by the formula: WC = (FW – DW)/FW × 100.

**HPLC analysis of LMW thiols and disulphides**

At nine intervals up to 48 h after the onset of imbibition, whole seeds (*n* = 4 replicates of 35 seeds), and dissected embryos and endosperms isolated after 15 h or 48 h of imbibition (*n* = 4 replicates of 40 seeds) were analysed by HPLC. Freeze-dried powder from whole seeds (70 ± 6 mg), endosperms (50 ± 0.5 mg), or embryos (25 ± 5.0 mg) were extracted at 4°C in 1 mL of 0.1 M HCl, vortexed at full speed for 1 min before centrifugation at 20,000 × g for 20 min at 4°C. An aliquot of 120 µL of the supernatant was used for the determination of total LMW thiols and disulphides, and 400 µL for assessing LMW disulphides according to Kranner (1998) [38], and described in detail by Bailly and Kranner (2011) [39]. The pH of the extracts was adjusted to values between 8.0 and 8.3 with 200 mM bicine buffer. To measure both LMW thiols and disulphides, the latter were reduced by 273 µM dithiothreitol (DTT, Applichem Gmbh, Darmstadt, Germany) for 1 h at room temperature, before labelling of thiols with 857 µM monobromobimane (mBBr) for 15 min at room temperature, and stopping the reaction with 0.104% (v/v) methanesulfonic acid. To measure disulphides, thiols were blocked with 583 µM N-ethylmaleimide (NEM) for 15 min at room temperature, before excess NEM was removed five times with toluene, and disulphides were reduced with DTT, then labelled with mBBr as for measuring total LMW thiols and disulphides. Labelled LMW thiols were separated by reversed-phase HPLC using an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) on a ChromBudget 120-5-C18 column (5.0 µm, BISCHOFF GmbH, Leonberg, Germany), and detected by a fluorescence detector (the excitation wavelength was set at 380 nm and the emission wavelength at 480 nm). Data were calculated using individual calibration curves for each LMW thiol that were linear over the range measured. The concentrations of LMW thiols were calculated by subtracting the concentrations of LMW disulphides from those of total LMW disulphides and thiols.

**Calculation of the cellular LMW thiol-disulphide based redox environment**

The half-cell reduction potential (E$_{\text{red}}$) for each thiol-disulphide couple was calculated according to the Nernst equation, and mathematically combined into the
LMW thiol-disulphide redox environment (E_{thiol-disulphide}) [2,37]:

$$E_i = \frac{E^0 - RT}{nF} \ln \frac{[\text{LMW thiol}]}{[\text{LMW disulphide}]}$$

(1)

$$E_{\text{thiol-disulphide}} = \sum_{j=1}^{n} E_j \times [\text{reduced species}]$$

(2)

where $R$ is the gas constant (8.314 J K$^{-1}$ mol$^{-1}$); $T$, temperature in K; $n$, number of transferred electrons (2, GSH $\rightarrow$ GSSG + 2H$^+$ + 2e$^-$); $F$, Faraday constant (9.6485 $\times$ 10$^4$ C mol$^{-1}$); $E^0_{i}$, standard half-cell reduction potential of a thiol-disulphide redox couple at an assumed cellular pH of 7.3 ($E^0_{\text{GSSG/GSH}} = -258$ mV); $E^0_{\text{CySS/2Cys}} = -244$ mV; $E^0_{\text{Cys-bis-Gly/2Cys-Gly}} = -244$ mV; $E^0_{\text{bis-Glu-Cys/2-Glu-Cys}} = -252$ mV [37]. $E_i$ is the half-cell reduction potential of an individual redox couple $i$, and [reduced species] is the concentration of the reduced species in that redox pair. The molar concentrations of LMW thiols and disulphides for each redox couple were calculated based on seed WC calculated as g H$_2$O g$^{-1}$ DW.

**Oxalate oxidase activity measurements and quantification of extracellular H$_2$O$_2$ production**

Oxalate oxidase (EC 1.2.3.4, oxalate: oxygen oxidoreductase, OXO) activity of whole seeds was determined spectrophotometrically, using a modification of the method of Laker et al. (1980) [40]. OXO catalyses the oxidative breakdown of one mole of oxalate to one mole of H$_2$O$_2$ and two moles of CO$_2$. Briefly, H$_2$O$_2$ was indirectly quantified via the formation of indamine, catalysed by the H$_2$O$_2$-dependent horseradish peroxidase (HPOX), by following the increase in absorbance at 555 nm ($A_{555}$). Absorbance values were calibrated using a standard curve for H$_2$O$_2$ within a 2.4–120 μM range. All extraction steps were conducted at 4°C (on ice). 50 ± 0.5 mg of seed powder were homogenised in 0.4 mL of 50 mM succinate buffer (pH 3.8) containing 1 mM EDTA and 9.6 mM oxalic acid in 2 mL Eppendorf tubes with two 3 mm glass beads (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), using a TissueLyser II (Qiagen, Hilden, Germany) for 1 min at 30 Hz. After centrifugation at 20,000 $\times$ g for 5 min at 4°C, the supernatant was collected and centrifuged again at 20,000 $\times$ g for 2 min. OXO activity was measured in 96-well BRANDplates® (pureGRADE™ S-clear, Sterile R, BRAND GmbH + CO KG, Wertheim, Germany), using a Synergy-HTX plate reader (BioTek® Instruments, Inc., Winooski, VT) at 35°C after pre-incubating 51.2 μL of protein extract and oxalic acid for 15 min. The increase in A$_{555}$ was followed for 10 min after adding 185 μL of a chromogenic solution (790 μM of N,N-dimethylalanine and 110 μM of 3-methyl-2-benzothiazolinone hydrazone dissolved in 50 mM succinate buffer, pH 3.8) and 2.5 U/mL of HPOX in a total volume of 240 μL, with a final concentration of oxalic acid of 2 mM. A reaction mixture without protein extract and oxalic acid was used as a blank. Three technical replicates for each biological replicate ($n = 4$) were measured.

H$_2$O$_2$ was quantified using a “Red Hydrogen Peroxide Assay Kit”, following optimisation of instructions by the manufacturer (Enzo Life Sciences Inc., Farmingdale, NY). Whole seeds and seedlings were incubated in 1 mL of two times concentrated reaction mixture containing 10 μL of red peroxidase substrate stock solution in dimethyl sulphoxide (DMSO), 40 μL of 20 U/mL HPOX stock solution, and 950 μL of assay buffer in darkness at room temperature for 30 min, then diluted 1:2 with the reaction buffer. Fluorescence of the HPOX- and H$_2$O$_2$-catalysed formation of Resorufin (excitation wavelength = 540 ± 35 nm, emission wavelength = 590 ± 20 nm) was measured with a Synergy-HTX plate reader (BioTek® Instruments, Inc., Winooski, VT). Data were corrected for background fluorescence occurring in the presence of pure assay buffer. Three technical replicates were measured for each biological replicate ($n = 4$, each containing 25 seeds). The content of extracellular H$_2$O$_2$ produced was calculated from a standard curve for H$_2$O$_2$ (0–3 μM range), and data are expressed as nmol g$^{-1}$ DW s$^{-1}$.

**Statistical analysis**

Data were analysed for significance ($\alpha = 0.05$) by one-way ANOVA in combination with Tukey’s HSD (Honest Significant Difference) test for post-hoc comparisons of means ($p$-value $\leq 0.05$), using the IBM SPSS Statistics 21 software package. Arcsine transformation was applied to germination and WC values to simulate normal distribution of data. The assumption of normal distribution was assessed via the Shapiro–Wilk test, and further verified with QQ-plots. The assumption of homoscedasticity of variances across groups was checked through Levene’s test, and, whenever not respected, the appropriate mathematical transformations were applied to the data. To compare different seed structures at the same time interval a one-sample $t$-test was applied ($p$-value $\leq 0.05$).

**Results**

**Seed germination and water uptake**

The first radicle protruded 12 h after the onset of imbibition, and 50% of total germination (TG) was reached...
after approximately 22 h (Figure 1). Within 48 h, 96% of seeds had germinated. The radicle and two seminal roots developed (in wheat this is indicative of healthy seedlings), and the coleoptile (a protective sheath covering the emerging shoot in monocotyledon plants) could be clearly distinguished in most seedlings. After 8 h of imbibition, the water uptake slowed down (Figure 1), typical for Poaceae seeds [41,42].

After 15 h from the onset of imbibition, it was first possible to accurately dissect the embryo from the endosperm; embryos and endosperms isolated from germinated (G) seeds and non-germinated (NG) seeds were analysed separately. After 15 and 48 h from the onset of imbibition, the embryo made up only 3.1 ± 0.2% and 4.9 ± 0.2% (means ± SE) of seed DW, respectively. Embryos and endosperms took up water at different rates, and the former always contained more water than the latter (Figure 2).

Changes in LMW thiols and disulphides in whole seeds during germination and early seedling growth

GSH + GSSG [hereafter termed “total glutathione”] and Cys + CySS [hereafter referred to as “cyst(e)ine”] dominated the LMW thiol-disulphide redox pool in dry whole seeds, whereas the concentrations of Cys-Gly + Cys-bis-Gly [hereafter termed as “cyst(e)inyl-(bis)-glycine”] and γ-Glu-Cys + bis-γ-Glu-Cys [hereafter referred to as “(bis)-γ-glutamyl-cyst(e)ine”] were lower by one order of magnitude (Figure 3(A–D)). Dry wheat seeds contained (bis)-γ-glutamyl-cyst(e)ine, respectively (Figure 3(A–D)). Disulphide contents in dry whole seeds, expressed as a percentage of total thiols and disulphides, were 30% for bis-γ-Glu-Cys, 36% for GSSG, 40% for Cys, and 41% for Cys-bis-Gly (Figure 3(A–D)). Species of the Poaceae may also contain a GSH homologue termed hydroxy-methylglutathione (hGSH, γ-L-glutamyl-L-cysteinyl-β-serine) [43], but no corresponding peak was detected in seeds or seedlings during the first 48 h after the onset of imbibition.

Total glutathione concentrations rose 1.3-fold within the first 8 h of imbibition, GSH increased by 43%, and GSSG decreased by 42%. A further significant increase in total glutathione, mostly due to GSH, was recorded during early seedling growth (i.e. between 24 h and 48 h after the onset of imbibition) (Figure 3(A)).

Cyst(e)ine concentrations steadily increased within 24 h, after which concentrations greatly increased (Figure 3(B)). Hence, after 48 h, cyst(e)ine was the most abundant LMW thiol-disulphide redox couple, and its concentration became 1.8 times higher than that of total glutathione (Figure 3(A,B)). Within the first 12 h of imbibition, cyst(e)inyl-(bis)-glycine decreased 2.3-fold, (bis)-γ-glutamyl-cyst(e)ine increased 2.8-fold, then both plateaued for the subsequent 12 h (Figure 3(C,D)). From 24 h of imbibition, when approximately 62% of seeds had germinated (Figure 1), cyst(e)inyl-(bis)-glycine and (bis)-γ-glutamyl-cyst(e)ine both increased progressively.
Changes in LMW thiol-disulphide redox couples in endosperm and embryo on a DW and on a seed basis

After 15 and 48 h from the onset of imbibition, seeds were sorted into two lots, G and NG, and embryos and seedlings were separated from the endosperms. At these intervals, we characterised the concentrations of LMW thiols and disulphides, expressed on a DW basis (Figure 4(A–D)), and their corresponding Ehcs in the two seed structures (Figure 4(E–H)). As in whole seeds, total glutathione and cyst(e)ine were the predominating LMW thiol-disulphide redox couples in both endosperm and embryo. Fifteen hours after the onset of imbibition, the endosperms isolated from NG seeds (termed "NG15_endosperms") contained the same concentrations of LMW thiols and disulphides as the endosperms isolated from germinated seeds (termed "G15_endosperms"). By contrast, embryos isolated from NG seeds 15 h after the onset of imbibition (termed "NG15_embryos") contained 3–19 times higher concentrations of all LMW thiols than whole dry seeds. Embryos with radicles protruding at least 1 mm and isolated from germinated seeds 15 h after the onset of imbibition (termed "G15_embryos") had about one third more total glutathione, one forth more cyst(e)inyl-(bis)-glycine, and five times more (bis)-γ-glutamyl-cyst(e)line than NG15_embryos. In particular, G15_embryos contained more disulphides than NG15_embryos (p-value ≤.05), except for CySS (Figure 4(A–D)).

After 48 h from the onset of imbibition, 96% of seeds had germinated (Figure 1). Only germinated seeds were characterised for their concentrations of LMW thiols and disulphides in isolated seed structures 48 h after the onset of imbibition. Total glutathione in the endosperms isolated from germinated seeds 48 h after the onset of imbibition (termed "G48_endosperms") dropped by 16% compared to G15_endosperms (p-value ≤.05). Conversely, cyst(e)ine, cyst(e)inyl-(bis)-glycine, and (bis)-γ-glutamyl-cyst(e)line increased about five-, two-, and four-fold, with high disulphide percentages (52, 44, and 40%, here arranged from the highest to the lowest contribution to each thiol-disulphide redox pool). In seedlings with coleoptile, radicle, and two seminal roots isolated from germinated seeds 48 h after the onset of imbibition (termed "G48_embryos"), total glutathione, cyst(e)inyl-(bis)-glycine, and
Figure 4. Concentration, on a DW basis, and redox state of four low-molecular-weight (LMW) redox couples in structures isolated from *Triticum aestivum* seeds upon imbibition. After 15 h from the onset of imbibition, white bars for LMW thiols and black bars for their corresponding disulphides show dry weight-based concentrations in endosperms and embryos isolated from NG seeds or G seeds. After 48 h from the onset of imbibition, bars show thiol and disulphide DW-based concentrations for endosperms and seedlings isolated from germinated seeds (panels A–D). Bars on the right side show the half-cell reduction potentials of the four LMW thiol-disulphide redox couples in the endosperm (dark grey bars) and embryo or seedling (light grey bars), respectively (panels E–H). Data for endosperms and embryos or seedlings were tested for significance using one-way ANOVA analyses followed by post-hoc Tukey's HSD test for thiols (lower case letters) and disulphides (upper case letters). Data points labelled with the same letter do not differ significantly (*p*-value ≤ 0.05; italics is used for comparing the endosperms). Data are means ± SE (*n* = 4 replicates of 40 endosperms and embryos or seedlings per condition). NG: non-germinated; G: germinated; 15 and 48 indicate the hours after the onset of imbibition.
(bis)-γ-glutamyl-cyst(e)ine concentrations dropped two-, three-, and two-fold compared to G15 embryos, whereas cyst(e)ine almost doubled. Low disulphide percentages were found for total glutathione (0.1%), cyst(e)inyl-(bis)-glycine (1.5%), and (bis)-γ-glutamyl-cyst(e)ine (1.8%), but not for (bis)-γ-glutamyl-cyst(e)ine, which was found as bis-γ-Glu-Cys for 20.4% (Figure 4(A–D)). In summary, G15 embryos showed markedly higher concentrations of LMW thiols and disulphides than NG15 embryos, but this trend was not evident in NG15 endosperms and G15 endosperms. NG15 embryos, G15 embryos and G48 embryos had far higher thiol-disulphide ratios than NG15 endosperms, G15 endosperms and G48 endosperms (except for the redox couple γ-Glu-Cys/bis-γ-Glu-Cys in G15 embryos) (Figure 4(A–D)). Thereafter, a phase of early seedling growth was identified by comparing G15 embryos and G48 embryos. In both G48 embryos and G48 endosperms, total glutathione declined, while cyst(e)ine increased. In contrast, cyst(e)inyl-(bis)-glycine and (bis)-γ-glutamyl-cyst(e)ine increased in G48 endosperms, but decreased in G48 embryos (Figure 4(A–D)).

When the total contents of LMW thiol-disulphide redox couples were expressed on a seed basis, their concentrations and proportions in the overall LMW thiol-disulphide redox pool changed distinctly (Figure S1). In particular, NG15 endosperms and G15 endosperms had the same concentrations and proportional distributions of the four LMW thiol-disulphide redox couples. In G48 endosperms, the total LMW thiol-disulphide redox pool increased 1.5-fold, and cyst(e)ine became the dominant LMW thiol-disulphide redox couple (Figure S1A). By contrast, after 15 h, the total LMW thiol-disulphide redox pool in G15 embryos was greater than in NG15 embryos, with a larger proportion represented by (bis)-γ-glutamyl-cyst(e)ine (Figure S1B). The total LMW thiol-disulphide redox pool further increased in G48 embryos compared to G15 embryos, with a decline in cyst(e)inyl-(bis)-glycine, and an increase in cyst(e)ine proportions, but total glutathione remained the dominant LMW thiol-disulphide redox couple (Figure S1B).

**Changes in LMW thiol-disulphide half-cell reduction potentials in endosperm and embryo**

The tissue WCs (g H₂O g⁻¹ DW; Figure 2) were used to calculate the molar concentrations of all thiols and disulphides (Figure S2), which are required for the individual EHcs of the four thiol-disulphide redox couples (Figure 4(E–H)). The EGSSG/2GSH⁺ half-cell reduction potential of the cysteinyl-glycine/cystinyl-bis-glycine redox couple (ECys-bis-Gly/2Cys-Gly), and the half-cell reduction potential of the γ-Glu-Cys/bis-γ-Glu-Cys redox couple (Ebis-γ-Glu-Cys/2γ-Glu-Cys) did not differ between NG15 endosperms and G15 endosperms. However, values of the half-cell reduction potential of the CySS/2Cys redox couple (ECySS/2Cys) were more oxidising in NG15 endosperms. ECySS/2Cys shifted back to more negative values in G48 endosperms compared to G15 endosperms (Figure 4(F)), and E_GSSG/2GSH and ECySS/2Cys values were by 19 mV and 15 mV more positive than in NG15 embryos. These values shifted back to more negative values (by 41 mV and 6 mV) in G48 embryos (Figure 4(E,G)). Values of ECySS/2Cys did not differ significantly between NG15 embryos and G15 embryos or G15 embryos and G48 embryos. The Ebis-γ-Glu-Cys/2γ-Glu-Cys did not differ between NG15 embryos and G15 embryos, but in G48 embryos, it shifted to a more negative value than that of NG15 embryos.

Due to their highest molar concentrations (Figure S2), EGSSG/2GSH and ECySS/2Cys were the most influential contributors to the mathematically combined Ethiol-disulphide of dry whole seeds, NG15 endosperms, G15 endosperms, G48 endosperms, and NG15 embryos, G15 embryos and G48 embryos (i.e. all measured samples), whereas ECys-bis-Gly/2Cys-Gly, and Ebis-γ-Glu-Cys/2γ-Glu-Cys together contributed less than 8% (Table 1). ECySS/2Cys contributed more to Ethiol-disulphide in endosperms than in embryos or seedlings, and the contributions of ECySS/2Cys increased in both seed structures during early seedling growth.

**Changes in the H₂O₂ production during germination and early seedling growth**

The H₂O₂ production rate decreased three-fold from the first imbibition interval to 16 h after the onset of imbibition (Figure 5). Between 16 and 32 h after the onset of imbibition, when the majority of seeds germinated (Figure 1), the rate of H₂O₂ production increased by five-fold and further increased after 48 h (Figure 5). Similar to the pattern of H₂O₂ production, the activity of OXO decreased in the first 16 h of imbibition and then increased, but the trend was much less pronounced (Figure 5).

**Discussion**

**Early seedling growth is accompanied by GSH synthesis and mobilisation of Cys**

In this paper, we report on LMW thiol-disulphide conversions involved in the transition from a seed to a
Table 1. Relative contribution of the individual half-cell reduction potentials (E_{1/2}) of the four LMW thiol-disulphide redox couples to the LMW thiol-disulphide redox environment (E_{thiol-disulphide}) in dry whole seeds and isolated *Triticum aestivum* seed structures upon imbibition. Values for the individual contributions of the thiol-disulphide redox couples are expressed as percentages of E_{thiol-disulphide} calculated for dry whole seeds and seed structures isolated after 15 or 48 h from the onset of imbibition. Data show means ± SE (n = 4). G: germinated; NG: non-germinated.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Physiological state</th>
<th>Seed structure</th>
<th>E_{GSSG/2GSH} (%)</th>
<th>E_{CySS/2Cys} (%)</th>
<th>E_{Cys-bis-Glu/2Cys-Gly} (%)</th>
<th>E_{bis-Cys-Gly} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>dry</td>
<td>Whole seed</td>
<td>84.0 ± 1.3</td>
<td>8.0 ± 1.2</td>
<td>7.0 ± 1.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>15</td>
<td>NG</td>
<td>Endosperm</td>
<td>86.7 ± 1.0</td>
<td>9.9 ± 0.8</td>
<td>3.3 ± 0.3</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td></td>
<td>NG</td>
<td>Embryo</td>
<td>93.3 ± 0.6</td>
<td>4.5 ± 0.5</td>
<td>2.2 ± 0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>Endosperm</td>
<td>89.1 ± 1.4</td>
<td>6.3 ± 0.9</td>
<td>3.2 ± 0.2</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>Embryo</td>
<td>93.0 ± 0.7</td>
<td>4.4 ± 0.3</td>
<td>2.0 ± 0.2</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>48</td>
<td>G</td>
<td>Endosperm</td>
<td>58.2 ± 2.4</td>
<td>34.9 ± 1.9</td>
<td>3.3 ± 0.4</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>Seedling</td>
<td>89.7 ± 0.6</td>
<td>8.5 ± 0.5</td>
<td>0.8 ± 0.0</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
</table>

In wheat, GSH-dependent redox activity is apparently required for storage protein mobilisation, one of the key events in early seedling growth [21,47]. In dry seeds, 36% of total glutathione was present as GSSG (Figure 3(A)), which typically accumulates during seed maturation drying, and has been related to the protection of PSH from oxidation upon desiccation [8,10]. These relatively high GSSG contents, which have potential to inhibit protein synthesis [8,25,48–50], were rapidly reduced within the first 4 h of imbibition, in agreement with earlier studies [25,26]. Cysteinyl-bis-glycine was the only other disulphide that degraded before radicle protrusion (Figure 3(C)), but the role of Cys-Gly in plant metabolism is far from understood. In whole seeds, GSH contents increased within the first 4 h, likely due to GSSG reduction and GSH synthesis (Figure 3(A)). Furthermore, both Cys and γ-Glu-Cys steadily increased within 12 h of imbibition, when the first radicles protruded, and Cys concentrations further rose up to 32 h. A further significant increase of Cys and γ-Glu-Cys occurred between 32 and 48 h, when all viable seeds had developed into seedlings (Figure 3(B,D)). Further to being a precursor of GSH, γ-Glu-Cys is a by-product from glutathione-associated catabolism via cytosolic γ-glutamyl cyclotransferases or vacuolar γ-glutamyl transpeptidases [5]. Forty-eight h after the onset of imbibition, the approximately four-fold increase in γ-Glu-Cys argues for GSH synthesis. However, at this interval Cys concentrations were much higher than GSH concentrations, suggesting that sources other than GSH metabolism were implicated in Cys production. Notably, the endosperm of wheat seeds contains gluten proteins, 75% of which are Cys-rich prolamins [19], and their mobilisation and degradation after 48 h are a likely source for the raised Cys, and possibly Cys-Gly, concentrations. Because of its potential toxicity [51–53], plants readily incorporate Cys into GSH, which is the main transport form of reduced sulphur [4]. The high Cys concentrations found in wheat seeds are unusual. Therefore, we assessed whether the LMW thiols and disulphides in whole seeds were located in the
endosperm or the embryo. The embryo and endosperm took up water at different rates (Figure 2). As the \( E_{nc} \) (Figure 4(E–H)) are calculated from molar concentrations [34], it is helpful to show thiol and disulphide concentrations on a molar basis (Figure S2). In the Nernst equation, the molar concentrations of the thiols are squared terms (Equation (1)), and therefore the \( E_{nc} \) of a thiol-disulphide redox couple depends on both the thiol-disulphide ratio and the molar thiol concentrations [34]. Finally, considering that the embryo makes up only 3% of the DW of a seed, we also show the LMW thiol-disulphide concentrations on a seed basis (Figure S1), which is useful when considering the total pool sizes of each redox couple.

**Changes in \( E_{GSSG/2GSH} \) during the transition from seed to seedling**

Changes in the cellular redox environment appear to be intricately involved in the control of the life cycle in animal and plant cells, from the first mitotic division, through differentiation, up to cell death [34,54,55]. Before the growth of the radicle and the extension of the coleoptile, seed germination mostly occurs by cell expansion rather than cell division [56]. The net production of total glutathione, supported by elevated concentrations of GSH intermediates (\( \gamma \)-Glu-Cys and Cys-Gly), was observed in G15_embryos compared to NG15_embryos (Figure 4(A–D)). These data suggest that radicle protrusion and early seedling growth could be supported by de novo synthesis of GSH. Elevated GSH contents were reported to occur in meristematic regions in rapidly growing tissues by Bielawsky and Joy (1986) [57]. A glutathione-dependent pathway was shown to control the initiation and maintenance of cell division in the roots of the model plant *Arabidopsis thaliana* [58], and specifically in the root apical meristem [58–60]. However, in G15_embryos of wheat this net GSH production was accompanied by net GSSG, Cys-bis-Gly and bis-\( \gamma \)-Glu-Cys accumulation (Figure 4(A,C,D)). Because the molar concentrations of GSH and Cys-Gly did not differ between NG15_embryos and G15_embryos (Figure S2(A)), partly due to a higher WC in the latter, the resulting \( E_{GSSG/2GSH} \) and \( E_{Cys-bis-Gly/2Cys-Gly} \) values were more oxidising in G15_embryos compared to NG15_embryos (Figures 4(E,G)). A shift towards more oxidising conditions was reported to enhance histodifferentiation and post-embryonic growth in somatic embryos of various plants species [35,61]. Taken together, increased GSSG levels, in conjunction with more oxidising conditions in G15_embryos, suggest that cell differentiation took place.

Upon early seedling growth, between 15 and 48 h after the onset of imbibition, the concentrations of GSH, \( \gamma \)-Glu-Cys and Cys-Gly decreased, and \( E_{GSSG/2GSH} \) and \( E_{Cys-bis-Gly/2Cys-Gly} \) shifted towards more reducing conditions (Figure 4(E,G,H)). This is in agreement with the requirement of the growing seedling for conditions that support cell division, and consistent with reports on cell cultures. For example, exogenous treatment of white spruce somatic embryos with GSH led to more reducing conditions and increased mitotic activity over cellular expansion [62]. Furthermore, the formation of pro-embryogenic masses, requiring cell division, before the formation of somatic orchard grass embryos correlates with reducing cellular conditions [35]. Therefore, more reducing conditions in G48_embryos (significant for all LMW thiol-disulphide redox couples, except for cyst(e)ine) could be associated with progressive proliferation required for organ growth.

**\( E_{CySS/2CyG} \) and \( E_{GSSG/2GSH} \) related to physiological processes in the endosperm**

The endosperm of a mature wheat seeds consists mainly of dead tissue, except for the aleurone layer, which also undergoes PCD from approximately 48 h after the onset of imbibition [44]. Therefore, the finding that in all conditions studied the \( E_{nc} \) of all four thiol-disulphide redox couples in the endosperm were more oxidising than those measured in the embryos and seedlings (Figure 4(E–H)) is consistent with the positive correlation between PCD and shifts towards more oxidising conditions [2].

Already after 15 h, significant increases in the thiol-disulphide redox couples were found between NG15_embryos and G15_embryos, but no significant increases were recorded for NG15_endosperms and G15_endosperms. However, upon seedling growth (between 15 and 48 h), the two dipeptide thiols (and their corresponding disulphides) and cyst(e)ine accumulated at higher concentrations than can be explained from GSH degradation at this time period (Figure 4(A–D)). This trend became particularly clear when data were expressed on a seed basis (Figure S1). A possible interpretation for the accumulation of the intermediates of GSH metabolism could be the de-glutathionylation of PSSG. After 48 h, proteolytic activities are detected in wheat and are crucial for the mobilisation Cys-rich prolams located in the endosperm [63,64], which are a likely source for the increase in cyst(e)ine (Figure 4B).

The role of \( E_{CySS/2CyG} \) did not receive attention by plant scientists. However, in animal cells the redox state of Cys/CySS is viewed as an essential part of thiol-disulphide conversions [65,66]. Upon early seedling...
growth, the difference between $E_{GSSG/2GSH}$ and $E_{CySS/2Cys}$ in the endosperm decreased (Figures 4(A,B,E,F) and 6). The significant shift towards more reducing $E_{CySS/2Cys}$ values between G15_endosperms and G48_endosperms resulted from the increase in Cys concentrations (Figure 4(B,F)). By contrast, the equivalent $E_{GSSG/2GSH}$ values showed a significant oxidative shift (Figure 4(E)), which could be due to processes such as de-glutathionylation of storage proteins that releases GSSG, or GRXs-mediated reduction of TRX $h$. In wheat, TRX $h$ is directly involved in the reduction of prolamins, an essential step to increase storage protein susceptibility to proteolytic degradation during the first 48 h after the onset of imbibition [67]. GRX/GSH-mediated reduction of TRX $h$ has been shown to occur in different plant systems [68,69], but a role for GRX/GSH in storage protein mobilisation in cereals has yet to be confirmed. Finally, the oxidative shift in $E_{GSSG/2GSH}$ could also mark the commencement of the apoptotic-like PCD programme in the aleurone layer after the release of hydrolases [44].

For comparison with previous work, we also calculated $E_{thiol-disulphide}$ as suggested by Schafer and Buettner [34]. $E_{thiol-disulphide}$ is perhaps a crude estimate of the cellular redox environment, which would benefit from including other redox couples, such as NADPH/ NADP$^+$, but $E_{thiol-disulphide}$ may suffice to take a “snapshot” of the metabolic state. $E_{thiol-disulphide}$ was useful for assessing seed ageing [35] and the capability for somatic embryogenesis [37]. The current study shows that $E_{CySS/2Cys}$ contributed more to $E_{thiol-disulphide}$ in endosperms than in embryos or seedlings, reinforcing the role of $E_{CySS/2Cys}$ in the endosperm, and in both embryo and endosperm during early seedling growth.

**A role for $H_2O_2$ production in altering $E_{2GSH/GSSG}$?**

Production of $H_2O_2$ increased more than five-fold after 16 h of imbibition, similar to elevated ROS production in imbibed pea seeds [30]. Oxidation of protein cysteine thiolates by $H_2O_2$ is key to redox signalling in mammalian cells [70,71]. Interestingly, the only protein known to be synthesised de novo upon the first hours of cereal seed imbibition was called “germin” for several years. Later, germin was characterised as an OXO, a main source of $H_2O_2$ production during wheat seed germination and seedling growth [56,72]. The rates of $H_2O_2$ production between 0 and 16 h of imbibition paralleled the activity of OXO (Figure 5), which is located in the cell wall [73]. Therefore, $H_2O_2$ production could be related to OXO activity within the first 16 h of imbibition. Notably, the lowest $H_2O_2$ production rates at 16 h were close to the time interval when values of $E_{GSSG/2GSH}$ in G15_embryo were the most oxidising. The increase in $H_2O_2$ production between 16 to 48 h of imbibition was higher than could be expected from OXO activity (Figure 5), indicating that alternative sources of $H_2O_2$ production, such as NADPH oxidases, were active [30,74]. From 15 to 48 h after the onset of imbibition, the majority of seedlings had developed a radicle, two seminal roots and a coleoptile. While $H_2O_2$ progressively increased over this period, $E_{GSSG/2GSH}$ values shifted to more oxidising conditions in the endosperm and more reducing conditions in the seedling (Figures 4(E) and 5), as summarised in (Figure 6). Considering that $H_2O_2$ is a pro-oxidant, $H_2O_2$ levels were closer linked to the $E_{GSSG/2GSH}$ of the endosperm than the embryo. At around 48 h after the onset of the imbibition, the aleurone surrounding the endosperm initiates PCD [44], which could be related to the elevated $H_2O_2$ concentrations. In conclusion, this study shows that $E_{GSSG/2GSH}$ was the most influential LMW thiol-disulphide redox couple in growing seedlings, as expected for the dominant role of GSH/GSSG in the cell.
cycle [54]. However, the major increase of cyst(e)ine, which became the dominant LMW thiol-disulphide redox couple of the endosperm in germinated seeds, highlights the relevance of this redox couple to early seedling growth of bread wheat.

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