Regulatory functions of ROS dynamics via glutathione metabolism and glutathione peroxidase activity in developing rice zygote

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SUMMARY

Reactive oxygen species (ROS) play essential roles in plant development and environmental stress responses. In this study, ROS dynamics, the glutathione redox status, the expression and subcellular localization of glutathione peroxidases (GPXs), and the effects of inhibitors of ROS-mediated metabolism were investigated along with fertilization and early zygotic embryogenesis in rice (Oryza sativa). Zygotes and early embryos exhibited developmental arrest upon inhibition of ROS production. Egg cells accumulated high ROS levels, and, after fertilization, intracellular ROS levels progressively declined in zygotes in which de novo expression of GPX1 and 3 was observed through upregulation of the genes. In addition to inhibition of GPX activity, depletion of glutathione impeded early embryonic development and led to failure of the zygote to appropriately decrease H2O2 levels. Moreover, through monitoring of the glutathione redox status, the developing zygotes exhibited a progressive glutathione oxidation, which became extremely delayed under inhibited GPX activity. Our results provide insights into the importance of ROS dynamics, GPX antioxidant activity, and glutathione redox metabolism during zygotic/embryonic development.

Keywords: reactive oxygen species dynamics, glutathione peroxidases, glutathione, zygote, embryogenesis, Oryza sativa.

INTRODUCTION

In angiosperms, after successful pollination, two sperm cells derived from a pollen are delivered into the embryo sac via a pollen tube (Drews and Yadegari, 2002; Huang and Russell, 1992). One sperm cell fuses with the egg cell to form a zygote and the other fuses with the central cell to form a triploid primary endosperm cell. The zygote and the primary endosperm cell develop into an embryo that inherits the parental genetic material and an endosperm that nourishes the developing embryo/seedling, respectively (Dresselhaus et al., 2016; Guignard, 1899; Nawaschin, 1898). The egg cell is specially differentiated for fertilization and subsequent embryogenesis, and it has been reported that the egg cell is halted at the G1 phase of the cell cycle and fusion with the sperm cell triggers conversion of the egg cell from a quiescent state at the G1 phase into an active state toward cell cycle progression (Sukawa and Okamoto, 2018; Zhao et al., 2017). However, our understanding of the cellular mechanisms underlying the maintenance of the egg cell at the quiescent G1 phase and subsequent progression into active zygotic states is still limited.

In somatic tissues, such as root apical meristem (RAM) cells, it has been reported that high intracellular reactive oxygen species (ROS) levels are related to maintenance of the cellular status in a quiescent state or at the G1 phase (Jiang et al., 2003; de Simone et al., 2017). ROS are reactive molecules derived from oxygen, which are even more reactive than molecular oxygen (O2) itself (Mittler, 2017). In plants, ROS are produced via basal metabolic pathways in various subcellular compartments, including photosynthesis in chloroplasts, photosynthesis in peroxisomes, aerobic respiration in mitochondria, and apoplastic ROS production by plasma membrane-localized NADPH oxidase (respiratory burst oxidase homologs [RBOHs]) (Waszczak et al., 2018). Overproduction of ROS can be harmful to the cells and lead to oxidative damage in cellular compartments. Plant cells have, in turn, evolved efficient redox
antioxidant defense system to maintain a balance of ROS and antioxidant capacity (Mittler, 2017; Waszczak et al., 2018). However, the paradigm regarding the destructive effect of ROS has been recently shifted toward the beneficial roles of ROS in signaling mechanisms (Foyer et al., 2017). The antioxidant system is nowadays perceived to involve processing ROS into the most potent and stable forms that are suitable for the redox signaling module (Waszczak et al., 2018). For example, superoxide (O$_2^-$), generated by the mitochondrial electron transport chain (ETC) or RBOHS, is converted to hydrogen peroxide (H$_2$O$_2$) by superoxide dismutases (SODs) (Aldrich et al., 2002). Then, H$_2$O$_2$ is reduced into water by various enzymatic antioxidants, including catalases (CATs), ascorbate peroxidases (APXs), glutathione peroxidases (GPXs), and thioredoxin peroxidases (TPXs) (Waszczak et al., 2018). In addition, non-enzymatic antioxidants, such as ascorbate and reduced glutathione (GSH), are known to be involved in ROS detoxification processes (Foyer and Noctor, 2011).

The interplay between ROS and their partner antioxidants has been shown to play essential roles in signaling pathways during plant development and environmental stress responses (Meinhard et al., 2002).

It has been indicated that ROS are involved in reproductive processes such as megagametogenesis (Martin et al., 2013a), programmed cell death of tapetal cells (Xie et al., 2014), pollen–pistil interaction, pollen tube growth and reception, and degeneration of synergids (Martin et al., 2013b; Duan et al., 2014; see Zhang et al., 2020). As for pre- and post-gamete fusion between egg and sperm cells, direct assessment of ROS levels in developing zygotes/embryos has not yet been performed, although decreased ROS levels in the fertilized embryo sac are proposed to promote proper embryonic development (Martin et al., 2013b). However, after gamete fusion, developmental events, such as egg activation, cell cycle restart, karyogamy, and reorganization of cell polarity, sequentially progress in zygotes, and it can be estimated that the ROS signal is related to this developmental machinery essential for proper zygotic growth. The female reproductive apparatus of angiosperms, the embryo sac, is deeply embedded in ovular tissues, which impedes the investigation of important molecular events induced by fertilization in gametes and zygotes. Therefore, in this study, using isolated rice (*Oryza sativa*) egg cells and zygotes produced by *in vitro* fusion (Uchiumi et al., 2007), intracellular ROS dynamics were directly monitored with specific ROS probes. In addition, ROS production inhibitors specific to subcellular locations were employed for the observation of the developmental profile of zygotes/embryos under defective ROS metabolism. Using these approaches, we were able to demonstrate important roles for GSH metabolism and GPX expression and activity, which provided insights into ROS and antioxidant functions during early development in rice.

## RESULTS

### Depletion of intracellular ROS inhibits first cleavage of rice zygotes

ROS have been recently reported to act as regulators of cell cycle progression and embryonic development in both animals and plants (de Simone et al., 2017; Han et al., 2018). Due to the lack of developed chloroplasts, intracellular ROS in non-photosynthetic plant embryos are primarily produced by the mitochondrial ETC (mETC), mainly through Complexes I, II, and III, and/or plasma membrane-localized NADPH oxidases (respiratory burst oxidase homologs [RBOHs]) (Huang et al., 2016; Kadota et al., 2015). To address whether intracellular ROS are involved in the process of zygotic development in plants, we first treated rice zygotes produced by electrofuson with 3.5 mM malonate, a reversible inhibitor of mitochondrial Complex II, since ROS production by the mETC is suppressed by malonate (Han et al., 2018). The concentrations of ROS inhibitors used in the present study were preliminarily optimized by qualitative assessment of the inhibitory effects on zygotic development and viability (Table S1). In this experiment, a sperm cell expressing H2B-GFP was fertilized with a wild-type egg cell to allow visualization of nuclei during zygotic/embryonic development (Figure 1a). Without inhibitor treatment, a rice zygote divides into a two-celled embryo and a multicellular embryo at 1 day and 2 days after fertilization (DAF), respectively. However, when rice zygotes were continuously treated with malonate during culture, zygotes were at the one-cell stage without division (Figure 1b). Importantly, when the inhibitor was removed at 1 DAF, cell division of zygotes was restored (Figure 1b). Moreover, the inhibitory effect of malonate seems to disturb the efficiency of the mETC, and the production of ATP by the mETC might be lessened. A study in *Xenopus* oocytes demonstrated that malonate effectively depleted intracellular ROS levels; however, the production of ATP was not significantly declined upon malonate exposure (Han et al., 2018). Therefore, it is estimated that ATP production was also not affected by the malonate treatment in rice zygote/embryo in our study. This indicates that the arrest of zygote development by 3.5 mM malonate treatment is not due to any toxic effect of the inhibitor but due to inhibition of the putative mETC in the rice zygote. The results suggest the essential role of intracellular ROS metabolism in possible activation of developmental mechanisms toward the first division of the rice zygote. We further examined the effects of a malonate inhibitor on intracellular ROS levels in egg cells using the cell permeant 2’,7’-dichlorodihydrofluorescein diacetate (H$_2$DCFDA), a putative ROS-sensitive probe. The results indicated that malonate depleted intracellular ROS in egg cells to approximately 50% compared to the control (Figure 1c,d). Since there might be a possibility that treatment...
Figure 1. Developmental profiles of zygotes treated with ROS production inhibitors. (a) A schematic illustration of karyogamy between gametic nuclei and chronological progression of developmental stages in rice zygotes/embryos. Green color indicates H2B-GFP-derived fluorescence in nuclei. This diagram was created based on Ohnishi et al., 2014. (b) Developmental profile of rice zygotes treated with malonate. Zygotes produced by electrofusion between a transgenic H2B-GFP sperm cell and a wild-type egg cell were treated with 3.5 mM malonate throughout the culture period (continuous treatment). Alternatively, zygotes were treated with malonate for 1 day after fusion and then transferred into fresh culture medium without the inhibitor (1-day treatment). The embryonic development was imaged at 1, 2, and 4 days after fertilization (DAF). Top and bottom panels represent fluorescent and merged fluorescent and bright-field images, respectively. Scale bars: 20 µm. (c) Isolated egg cells were treated with 3.5 mM malonate for 4 h and stained with H2DCFDA. The intracellular DCF fluorescent signals in treated and untreated egg cells were observed using a confocal laser scanning microscope, and representative images are presented. Scale bars: 20 µm. (d) Fluorescence intensity derived from DCF in egg cells treated with or without malonate was quantified and expressed as mean fluorescence intensity. The data are mean ± SD of treated (n = 12) and untreated (n = 8) egg cells. (e) Staining profiles of egg cells treated with or without 3.5 mM malonate for 4 h using fluorescent diacetate (FDA). Intracellular fluorescent signals in treated and untreated cells were imaged using a confocal laser scanning microscope, and representative images are presented. Scale bars: 20 µm. (f) Fluorescence intensity derived from fluorescein in egg cells treated with or without malonate was quantified and expressed as mean fluorescence intensity. The data are mean ± SD of treated (n = 8) and untreated (n = 6) egg cells.
of egg cells with malonate affects the penetration rate of H$_2$DCFDA into the cells (Figure 1c,d), fluorescein diacetate (FDA), a cellular viability marker structurally similar to H$_2$DCFDA, was also used to investigate the penetration of fluorescent probes into egg cells treated with or without malonate (Figure 1e,f). The comparable fluorescein-derived fluorescence levels between the treated and untreated cells indicates that the decline of ROS levels in malonate-treated egg cells (Figure 1c-f) is directly due to the inhibitory activity of malonate on ROS production. Taken together, it was estimated that malonate can be utilized as an effective inhibitor for ROS production in plant cells and that developmental arrest of rice zygotes caused by malonate treatment is due to continuous depletion of intracellular ROS. In addition, diphenyleneiodonium (DPI) treatment of rice zygotes resulted in cessation of the first cleavage of the zygotes (Figure S1), indicating the essential role of cytosolic ROS metabolism for possible activation of developmental mechanisms toward the first division of rice zygotes.

Dynamics of intracellular ROS levels during development of rice zygotes

We next measured intracellular ROS levels using H$_2$DCFDA in egg cells, zygotes, and early embryos. After electrofusion between wild-type egg and sperm cells, the produced zygotes were cultured and stained with H$_2$DCFDA at different time points to observe DCF-derived fluorescence intensity. In egg cells, a strong fluorescent signal was observed (Figure 2a), suggesting high ROS levels. Although the ROS-derived fluorescence level was constant in zygotes at 1 h after fusion (HAF), the total intracellular ROS levels in 4 HAF zygotes decreased approximately 40% compared with that in egg cells (Figure 2a,b). The ROS levels reached a minimum in 8 HAF zygotes (Figure 2a,b). Thereafter, we observed gradual increases of the total ROS levels in 16 HAF zygotes and two-celled and globular-like embryos (Figure 2a,b). These results suggested that ROS levels decline during the early developmental stage of the zygote and that active ROS production reoccurs prior to and during cell division of the zygote. In addition to H$_2$DCFDA, FDA was also used to stain egg cells, zygotes, and early embryos, since the possibility could not be excluded that the cell wall around zygotes, which is formed after fertilization (Ohnishi et al., 2019; Uchiumi et al., 2007), affects the rate of H$_2$DCFDA penetration into developing zygotes, resulting in possible underestimation of DCF fluorescence intensity. FDA-stained egg cells, zygotes, and embryos showed unchanged fluorescent signal profiles (Figure 2c), indicating that electrofusion and cell wall formation of zygotes do not affect H$_2$DCFDA reactivity and that changes in DCF-derived fluorescence intensity during zygotic development reflect changes in intracellular ROS levels. Moreover, zygotes which were fertilized within the embryo sac were isolated from flowers at 4 h after pollination (HAP) (Abiko et al., 2013a) and subjected to H$_2$DCFDA staining to confirm that the decline in ROS levels also occurs in zygotes produced in planta and to evaluate the possibility of ROS level alteration induced by mechanical stress emanating from gamete isolation. The results indicate remarkably low ROS levels in 4 HAF in planta zygotes compared with that in egg cells (Figure S2), suggesting that cell isolation does not induce a mechanical stress-mediated ROS burst and that the ROS level dynamics detected in zygotes produced in vitro reflect those in zygotes that emerged in the embryo sac (Figure 2a).

In addition to changes in total ROS levels during the development of rice zygotes, the intracellular distribution of fluorescent signals from DCF appeared to change through the conversion of egg cells into zygotes. In egg cells, the ROS signal was detected in the nucleus, cytoplasm, and mitochondria, which densely localize at the periphery of the nucleus (Figure 2d). The ROS signal in the nucleus gradually diminished after fertilization in zygotes and early embryos, whereas the ROS signal in mitochondria seemed to be unchanged in zygotes and to increase to a large extent in cells of early embryos (Figure 2a,d).

Intracellular O$_2^{•−}$ level dynamics in developing rice zygotes

In plants, it has been shown that each species of ROS particularly functions in different redox-sensitive signaling pathways in response to various factors, such as environmental stresses and developmental cues (see Mhamdi and Van Breusegem, 2018), and the biological functions of ROS in signaling mechanisms are largely dependent on their chemical properties and lifetime (see Waszczak et al., 2018). Based on its moderate longevity and reactivity, we first investigated whether O$_2^{•−}$ plays an important role in early development of rice. For monitoring total intracellular O$_2^{•−}$ levels, zygotes produced by electrofusion were stained with BES-So-AM, a non-oxidative O$_2^{•−}$-specific fluorescent probe (Maeda et al., 2005). In addition, the mitochondrial O$_2^{•−}$ levels were also examined using MitoSOX, a fluorescent probe specifically oxidized by O$_2^{•−}$ in the mitochondrial matrix (Robinson et al., 2008). In the case of BES-So-AM staining, the egg cells exhibited high total intracellular O$_2^{•−}$ levels (Figure 3a,b). Upon fertilization, the O$_2^{•−}$ levels slightly decreased in 1 HAF zygotes and rapidly dropped by approximately 80% in 4 and 8 HAF zygotes compared to egg cells (Figure 3a,b). In 16 HAF zygotes, the intracellular O$_2^{•−}$ levels reached a minimum compared to the other observed time points (Figure 3b). The low total O$_2^{•−}$ levels continued until the two-celled embryo stage, and recovery of total O$_2^{•−}$ levels was detected in globular-like embryos (Figure 3a,b).
Despite a gradual decline detected in the total O₂⁻/\textsuperscript{●}/C0 levels in developing zygotes (Figure 3a,b), the mitochondrial O₂⁻/\textsuperscript{●}/C0 levels monitored with MitoSOX abruptly declined by approximately 62% at only 15 min after fertilization (MAF) compared with egg cells (Figure 3c,d). Importantly, this immediate decrease in O₂⁻/\textsuperscript{●}/C0 levels in mitochondria was not observed in egg cells to which a DC pulse, an electrical stimulus equivalent to gamete fusion conditions, was applied or with which an additional egg cell was fused (Figure S3) (Ohnishi et al., 2019). This suggests that the entry of a sperm cell into an egg cell immediately triggers the decrease of O₂⁻/\textsuperscript{●}/C0 levels in mitochondria and that fertilization-induced conversion of O₂⁻/\textsuperscript{●}/C0 into H₂O₂ occurs in mitochondria. Low levels of mitochondrial O₂⁻/\textsuperscript{●}/C0 were consistently observed through the remaining time points from the 1 HAF zygote to the two-celled embryo, and a rebound of high mitochondrial O₂⁻/\textsuperscript{●}/C0 levels was found in globular-like embryos (Figure 3c,d).

Intracellular H₂O₂ level dynamics in developing rice zygotes

Based on its chemical stability and mobility, H₂O₂ has been considered as the most potent redox signaling molecule with the ability to transduce signals via oxidation of other signaling intermediates (Mullineaux et al., 2018; Waszczak et al., 2018). To monitor H₂O₂ levels in developing zygotes and early embryos, BES-H₂O₂-Ac, a non-oxidative H₂O₂-specific fluorescent probe, was...
employed for quantitative measurement of intracellular H$_2$O$_2$ levels (Maeda et al., 2004). Similar to other ROS species, H$_2$O$_2$ was observed to be highly accumulated in egg cells (Figure 4). After fertilization, the intracellular H$_2$O$_2$ levels in zygotes progressively decreased, and a rebound of H$_2$O$_2$ levels in early embryos was not observed (Figure 4). Interestingly, temporal and spatial profiles of the decrease in H$_2$O$_2$ levels during zygotic development indicated that fluorescent foci representing mitochondrial H$_2$O$_2$ signals were detectable throughout zygotic development until the two-celled embryo stage, although cytosolic signals had mostly disappeared in 8 HAF zygotes (Figure 4a), suggesting that the decrease in cytosolic H$_2$O$_2$ levels is much faster than that in mitochondria. The detection of BES-H$_2$O$_2$Ac-labeled mitochondria in developing zygotes is consistent with the possible conversion of mitochondrial O$_2^{-\cdot}$ into H$_2$O$_2$ in zygotes (Figure 3c).

Through monitoring the effects of inhibition of ROS metabolism (Figure 1) and the dynamics of ROS levels using several fluorescent probes reactive to total ROS, O$_2^{-\cdot}$, and H$_2$O$_2$ (Figures 2–4), it could be observed that ROS are highly accumulated in egg cells and that a fertilization-dependent decrease in ROS levels in zygotes triggers proper development of the zygote and early embryo. Notably, during the course of observation of ROS levels in zygotes, we found that approximately 10% of the produced zygotes did not show a decrease in total ROS levels (Figure S4a), total intracellular O$_2^{-\cdot}$ levels (Figure S4b), and total intracellular H$_2$O$_2$ levels (Figure S4c) and that all these zygotes exhibited developmental arrest or delayed cell division. In this study, the data of ROS levels for these zygotes were not included in the calculation of relative fluorescence intensity in Figures 2–4. In addition to the persistent endogenous ROS levels in undeveloped zygotes, a short-term exposure of zygotes to exogenous H$_2$O$_2$ after fertilization was also shown to induce a significant delay in cell division in zygotes (Figure S5). These results also support the importance of decreased ROS levels in zygotes for their development.
Among ROS species, we further investigated H$_2$O$_2$ metabolism in zygotes, since O$_2^*$ is quickly converted into H$_2$O$_2$ and, relative to other ROS, H$_2$O$_2$ is widely accepted as an ideal redox signaling molecule due to its versatile chemical properties (Waszczak et al., 2018).

Possible involvement of GPXs in zygotic development and importance of glutathione-mediated redox signaling in zygotes

As for enzymes that can be involved in H$_2$O$_2$ metabolism during zygotic development, we focused on GPX1 and GPX3, since it has been reported that, among five putative rice GPXs (Islam et al., 2015), GPX1 and 3 are highly expressed in rice sperm cells and their expression levels in zygotes are upregulated to an enormous extent by fertilization (Rahman et al., 2019) (Table S2). To address the contribution of GPX1 and 3 transcripts in a sperm cell to the GPX transcript levels in a fused egg cell (a zygote) and to monitor changes in expression levels of GPXs during early zygotic development, semi-quantitative reverse transcription-PCR (RT-PCR) was conducted using cDNA from egg cells, sperm cells, and zygotes at 15 and 30 MAF and 1, 2, and 4 HAF as templates. Expression levels of GPX1 and 3 in both sperm cells and egg cells were measured (Figure 5a). Notably, upon the fusion of gametes, an immediate increase of GPX1 transcripts was not detected in zygotes at 15 and 30 MAF (Figure 5a) compared with GPX1 expression in egg cells, suggesting that GPX1 transcripts in sperm cells do not contribute to the increase in GPX1 transcript levels in zygotes. GPX1 expression in zygotes seems to be mainly upregulated at 1 HAF onwards (Figure 5a), indicating that upregulation of GPX1 in zygotes occurs under global de novo gene expression upon zygotic genome activation (Ohnishi et al., 2014). The expression profile of GPX3 in gametes and early zygotes showed a very similar pattern to that of GPX1 (Figure 5a).

Next, to investigate the importance of catalytic activity of GPXs during early development, we inhibited the enzymatic activity of GPXs in zygotes using mercaptosuccinic acid (MSA), a potent GPX inhibitor (King et al., 2010). The in vitro enzymatic analysis of recombinant non-selenocysteine GPX1 from cyanobacteria Synechocystis PCC 6803 showed that 50% GPX1 activity was inhibited by MSA treatment (Gaber et al., 2001). The amino acid sequence of rice GPX1 shows 46% identity to cyanobacterial GPX1 with conserved positions of possible catalytic cysteine residues. These suggest that MSA treatment can inhibit non-selenocysteine GPX1 activity probably via a similar mechanism in both rice and cyanobacteria. Interestingly, we found that the inhibition of GPX activity by 1.5 mM MSA significantly delayed cell division in rice zygotes and early embryos compared to zygotes without inhibition.
inhibitor (Figure 5b), suggesting the important role of GPXs in initiating cell division and maintaining appropriate embryonic development. Since GPXs have been shown to be highly upregulated in response to oxidative stress (Sugimoto et al., 2014) and function as important antioxidant enzymes that convert H$_2$O$_2$ and organic hydroperoxides into water or respective alcohols (Bela et al., 2015), we then hypothesized that the dramatic decline in intracellular H$_2$O$_2$ levels in 8 HAF zygotes (Figure 4) is due to the increased activity of GPXs in developing zygotes. To address this hypothesis, we measured intracellular H$_2$O$_2$ levels in 8 HAF zygotes continuously cultured with 1.5 mM MSA. We found that the drastic decline in H$_2$O$_2$ levels did not occur in MSA-treated 8 HAF zygotes (Figure 5c,d). This suggests a crucial role of GPX activity in the reduction of H$_2$O$_2$ levels during zygotic development to accomplish proper embryogenesis without a delay (Figures 4 and 5b,c).

GSH is the most abundant thiol-based non-enzymatic antioxidant, which has been documented as an important cell cycle regulator in plants (Diaz Vivancos et al., 2010). We therefore asked whether an alteration of GSH levels in zygotes affects the developmental profile of zygotes by using buthionine sulfoximine (BSO), an inhibitor of glutamate-cysteine ligase that catalyzes the first step of GSH biosynthesis and mainly depletes cytosolic GSH (Markovic et al., 2009). The inhibitory effect of BSO on GSH biosynthesis was first examined in egg cells by visualization of intracellular GSH levels using monochlorobimane (MCB), a GSH detection fluorescent probe. When egg cells were treated with 2 mM BSO for 4 h, the intracellular GSH levels during zygotic development to accomplish proper embryogenesis without a delay (Figures 4 and 5b,c).
dramatically diminished by approximately five times compared to the control (Figure 6a,b). We then investigated the effect of GSH depletion on zygotic development by culturing zygotes in 2 mM BSO. The BSO-treated zygotes divided into two-celled embryos; however, the development was arrested at the two-celled embryo stage and never proceeded further into the globular-like embryo stage (Figure 6c). Since it is known that BSO cannot effectively deplete nuclear GSH, we then employed diethyl maleate (DEM), a non-specific thiol-binding reagent that conjugates with GSH via glutathione-S-transferase activity forming DEM-GSH adducts, to deplete both nuclear and cytosolic GSH pools (Markovic et al., 2009). Notably, the GSH levels in the nucleus were highly diminished after 4 h of treatment with 0.5 mM DEM in egg cells (Figure 6d,e). Next, we further confirmed the role of nuclear and cytosolic GSH in early zygotic development by culturing zygotes in media containing 0.5 mM DEM. Although karyogamy was normally completed, the treated zygotes were arrested at the one-celled stage and remained undivided, indicating that cell division of zygotes was severely affected by DEM-induced GSH depletion (Figure 6f). These results suggest that both cytosolic and nuclear GSH play an important role in early embryonic development, possibly as non-enzymatic antioxidant for conversion of H₂O₂. Therefore, we then asked whether the intracellular redox status of glutathione is altered during development in rice zygotes after fertilization.

Transgenic rice stably expressing Grx1-roGFP2 was produced to allow fluorometric real-time observation of the glutathione redox status in the nucleus and cytosol of a single rice zygote and embryo (Gutsch er et al., 2008). The results were expressed as ratiometric imaging between oxidized and reduced Grx1-roGFP2, which is derived from the ratio of emissions in the green channel (500–530 nm) excited with 408- and 488-nm lasers, respectively (Gutscher et al., 2008). We first investigated the redox sensing properties of Grx1-roGFP2 in rice egg cells by simultaneous treatment with H₂O₂, an oxidizing agent, and dithiothreitol (DTT), a reducing agent. Strong glutathione oxidation in the egg cell was immediately induced after 5 mM H₂O₂ treatment and could be rapidly reversed to a highly reduced state upon the addition of 20 mM DTT (Figure S6), indicating that heterologously expressed Grx1-roGFP2 can be utilized to monitor the oxidation degree or estimate the availability of glutathione in egg cells and zygotes upon the transition of cellular events (Meyer et al., 2007). Upon gamete fusion, a highly reduced redox status of glutathione was observed in the 1 HAF zygote (Figure 6g), which was equivalent to that in the unfertilized egg cell (Figure S6). Notably, as the development of the zygote progressed, the oxidation degree of glutathione gradually increased, reaching its highest level at 8 HAF. The cytosolic glutathione seemed to be oxidized more rapidly compared to the nuclear glutathione (Figure 6g). Thereafter, a subsequent decline in levels of glutathione oxidation was detected in the two-celled embryo and globular-like embryo stages (Figure 6g). These results suggested that after gamete fusion, oxidation of glutathione progresses in early developing zygotes and, since de novo GSH biosynthesis is required for S to G2 phase progression (Poot et al., 1995), the markedly reduced glutathione redox state during/after the development of the zygote into an early embryo could result from an increased GSH production (Figure 6g). In addition, the effect of GPX activity inhibition on glutathione oxidation dynamics during zygotic/embryonic development was further examined to make clear whether GPX activity is involved in oxidation of glutathione. When oxidation levels of glutathione were monitored in zygotes expressing Grx1-roGFP2 cultured with or without 1.5 mM MSA, despite the highly oxidized glutathione in 8 HAF zygotes under physiological conditions, MSA-induced GPX activity inhibition significantly delayed glutathione oxidation in MSA-treated zygotes along with the largely delayed cell division (Figure 6h). These results strongly suggest a correlation between oxidation of glutathione and GPX-mediated H₂O₂ processing upon early zygotic and embryonic development.

**Nuclear localization of GPX1 in rice egg cell and zygote**

In rice egg cells, zygotes, and cells of early embryos, H₂O₂ was detected in the cytosol, mitochondria, and nucleus (Figure 4a), and GPX1 and 3 were considered to mediate H₂O₂ metabolism during zygotic development (Figures 5 and 6h). Therefore, the subcellular localization of GPX1 and GPX3 is essential information to judge whether these enzymes can catalyze H₂O₂ that was accumulated/produced in the cytosol, mitochondria, or nucleus. When their sublocalizations were estimated *in silico* using TargetP-2.0 (www.cbs.dtu.dk/services/TargetP/), GPX3 was predicted to localize in mitochondria (likelihood = 0.3661), while GPX1 showed no specific targeting subcellular location. Although the mitochondrial localization of both GPX1 and 3 was previously examined in rice by transient expression of OsGPX1-YFP and OsGPX3-YFP fusion proteins in leaf protoplasts (Passaia et al., 2013), the resolution of fluorescent images of leaf protoplasts expressing these fusion proteins appeared to be insufficient probably due to low expression levels of these fusion proteins in protoplasts. Moreover, to our knowledge, the intracellular localization of GPX1 and 3 in female gametes and zygotic cells has not been reported. Therefore, we addressed the intracellular localization of GPX1 and 3 in rice egg cells and zygotes using polyethylene glycol (PEG)-Ca²⁺ transfection of egg cells or zygotes with plasmid vectors harboring an OsGPX1-GFP or OsGPX3-GFP expression cassette to deliver these plasmids into the cells. After a 1-day incubation, the treated egg cells or zygotes were stained with
Figure 6. Effects of inhibition of glutathione biosynthesis on rice embryonic development (a–f), dynamics of redox status of glutathione during zygotic development (g), and effects of GPX activity inhibition on the redox status of glutathione (h).

(a) Isolated egg cells were incubated with or without 2 mM buthionine sulfoximine (BSO) for 4 h prior to the detection of intracellular GSH using 100 µM monochlorobimane (MCB). Fluorescent signals were imaged by a confocal laser scanning microscope as described in the Experimental Procedures section.

(b) The fluorescence intensity derived from GSH-MCB adduct in egg cells treated with or without BSO was quantified and expressed as mean fluorescence intensity. The data are mean ± SD of treated (n = 10) and untreated (n = 8) egg cells.

(c) Zygotes produced by electrofusion between a transgenic H2B-GFP sperm cells and a wild-type egg cell were cultured with or without 2 mM BSO. The developmental profiles of BSO-treated and untreated zygotes/embryos were monitored 1, 2, and 3 days after fertilization (DAF) using a fluorescence microscope. Left, middle, and right panels represent fluorescent, bright-field, and merged images, respectively. Scale bars: 20 µm.

(d) Isolated egg cells were incubated with or without 0.5 mM diethyl maleate (DEM) for 4 h prior to the detection of intracellular GSH using 100 µM MCB. Fluorescent signals were imaged by a confocal laser scanning microscope as described in (a).

(e) The fluorescence intensity derived from GSH-MCB adduct in egg cells treated with or without DEM was quantified and expressed as mean fluorescence intensity. The data are mean ± SD of treated (n = 12) and untreated (n = 9) egg cells.

(f) Zygotes produced by electrofusion between a transgenic H2B-GFP sperm cell and a wild-type egg cell were cultured with or without 2 mM DEM. The developmental profiles of DEM-treated and untreated embryos were monitored 1 to 3 DAF using a fluorescence microscope. Left, middle, and right panels represent fluorescent, bright-field, and merged images, respectively. Scale bars: 20 µm.

(g) Zygotes produced by electrofusion between a transgenic Grx1-roGFP2 egg cell and a wild-type sperm cell were cultured as described in the Experimental Procedures section. Single fertilized egg cells, zygotes at 1, 4, and 8 h after fertilization, two-celled embryos, and globular-like embryos were sequentially excited with 408-nm and 488-nm lasers and the ratiometric images of the serial observations were created based on the ratio of emissions in the green channel (500–530 nm) from both excitation wavelengths (ratio 408/488 nm). Scale bars: 20 µm.

(h) Zygotes produced by electrofusion between a transgenic Grx1-roGFP2 egg cell and a wild-type sperm cell were cultured with or without 1.5 mM MSA. Single fertilized egg cells, zygotes at 1, 4, and 8 h after fertilization, two-celled embryos, and globular-like embryos were serially imaged as described in (g). Scale bars: 20 µm.
MitoTracker Red CMXRos, a mitochondrial probe, and then observed with confocal microscopy. Notably, fluorescent signals from GPX1-GFP were mainly localized in the nucleus and cytosol of egg cells and zygotes, but the areas stained with MitoTracker Red did not overlap with GPX1-GFP-derived fluorescence foci (Figure 7). These results indicate that GPX1 mainly localizes in the nucleus and cytosol of egg cells and zygotes. In contrast, the signal from GPX3-GFP fusion proteins mostly merged with that from MitoTracker Red-stained foci (Figure 7). The mitochondrial localization of GPX3 was consistent with a previous report (Passaia et al., 2013) and TargetP-based estimation.

DISCUSSION

Intracellular ROS metabolism and dynamics in developing rice zygote

It has been previously shown that early zygotic development in rice is modulated by multiple regulatory events such as a fertilization-dependent increase in cytosolic Ca\(^{2+}\) levels, chromatin decondensation during karyogamic stages, preferential paternal allele-dependent gene expression, and reorganization of cellular polarity (Dresselhaus et al., 2016; Ohnishi et al., 2014; Ohnishi et al., 2019; Rahman et al., 2019; Sato et al., 2010; Zhao et al., 2017). Correlation between Ca\(^{2+}\) wave propagation triggered by sperm entry during fertilization and elevated ROS production in early zygotes was well established in animal models (Whitaker, 2012; Wong et al., 2004). In Xenopus, fertilization-induced Ca\(^{2+}\) wave generation is reported to act upstream of H\(_2\)O\(_2\) production upon entry of sperm, and ROS production seemed essential for cell cycle progression, thereby promoting proper embryonic cell division (Han et al., 2018). In this study, the importance of ROS metabolism after fertilization in early developing rice zygotes/embryos was elucidated, since we found that ROS depletion by malonate and DPI in zygotes caused cell division arrest (Figure 1b; Figure S1). These results are consistent with a previous study showing the inhibitory effect of ROS perturbation on cell proliferation during somatic embryogenesis in cotton (Gossypium hirsutum) (Zhou et al., 2016).

Although our knowledge of the functional profile of ROS in fertilized zygotes, which are deeply embedded in ovular tissues, has been limited, the present study successfully demonstrated that alterations in ROS metabolism can lead to developmental defects not only in somatic embryogenesis but also in zygotes/embryos generated by fertilization between egg and sperm cells.

ROS level dynamics were also monitored in developing rice zygotes using different kinds of probes to detect specific ROS species in different cellular compartments. In contrast to the rapid rise of H\(_2\)O\(_2\) levels observed in Xenopus zygotes (fertilized oocytes) after fertilization.
(Han et al., 2018), we found a decline in intracellular total ROS, $O_2^{\bullet-}$, and $H_2O_2$ levels in rice zygotes at 4 HAF (Figures 2–4), the time point at which karyogamy is mostly completed and subsequent de novo gene expression occurs (Ohnishi et al., 2014). This suggests that the drop in ROS levels in rice zygotes might result from upregulation of genes involved in redox homeostasis and that the ROS-mediated signaling mechanism is probably activated during the de novo gene expression period in rice zygotes. Interestingly, zygotes, in which a drop in ROS levels was not detected after fertilization, showed arrest or delay in their cell division (Figure S4), providing the possibility that proper zygotic/embryonic development is associated with a preceding decline in ROS levels during developmental progression of the zygote into an early embryo. Notably, similar to those produced by in vitro fertilization, zygotes fertilized in embryo sacs also exhibited a significant drop in intracellular ROS levels (Figure S2), indicating that the decline in ROS levels in developing zygotes produced in vitro constitutes the occurrence of the same event in sexual reproduction in planta.

Despite the decrease in $H_2O_2$ levels in the cytoplasm, persistent levels of mitochondrial $H_2O_2$ were detected in developing zygotes (Figure 4a). This preferential detection of $H_2O_2$ in mitochondria might be consistent with the report that fertilization-induced Ca$^{2+}$ waves in Xenopus zygotes induce $H_2O_2$ production in mitochondria and a subsequent intracellular ROS burst (Han et al., 2018). The importance of $H_2O_2$ production in mitochondria for zygotic development is also supported by our results showing the failure of conversion from zygote into early embryo by long-term inhibition of mitochondrial ROS synthesis (Figure 1b). The possible rapid conversion of mitochondrial $O_2^{\bullet-}$ into $H_2O_2$ in rice zygotes (Figures 3c and 4a) might be a mechanism for $H_2O_2$ production in mitochondria. Mn-independent SOD (Mn-SOD) can be considered as a candidate enzyme for catalyzing this conversion, since this enzyme was identified as one of the proteins enriched in egg cells (Abiko et al., 2013a). Interestingly, it is known that Mn-SOD activity can be increased post-translationally by a variety of mechanisms, including nitration, acetylation, glutathionylation, methylation, phosphorylation, and metal incorporation (Candas and Li, 2014). Rapid disappearance of mitochondrial $O_2^{\bullet-}$ after sperm entry in the rice zygote (Figure 3c,d) may result from post-translational activation of Mn-SOD catalytic activity induced by fertilization. Taken together, we speculated that in rice, the egg cell activation process is induced after fertilization via an increase in intracellular Ca$^{2+}$ levels, which, later, lead to mitochondrial $H_2O_2$ production to possibly initiate redox-mediated signaling mechanisms to promote physiological alterations during egg-to-zygote conversion.

**Functions of ROS in cell cycle of rice zygote and early embryo**

Despite the remarkably decreased levels of all ROS species in zygotes after fertilization, we consistently detected high levels of total ROS, $O_2^{\bullet-}$, and total $H_2O_2$ in rice egg cells (Figures 2–4). The maintenance of high ROS levels in the egg cell will be required to halt the cell cycle at the G1 phase. In maize (Zea mays) RAM, it has been shown that cellular characteristics of quiescent center (QC) cells existing in a prolonged G1 phase are attributable to their highly oxidizing environment, whereas actively dividing proximal cells are in a reduced state. Moreover, it has been reported that exogenous addition of reducing agents such as ascorbate to maize RAM perturbs the highly oxidized cellular redox state and triggers cell cycle progression from the G1 into the S phase (Kerk and Feldman, 1995). Interestingly, we previously reported that the rice egg is arrested at the G1 phase, and, through fusion with sperm, karyogamy, and possibly OsWEE1-mediated parental DNA integrity maintenance occurring in the zygote nucleus, the cell cycle in the zygote then progresses into the S and subsequently the M phase (Sukawa and Okamoto, 2018). The maintenance of a highly oxidizing cellular environment will enable the rice egg cell to sit in the G1 phase, and this cellular characteristic of high intracellular ROS levels may be a common egg cell feature among angiosperms, since the cell cycle phase of egg cells has been reported to be G1 in both monocots (maize, rice, and barley [Hordeum vulgare]) and dicots (Arabidopsis) (Liu et al., 2020; Mogensen and Holm, 1995; Mogensen et al., 1995; Sukawa and Okamoto, 2018). The transition of G1 to S phase of the cell cycle in rice zygotes occurs around 6–10 h after fertilization (Kowyama, 1983; Sukawa and Okamoto, 2018). Notably, we detected a drastic decline in all ROS species in zygotes at 8 HAF (Figures 2–4), suggesting that the cellular redox state in rice zygotes is shifted toward a highly reducing condition to initiate DNA replication in the S phase, at which OsWEE1 expression is upregulated (Sukawa and Okamoto, 2018). Interestingly, because OsWEE1 possesses nine exposed cysteine residues, which are prone to ROS exposure and post-translational modifications mediated by ROS (Foyer et al., 2018; Wani et al., 2014), it was hypothesized that the activity of OsWEE1, a redox-sensitive protein, is modulated upon oxidative post-translational modification and that OsWEE1 might be activated via cysteine oxidation in conjunction with the decreasing intracellular ROS levels after fertilization. In addition, since the progressive increases in nuclear and cytosolic oxidation degree of glutathione were detected in developing zygotes, a constant drop in both nuclear and cytosolic $H_2O_2$ levels might reflect the synergistic effect between the $H_2O_2$ scavenging activity of GSH and the fertilization-induced upregulation of GPX1, which occurs at the onset of the karyogamic stage in
zygotes (Figures 1a, 4, 5a, and 6g,h). Taken together, it seems that the interplay between the decline in H2O2 levels mediated by GSH redox metabolism and/or GPX activity and WEEl-dependent DNA integrity maintenance is remarkably important for proper cell division in early zygotic and embryonic development in rice.

Glutathione peroxidase 1 and 3 activity is crucial for faithful development of rice zygotes and embryos via possible oxidation of glutathione

We found that inhibition of GPX activity effectively impedes cell division in zygotes (Figure 5b) as well as somatic cells, which is consistent with a previous study demonstrating that OsGPX3 knockdown significantly decreased shoot and root length in rice, concomitant with high H2O2 levels (Passaia et al., 2013). In addition, our expression profile data of GPX1 and 3 also showed that these enzymes are abruptly upregulated in the very early phase of zygotic development (Figure 5a). Although high levels of H2O2 were detected in egg cells and early zygotes at 1 HAF (Figure 4), the primarily reduced glutathione redox state of egg cells and 1 HAF zygotes indicated that the progressive oxidation of glutathione in zygotes at 4 and 8 HAF stages appeared to be highly correlated with the activity of GPXs (Figure 6h) that are highly expressed in the zygotes (Figure 5a) and can contribute to the gradual decline in H2O2 levels as zygotic development progresses (Figure 4). The reduction of H2O2 level was also proved to be crucial for normal zygotic division and development, since the acute H2O2 treatment after gamete fusion significantly impeded cell division of developing zygotes (Figure S5). In addition, the detection of persistently high H2O2 levels in zygotes with deficient GPX activity also emphasizes the crucial role of GPXs in the reduction of H2O2 levels in the zygote (Figure 5c). These suggest that both antioxidant properties of glutathione and GPX functions are synergistically required for removal of excessive H2O2 that is accumulated in egg cells during zygotic/embryonic development and that maintenance of low H2O2 levels is essential for cell proliferation in rice zygotes. Importantly, it was indicated that GPX1 localizes not only in the cytosol but also in the nucleus of rice eggs and zygotes (Figure 7), in which GSH might already exist. The GSH localization in the nucleus of early zygotes is consistent with the previous report that GSH is recruited into the nucleus of proliferative somatic cells at the G1/S phase transition (Diaz Vivancos et al., 2010a, 2010b) (Figure 8). The putative recruitment of GSH into the nucleus is supportive of our findings on the nuclear localization of GPX1 and the increased oxidation degree of glutathione in the developing zygote (Figures 6g and 7), since coexistence of GPX1 and GSH in the nucleus might contribute to the faster decline of H2O2 levels in the nucleus compared with that in nuclear periphery-located mitochondria (Diaz Vivancos et al., 2010b) (Figure 4a). Interestingly, our results showed that GSH depletion by BSO-induced GSH biosynthesis inhibition causes cell division arrest at the two-celled embryo stage (Figure 6c), and high ROS accumulation in nuclei of GSH-depleted two-celled embryos was also detected (Figure S7). This developmental arrest in post-first zygotic division can be explained by cell cycle-dependent GSH localization and the mode of GSH depletion by BSO. In dividing cells, the majority of GSH is recruited into the nucleus during cell cycle progression from G1 to the S phase, which leads to a depletion of GSH in the cytosol (Diaz Vivancos et al., 2010b; Markovic et al., 2007). When the level of cytosolic GSH is largely diminished, compensatory GSH biosynthesis in cytoplasm is activated to replenish cytosolic GSH during S phase progression (Diaz Vivancos et al., 2010a, 2010b). BSO is known to effectively deplete the cytosolic but not the nuclear pool of GSH (Markovic et al., 2009). Taken together, our results suggest that depletion-resistant nuclear GSH is sufficient to drive the first cleavage in zygote; however, after the first cell division, a lack of newly synthesized GSH prevents subsequent recruitment of GSH into nuclei of the divided zygote. Furthermore, this decreased GSH level in the nucleus lowers the GSH-dependent H2O2 scavenging activity and subsequently leads to high nuclear ROS accumulation (Figure S7), resulting in an inability to proceed to the second round of cell division of the two-celled embryo (Figure 6c). In addition, when the nuclear pool of GSH in the zygote was depleted by DEM treatment (Figure 6d), the DEM-treated zygotes did not proceed to the first cleavage and remained in the single-celled stage after fertilization (Figure 6f). Moreover, a prominently reduced redox state of glutathione in two-celled and globular-like embryos was clearly elucidated in the present study (Figure 6g), suggesting that oxidized glutathione (GSSG) produced in the zygote might be reduced by glutathione reductase into GSH, which is required to promote subsequent cell division in embryo stages. These observations indicate that the existence of GSH in the nucleus is crucial for stimulating cell division not only in proliferative somatic cells (Diaz Vivancos et al., 2010b; Markovic et al., 2007) but also in the first cell division of the early fertilized zygote, which is a fundamental basis of sexual reproduction in angiosperms for establishing the initial apical–basal axis (Kimata and Ueda, 2020; Zhao et al., 2017). High ROS accumulation in zygotic/embryonic nuclei was induced by both depletion of glutathione and inhibition of GPX activity, indicating the possible cooperative roles of GSH and GPX1 in quenching of nuclear ROS to promote developmental progression in the zygote (Figure 5c and Figure S7). The coupling of a developmental defect with high intracellular ROS levels might be due to the dysfunction of GPX-dependent ROS scavenging activity in

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developing zygotes (Figure S4). In addition, the peak of glutathione oxidation degree was detected in 8 HAF zygotes, at which H$_2$O$_2$ levels became extremely low (Figure 4; Figure 6g), strongly suggesting that the redox activity of glutathione is required for H$_2$O$_2$ removal in zygotes. Notably, the GPX-mediated H$_2$O$_2$ scavenging reaction in developing zygotes appeared to be tightly related to glutathione oxidation, since glutathione oxidation in developing zygotes was extremely delayed when GPX activity had been inhibited (Figure 6h). These series of results provide the possibility that the reducing power of GSH might directly allow the GPX-catalyzed H$_2$O$_2$ reduction reaction in zygotes, although plant GPXs have been reported to preferentially utilize thioredoxin (TRX) instead of GSH (Bela et al., 2015). Alternatively, the possible relationship between glutathione redox status and GPX activity can be explained by interactions between GSH and TRX systems, since it has been demonstrated that glutathione exhibits compensatory activity when the TRX reduction system is impaired. Using Arabidopsis mutants for NADPH-dependent TRX reductase A and B genes (NTRA and NTRB), it was indicated that ntra ntrb homoygous mutants showed the root growth defect only when GSH in root cells was depleted by BSO treatment (Reichheld et al., 2007). Moreover, ntra ntrb embryos showed no developmental arrest and this normal embryogenesis in ntra ntrb was attributable to the undetectable expression level of NTRA and NTRB in Arabidopsis embryos (Reichheld et al., 2007). Interestingly, the expression levels of OsNTRA and OsNTRB genes in rice zygotes are also low (Table S3), and BSO treatment resulted in developmental arrest in zygotes and embryos (Figure 6c). These results suggest that the compensatory mechanism between GSH and TRX systems might also function during zygotic/embryonic development in rice.

In addition to GSH-dependent H$_2$O$_2$ scavenging mediated by GPX activity, the possibility could not be excluded that the progressive oxidation of GSH, along with the decline in H$_2$O$_2$ levels, might result from glutathione recycling via the ascorbate-glutathione (ASC-GSH) cycle. The process involves GSH utilization for the conversion of oxidized forms of ascorbate (ASC), including monodehydroascorbate (MDA) and dehydroascorbate (DHA), to the reduced form, after being used as an electron donor in H$_2$O$_2$ scavenging reactions catalyzed by APXs (see Hasanuzzaman et al., 2019). In rice egg cells and zygotes, ASC peroxidases (APXs) and glutathione-dependent DHA reductase (DHAR), as well as GPX1 and 3, are also highly expressed, and the expression levels of the gene encoding for MDA reductase 4 (MDAR4), an enzyme catalyzing the conversion of MDA into ASC, is highly upregulated after fertilization (Abiko et al., 2013b; Rahman et al., 2019). Taken together, these expression profiles of the enzymatic components in the ASC-GSH cycle and progressive GSH...
oxidation during zygotic development suggest that this cycle might be activated after gamete fusion to regenerate ASC from its oxidized form using GSH as electron donor to further proceed APX-mediated H$_2$O$_2$ scavenging mechanisms.

**The possible interplay among ROS dynamics, glutathione-mediated redox metabolism, and cell cycle regulation in promoting cell cycle progression in zygote**

In the present study, the decrease in nuclear ROS levels was found to be associated with the progression of the cell cycle and cell division of rice zygotes. The possible interplay among ROS dynamics, antioxidant activity of GPX1, and nuclear recruitment of GSH in rice zygotes has been schematically represented in Figure 8. Before gamete fusion, ROS are maintained at high levels in egg cells to halt the cell cycle at the G1 phase. Upon fertilization, both nuclear and mitochondrial O$_2^{**}$ is largely converted to H$_2$O$_2$ by SODs, which are primarily expressed and accumulated in egg cells. The resultant zygote tends to progress from G1 to the S phase of the cell cycle after gamete fusion. In the zygotic nucleus, where GPX1 and GSH are colocalized at high extents, H$_2$O$_2$ is gradually converted into water, resulting in a progressive decline of nuclear H$_2$O$_2$ levels. Along with the decline in nuclear and cytosolic H$_2$O$_2$ levels, karyogamy progresses within the fertilized zygote, in which paternal and maternal genomes are combined together in the zygote nucleus. Toward the end of the S phase, high-fidelity DNA replication is performed, and the zygote is allowed to progress further to mitosis. Moreover, the reducing power of GSH might be important for TRX-mediated H$_2$O$_2$ scavenging activity of GPX1 in zygotic nucleus, since the recycling of reduced TRX is known to be highly dependent on the presence of GSH (Reichheld et al., 2007).

**The rebound of intracellular superoxide anion levels in the embryo stage**

It seems that the decline in ROS levels after fertilization is a transient event, since we detected the rebound of total ROS in 16 HAF zygotes and two-celled and globular-like embryos and the restoration of cytosolic and mitochondrial O$_2^{**}$ levels in globular-like embryos (Figures 2 and 3). Among ROS species, H$_2$O$_2$ is not considered to contribute to the rebound of ROS levels, since H$_2$O$_2$ levels remain low in globular-like embryos (Figure 4). It is known that O$_2^{**}$ is required for supporting the meristematic properties in Arabidopsis shoot apical meristem (SAM) and RAM, while H$_2$O$_2$ suppresses cell proliferation and activates differentiation, suggesting the distinct roles of different ROS species in plant development (Tsukagoshi et al., 2010; Zeng et al., 2017). Interestingly, despite the absence of rebound in H$_2$O$_2$ levels (Figure 4), the preferential restoration of O$_2^{**}$ levels in early embryo stages indicates that O$_2^{**}$ is essential for the maintenance of the proliferative state of mitotically active globular-like embryos (Figure 3), in which the differentiation process remains suppressed. Moreover, the rebound of ROS levels in the late zygote and embryo might be essential to trigger proper embryonic development and maintain viability, since two-celled embryos treated with 5 mM DPI failed to develop into proper globular-like embryos and degenerated at 3 DAF (Figure S6). Results showing the requirement of ROS for developmental machineries in zygotic embryos are consistent with the report that ROS play an essential role in the emergence of somatic embryos from cotton explants (Zhou et al., 2016). Moreover, non-specific characteristics of H$_2$DCFDA reacting with a wide range of oxidants apart from ROS have been extensively reported (Kalyanaraman et al., 2012). The early restoration of DCF fluorescence intensity in 16 HAF zygotes might be due to other reactive species, such as reactive nitrogen species (RNS), and the possibly increased RNS levels with the concomitant ROS level rebound may contribute to proper development in the embryo stage. Investigation of RNS dynamics in rice zygotes/embryos will therefore provide further understanding of the ROS and RNS signaling framework in zygotic/embryonic development. In addition, information on redox signaling target proteins is limited and the majority of ROS-related signaling pathways also remain open. Although the role of ROS and redox signaling in early development remains to be largely elucidated, the present study provides insights into ROS dynamics and the involvement of both enzymatic and non-enzymatic antioxidants at a fundamental level of development in the zygotes of angiosperms.

**EXPERIMENTAL PROCEDURES**

**Plant materials and in vitro fertilization system**

*Oryza sativa* L. cv. Nipponbare plants were grown in an environmental chamber (K30-7248; Koito Industries, Yokohama, Japan) at 26°C under a 13 h light/11 h dark photoperiod. Transformed rice plants expressing histone H2B-GFP fusion protein were prepared as previously described (Abiko et al., 2013a). The isolation of egg cells and sperm cells from rice flowers and the electrofusion of isolated gametes for zygote production were conducted as previously described (Uchiumi et al., 2006, 2007). Zygotes fertilized in embryo sacs (in vivo zygotes) were also isolated from rice flowers 3–4 h after flowering (pollination) according to Abiko et al. (2013b).

**Microscopic observation**

Cellular features and developmental profiles of gametes, zygotes, and embryos were observed using a BX-71 inverted microscope (Olympus, Tokyo, Japan). The intracellular fluorescent signal of H2B-GFP proteins was observed under a BX-71–490 nm and an emission wavelength of 510–550 nm (U-MWIBA2 mirror unit; Olympus). Digital images of gametes, zygotes, and the resultant embryos were obtained using a cooled charge-coupled device camera (Penguin 600CL; Pixcera, CA, USA).
and InStudio software (Pixcera). In addition to the BX-71 inverted fluorescence microscope, egg cells, zygotes, and embryos expressing GFP fusion proteins or stained with fluorescent probes were observed using an LSM 710 CLS microscope (Carl Zeiss, Jena, Germany) with excitation and emission wavelengths specific to each type of fluorophore as described below.

Treatment of zygotes with ROS production inhibitor

An egg cell isolated from wild-type rice plants was fused with a sperm cell expressing H2B-GFP, and the resulting zygote was cultured in N6Z medium (Uchiumi et al., 2007) with or without inhibitors. Inhibition of intracellular ROS production was performed by treatment with 3.5 mM malonate (Sigma) and 5 or 10 μM DPI (Sigma, MO, USA). The zygotes were continuously cultured in inhibitor-containing medium or transferred to inhibitor-free medium after 1 day of treatment. The developmental profile and fluorescent signal of developing zygotes/embryos were monitored using a BX-71 inverted fluorescence microscope as described above.

Treatment of zygotes with GSH-depleting agents and GPX activity inhibitor

BSO (Sigma) and DEM (Sigma) were employed to stimulate depletion of intracellular GSH in zygotes with different modes of action. The zygotes produced by in vitro fusion were cultured in N6Z medium with 2 mM BSO or 0.5 mM DEM. In addition, the inhibition of GPX activity was conducted by treatment of zygotes with 1.5 mM MSA (Sigma) in N6Z medium. The developmental profile and fluorescent signal of the treated zygotes/embryos were monitored using an the BX-71 inverted fluorescence microscope as described above.

Transient expression of GPX1-GFP and GPX3-GFP proteins in egg cells and zygotes

A DNA cassette containing GFP linked to a NOS terminator was amplified by PCR using the pH2B-GFP plasmid vector (Koiso et al., 2017) as a template and primers (forward 5′-CTCTAGATTAATTTCTTGTACAAAGTGGTTC-3′ and reverse 5′-CGCCCTTGCGATGCCCCCCTATCTGGTAATCAATGATG-3′). The DNA fragment obtained from PCR was subcloned between the Ppd and AseI restriction sites of the pDsRed vector (Koiso et al., 2017) using an In-Fusion HD Cloning Kit (Takara Bio, Shiga, Japan) according to the manufacturer’s instructions, which resulted in the replacement of the DsRed2 sequence with the GFP sequence downstream of the maize ubiquitin promoter-ubiquitin intron, and the constructed vector was termed pUbiproc::GFP. DNA fragments encoding the OsGPX1 or OsGPX3 protein were amplified by PCR with primers with cDNA (Abiko et al., 2013b) and primers (OsGPX1, Os04g0556300: forward 5′-CTCTAGATTAATTTCTTGTACAAAGTGGTTC-3′ and reverse 5′-ACAGAAATTAAAGAGCTCCCAGCAGT-3′; OsGPX3, Os02g0866000: forward 5′-CTCTAGATTAATTTAAAGAGCTCCCAGTCTCGCACCGC-3′ and reverse 5′-ACAGAAATTAAAGAGCTCCCAGTCTCGCACCGC-3′). The amplified PCR fragments were subcloned into the Ppd site of pUbiproc-GFP using an In-Fusion HD Cloning Kit, generating plasmid vectors expressing OsGPX1-GFP or OsGPX3-GFP fusion protein under control of the maize ubiquitin promoter. After verification of the DNA sequence, the resultant plasmid vectors were used for transient expression of these fusion proteins in egg cells and zygotes.

The plasmids were delivered into isolated egg cells and/or zygotes produced by in vitro fusion via PEG-Ca²⁺-mediated transfection as previously described (Koiso et al., 2017). GFP-derived fluorescent signals were monitored one day after the transfection using a BX-71 inverted fluorescence microscope, and the cells showing a fluorescent signal were then transferred to droplets of 370 mM Osmol kg⁻¹ H₂O mannitol, which were placed on a glass bottom dish and covered with mineral oil. Subsequently, these cells were further transferred into a droplet of mannitol solution containing 2 μg ml⁻¹ MitoTracker Red CM-H₂XRos (Molecular Probes, New York, USA) to confirm subcellular localization of mitochondria in the egg cells or zygotes. After 10 min of incubation, cells were washed three times by transferring them into fresh mannitol droplets, and the fluorescent signal was observed using the LSM 710 microscope with an excitation wavelength of 488 nm and an emission wavelength of 505-530 nm for OsGPX1::GFP and OsGPX3::GFP and with an excitation wavelength of 543 nm and an emission wavelength of >560 nm for MitoTracker Red.

Measurement of intracellular ROS levels with fluorescent probes

Determination of intracellular ROS levels in egg cells, zygotes, and early embryos was conducted by employing oxidant-sensitive fluorescent probes specific to different kinds of ROS. Zygotes produced by in vitro fusion were cultured as described above for 1, 4, 8, and 16 HAF, and the zygotes were developed into the two-celled or globular-like embryo stage. The zygotes and early embryos were transferred to droplets of 450 mM Osmol kg⁻¹ H₂O mannitol solution on coverslips and then transferred to droplets of mannitol solution containing ROS detection probes. In addition to zygotes and early embryos, isolated egg cells were also subjected to staining with ROS detection probes. The egg cells, zygotes, and embryos were stained with permeant H₂DCFDA probe (Sigma) at the concentration of 10 μM for measuring the total intracellular ROS levels. Total and mitochondrial O₂⁻ levels were examined using 0.05 μM non-oxidant BES-So-AM (Wako, Osaka, Japan) and 2.5 μM MitoSOX (Invitrogen, Oregon, USA) probes, respectively. The H₂O₂-specific non-oxidant probe BES-H₂O₂-Ac (Wako) was also used at a concentration of 0.5 μM to measure total H₂O₂ levels. After staining for 15 min, cells and embryos were washed by transferring them into fresh mannitol droplets three times, and the fluorescent signal derived from H₂DCFDA, BES-So-AM, or BES-H₂O₂-Ac was detected using an LSM 710 CLS microscope with an excitation wavelength of 488 nm and an emission wavelength of 505-530 nm, while the fluorescent signal derived from MitoSOX was detected with an excitation wavelength of 510 nm and an emission wavelength of 580 nm. In the present measurement, each individual was stained with fluorescent probes at a single timepoint, and, after fluorescent observation, the cells and embryos were then cultured in fresh medium to verify their viability and development. After obtaining fluorescence images, fluorescence intensity was quantified using ImageJ software. The fluorescence intensity of the zygotes/embryos at different timepoints was normalized to that of egg cells stained in the same replicate. When zygotes showed a developmental defect, the fluorescent images obtained from these zygotes were not utilized for the above quantification.

Determination of intracellular GSH levels

The effect of BSO or DEM on intracellular GSH levels in egg cells was examined by MCB (Sigma) staining. Egg cells were incubated in droplets of mannitol solution, adjusted to 370 mM Osmol kg⁻¹ H₂O, with or without 2 mM BSO for 4 h. After 4 h, the cells were then transferred to inhibitor-free mannitol droplets containing 50 μM MCB and treated with each type of fluorophore as described above (Uchiumi et al., 2007). GFP-derived fluorescent signals were monitored one day after the transfection using a BX-71 inverted fluorescence microscope, and the cells showing a fluorescent signal were then transferred to droplets of 370 mM Osmol kg⁻¹ H₂O mannitol, which were placed on a glass bottom dish and covered with mineral oil. Subsequently, these cells were further transferred into a droplet of mannitol solution containing 2 μg ml⁻¹ MitoTracker Red CM-H₂XRos (Molecular Probes, New York, USA) to confirm subcellular localization of mitochondria in the egg cells or zygotes. After 10 min of incubation, cells were washed three times by transferring them into fresh mannitol droplets, and the fluorescent signal was observed using the LSM 710 microscope.
incubated for 15 min. After washing three times, the fluorescent signal was observed using an LSM 710 CLS microscope with an excitation wavelength of 358 nm and an emission wavelength of 461 nm.

Vector construction and preparation of transformants

To create transgenic rice stably expressing cytosolic Grx1-roGFP2, pLPCX cyto Grx1-roGFP2 was obtained as a gift from Tobias Dick (Addgene plasmid #64975; http://n2t.net/addgene:64975; RRID: Addgene_64975) (Gutacher et al., 2008). DNA fragments containing cyto Grx1-roGFP2 were amplified by PCR using pLPCX cyto Grx1-roGFP2 as a template with gene-specific primers (forward 5'-GGGCAAGATTGTGACAAAAAAGCAGGCTCCATGGCTCAAGAGT-3' and reverse 5'-GGGACCACTTTGTGACAGAAGAAGGCGTGCTTTACAGTACGTCGCTCA-3') and subcloned into the entry vector pENTR/D-TOPO using a Gateway BP reaction kit (Life Technologies, USA). To generate a suitable expression vector for rice gametes, the cloned Grx1-roGFP2 DNA fragment was transferred from the entry vector to the destination vector p2KGW (Miki and Shimamoto, 2004) by an LR reaction using Gateway LR Clonase enzyme mix (Invitrogen, USA) according to the manufacturer’s instructions. Agrobacterium tumefaciens LBA4404 was transformed with the Ubi promoter:cyto Grx1-roGFP2 vector, and transformed rice plants were prepared by cocultivation of rice calli with A. tumefaciens as previously described (Hiei et al., 1994). Egg cells isolated from the transgenic plants and zygotes which were produced by in vitro fusion between Grx1-roGFP2-expressing egg and WT sperm cells were serially observed using an LSM 710 CLS microscope and imaged line by line with sequential excitations by 408-nm and 488-nm lasers and the detection wavelength was set to 500-530 nm. Using ImageJ software, ratiometric analysis was conducted to create ratio images by dividing the 408 nm image by the 488 nm image pixel by pixel with the Hwada FRETratioFX plug-in. The background was subtracted, and a threshold was set to avoid ratio-created artifacts. The divided pictures were applied with the ImageJ lookup table ‘Fire’ for creating false-color ratio images. The ratio values were determined with the upper threshold of the ratiometric images set to three.

Semi-quantitative RT-PCR

The cDNAs of egg cells, sperm cells, and zygotes at 15 MAF, 30 MAF, 1 HAF, 2 HAF, and 4 HAF were synthesized as described previously (Rahman et al., 2019) using the SMART-Seq HT Kit (Takara Bio, USA) and used as templates for PCR with primers specific to OsGPX1 (forward 5’-CTCTAGATTATAATTGCGCCGCGCCTG-3’ and reverse 5’-ACAAGAAATTATATAAGAGCTCCAAGACAGT-3’) and OsGPX3 (forward 5’-CTCTAGATTTAAATTGCGCCGCGCCTG-3’ and reverse 5’-ACAAGAAATTATATAAGAGCCTCCAAGACAGT-3’). For PCR, 0.5 µl of cDNA (200 pg µl⁻¹) was used as the template in a 25-µl reaction volume with 0.3 µM of primers using KOD-FX DNA polymerase (Toyobo, Osaka, Japan) with the following program: 21 cycles of 94°C for 1 min, 55°C for 30 sec, and 72°C for 1 min. The expression of the ubiquitin gene (Os02g0161900) was monitored as an internal control using a gene-specific primer (forward 5’-GCCGGATCTTGTGACAAAAAAGCAGGCTCCATGGCTCAAGAGT-3’ and reverse 5’-GGGACCACTTTGTGACAGAAGAAGGCGTGCTTTACAGTACGTCGCTCA-3’). For PCR, 0.5 µl of cDNA (200 pg µl⁻¹) was used as the template in a 25-µl reaction volume with 0.3 µM of primers using KOD-FX DNA polymerase (Toyobo, Osaka, Japan) with the following program: 21 cycles of 94°C for 1 min, 55°C for 30 sec, and 72°C for 1 min. The expression of the ubiquitin gene (Os02g0161900) was monitored as an internal control using a gene-specific primer (forward 5’-GCCGGATCTTGTGACAAAAAAGCAGGCTCCATGGCTCAAGAGT-3’ and reverse 5’-GGGACCACTTTGTGACAGAAGAAGGCGTGCTTTACAGTACGTCGCTCA-3’).

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