RESEARCH PAPER

Glutathione redox state plays a key role in flower development and pollen vigour

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Received 2 May 2019; Editorial decision 6 August 2019; Accepted 10 August 2019

Abstract

The importance of the glutathione pool in the development of reproductive tissues and in pollen tube growth was investigated in wild-type (WT) Arabidopsis thaliana, a reporter line expressing redox-sensitive green fluorescent protein (roGFP2), and a glutathione-deficient cad2-1 mutant (cad2-1/roGFP2). The cad2-1/roGFP2 flowers had significantly less reduced glutathione (GSH) and more glutathione disulfide (GSSG) than WT or roGFP2 flowers. The stigma, style, anther, germinated pollen grains, and pollen tubes of roGFP2 flowers had a low degree of oxidation. However, these tissues were more oxidized in cad2-1/roGFP2 flowers than the roGFP2 controls. The ungerminated pollen grains were significantly more oxidized than the germinated pollen grains, indicating that the pollen cells become reduced upon the transition from the quiescent to the metabolically active state during germination. The germination percentage was lower in cad2-1/roGFP2 pollen and pollen tube growth arrested earlier than in roGFP2 pollen, demonstrating that increased cellular reduction is essential for pollen tube growth. These findings establish that ungerminated pollen grains exist in a relatively oxidized state compared with germinating pollen grains. Moreover, failure to accumulate glutathione and maintain a high GSH/GSSG ratio has a strong negative effect on pollen germination.

Keywords: Flower development, glutathione, oxidative stress, pollen germination, redox regulation, redox-sensitive green fluorescent protein (roGFP).

Introduction

The tripeptide thiol γ-glutamylcysteine glycine (glutathione, GSH) is the most abundant low-molecular-weight thiol in plants and animals (Noctor et al., 2011). GSH synthesis in the cytosol and plastids of plant cells (Wachter et al., 2005; Galant et al., 2011) occurs via the sequential action of two ATP-dependent enzymes, γ-glutamylcysteine synthetase (γ-ECS), also called γ-glutamylcysteine ligase, and glutathione synthase (GS) (Cairns et al., 2006; Pasternak et al., 2008; Galant et al., 2011; Noctor et al., 2011). In the first step, γ-glutamylcysteine (γ-EC) is produced from cysteine and glutamate by the action of γ-ECS. In the second step, glycine is added to γ-EC to form GSH by the action of GS (Noctor et al., 2002; Sugiyama et al., 2004; Wachter et al., 2005; Galant et al., 2011). GSH synthesis is regulated by the availability of cysteine or glycine and by the post-translational redox activation of γ-ECS via the formation of an intramolecular disulfide bond (Cys186-Cys406 in Arabidopsis) (Noctor et al., 2002; Gromes et al., 2008). In this way GSH synthesis is regulated by the redox...
environment of the chloroplast, allowing rapid responses to biotic and abiotic stresses.

Knockout γ-ECS mutants are lethal (Cairns et al., 2006). However, there are a number of Arabidopsis mutants with defects in γ-ECS that allow a lower level of GSH synthesis (Cobbett et al., 1998; Ball et al., 2004). These mutants have significantly lower levels of GSH accumulation than the wild type (WT), and have no marked shoot phenotypes but variable ef-

However, there are a number of Arabidopsis mutants with

GSH into mitochondria and other cellular compartments are
cytosol by CLT transporters, but the mechanisms of import of

plant cells and is directly involved in the maintenance of intra-
cellular redox homeostasis, acting as a redox buffer (Meister
et al., 1983; Schafer et al., 2014). GSH accumulates at high concentrations (5–10 mM) in
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and that oxidative challenges are rapidly counterbalanced by
the cytosol, nuclei, and mitochondria contain very little GSSG,
and GSSG are also involved in the control of gene expression
ensuring a stable redox environment within the cell. GSH
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A combination of different techniques has been used to investi-
gate the role of glutathione in fertilization and the ger-
mination of the pollen tube. Specific antibodies were used to
characterize the abundance of GSH in flowers during devel-


tude lines were used in the present study to estimate the redox state of the nuclei and cytosol of flower and pollen cells to provide

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Materials and methods

Plant material and growth conditions

Seeds of WT Arabidopsis thaliana accession Columbia-0 (Col-0), Col-0 expressing roGFP2 (de Simone et al., 2017), and cad2-1 mutants expressing roGFP2 (cad2-1/roGFP2) were surface sterilized and washed
twice with sterile water. After stratification for 2 days in the dark at 4 °C,
seeds were sown on soil and grown in controlled environment chambers
under 120–150 μmol m−2 s−1 irradiance with an 8 h/16 h day/night
period, 22 °C/18 °C day/night temperatures, and a relative humidity
of 60%. Samples were collected from 6-week-old plants bearing flowers
at different stages (Alvarez-Buylla et al., 2010). Samples were collected
from younger flower buds at flowering stage 6–7, with sepal that still
clasp buds and anthers (at meiosis) and tetrad. Flowers were also col-
clected from later stages of flowering, particularly flowering stage 11–12
when petals, stamens and pollen at the bicellular–tricellular transition are
present), flowering stage 13 (when stamens outstrip the gynoecium and
anthers delusae), and flowering stage 14 (when dehiscent anthers extend
above the stigma). Each experiment was repeated three times and results
are the average of three independent experiments.

Pollen germination

A total of 25–30 freshly opened flowers (at stage 14) of Arabidopsis were
collected and allowed to dry at room temperature for 30 min in a 1.5 ml
tube with the lid opened. A 1 ml volume of germination medium (Fan
et al., 2001) was added and the pollen was concentrated according to Li
(2011). The pollen grains were germinated at 28 °C and in the dark for
1, 2, and 3 h. Images of germinated pollen grains were taken in a Leica
DMI600B or a Nikon Eclipse TE2000-U inverted epifluorescence micro-
scope. The germination of the pollen was assessed by counting the ger-
mminated grains (those in which the pollen tube length was greater than
the pollen diameter) at the different germination times, and expressing the
number of germinated grains as a percentage of the total number of pollen
grains. The length of the pollen tubes was calculated using the Straight tool of ImageJ software. Data presented are the means and SD of a minimum of
nine different samples of each one of three experimental replicates.

Sample preparation for immunohistochemistry

Samples were collected at the different developmental stages iden-
tified in the figure legends and immersed in a fixative solution (4% paraformaldehyde and 0.2% glutaraldehyde in 0.05 M sodium cac-
dylate buffer, pH 7.2). Further dehydration in an ethanol series and
embedding in Unicryl resin followed by polymerization using UV
light at ~20 °C were performed. Thin sections (1 μm) were attached to
3-triethoxysilylpropylamine–treated slides.

Immunolabelling of glutathione

Thin sections were incubated in phosphate-buffered saline (pH 7.2) con-
taining 2.5% bovine serum albumin (Sigma-Aldrich) for 1 h at room
temperature and then treated with primary anti-GSH antibody (Agrisera prod. No. AS09 594) followed by secondary antibody (anti-rabbit IgG Alexa549-labelled secondary antibody; Molecular Probes). Negative controls were prepared by omitting the primary antibody. After washing three times, samples were mounted with citifluor (Ted Pella). Fluorescence microscopy observations were performed using a Nikon Eclipse TE2000-U inverted microscope fitted with a ProgRes MF Cool CCD microscope camera (Jenoptik). Fluorescence intensity was quantified using ImageJ software. Data presented are the average of a minimum of nine images obtained in three independent experiments.

Liquid chromatography–electrospray/mass spectrometry

Tissue glutathione contents were determined by liquid chromatography–electrospray/mass spectrometry (LC–ES/MS). Samples of open flowers and floral buds (green and white) of WT, roGFP2, and cad2-1/roGFP2 plants were frozen in liquid nitrogen, ground, and immediately homogenized in 1 ml 0.1 M HCl. After centrifugation, supernatants were extracted again. The supernatants were pooled and filtered through 0.45 μm polyvinylidene fluoride filters and analysed by LC–ES/MS on a Triple quadrupole mass spectrometer (Quattro micro) coupled to a liquid chromatograph (Waters Alliance 2695) with dual wavelength detector.

Confocal laser scanning microscopy and image analysis

Freshly opened flowers (day 0 according to Boavida et al., 2007) were incubated in 1 ml of germination medium (Fan et al., 2001) at 28 °C for 1 h in darkness. After the incubation, selected flowers were assembled on excavated slides containing a few drops of the germination medium. They were immediately observed with a C-1 confocal microscope (Nikon) fitted on a Nikon Eclipse TE2000-U inverted microscope, using an image capture schedule equivalent to that described by de Simone et al. (2017). Briefly, the image of reduced roGFP (green) was calculated by subtracting the image after excitation with 405 nm from the image obtained after excitation with 488 nm, by using ImageJ software. The final reduced roGFP/oxidized roGFP ratios were also determined with ImageJ, by using a threshold of 10. Finally, to better illustrate the redox potential differences in the images evaluated, the grayscale was converted to a false-colour scale image using the ImageJ ‘Fire’ query table. All measurements were taken with identical adjustments to be able to compare them in absolute terms (Meyer et al., 2007). The intensity per area was measured in each image by using ImageJ software, and these data were used to determine the degree of oxidation and the redox potential in each tissue examined according to the Nernst equation (de Simone et al., 2017). A minimum of 10 samples per treatment were analysed.

Statistical analysis

IBM SPSS Statistics software (version 21.0.0.0) was used to analyse the statistical significance of the results. The Shapiro–Wilk test combined with the Kolmogorov–Smirnov test was used to evaluate the normality of the data. A one-way ANOVA was performed in combination with a Duncan’s multiple range test and post hoc Bonferroni procedure to verify the statistical significance of the data.

Results

Immunolocalization of GSH during flower development

GSH was detected in the microsporocytes, tetrads, pollen grains, and anther tissues (including the tapetum) of the WT, roGFP2, and cad2-1/roGFP2 plants using anti-GSH antibody (Figs 1–3). GSH was also present in the gynoecium (including stigma), the embryo sacs, and other tissues of the ovary (Figs 4–6) of the WT and roGFP2 plants.

GSH was present in the nuclei, cytosol, apoplastic/cell wall compartment, tapetum, and embryo sacs of these lines. GSH and GSSG are transported throughout the plant, and both metabolites are readily taken up by the plasma membrane. Low levels of GSH and GSSG have been detected in the apoplastic fluid of leaf cells (Vanacker et al., 1998), consistent with our findings. The antibody–linked fluorescence was much less intense in all the flower tissues of the cad2-1/roGFP2 plants than either the WT or roGFP2 plants. This observation reflects the lower levels of GSH present in the cad2-1/roGFP2 plants than the other lines, which had similar levels of GSH during all developmental stages, especially at the end of anther development (Fig. 7).

LC–ES/MS determination of GSH and GSSG levels

The levels of GSH and GSSG were similar in WT (Fig. 8A) and roGFP2 (Fig. 8B) flowers at all stages of development. The highest levels of GSH were determined in flowers at stage 14, with high GSH/GSSG ratios maintained throughout flower development in the WT (Fig. 8A) and roGFP2 (Fig. 8B) plants. In contrast, the flowers of cad2-1/roGFP2 plants had less than 50% of the GSH present in WT or roGFP2 lines at equivalent stages of flower development. Moreover, the levels of GSSG in cad2-1/roGFP2 flowers (Fig. 8C) were similar to or slightly higher than those detected in WT (Fig. 8A) and roGFP2 (Fig. 8B) flowers, resulting in lower GSH/GSSG ratios.

Redox state of the glutathione pool in vivo

roGFP fluorescence was readily detected in the anthers, pistils, and germinated pollen grains of roGFP2 and cad2-1/roGFP2 plants (Figs 9–11). The degree of oxidation was low in the stigma, style, anther, germinated pollen grains, and pollen tubes of roGFP2 flowers (Fig. 12), suggesting that the glutathione pool is highly reduced in these tissues. This finding is in agreement with the data obtained by biochemical analysis of the tissues described above. In contrast, the ungerminated pollen grains were significantly more oxidized than the germinated pollen grains (Fig. 12). The degree of oxidation was also higher in the ungerminated pollen grains of cad2-1/roGFP2 flowers compared with germinated pollen grains and other tissues (Fig. 12). All tissues (stigma, style, anther, mature and germinated pollen grains, and pollen tubes) of the cad2-1/roGFP2 flowers tended to be more oxidized than the respective tissues of roGFP2 flowers (Fig. 12). However, these differences were statistically significant only for stigma, anther, and mature pollen.

The glutathione redox potentials were similar in the stigma, style, anther, germinated pollen grains, and pollen tubes of the roGFP2 flowers (Fig. 13). However, the glutathione redox potential of the ungerminated pollen grains was significantly lower than that of the germinated pollen grains of roGFP2 (Fig. 13). The degree of oxidation was lower in the stigma, style, anther, germinated pollen grains, and pollen tubes of cad2-1/roGFP2 flowers than in roGFP2 plants (Fig. 13). The most positive glutathione redox potentials were determined in the ungerminated pollen grains. These values were significantly more positive than those calculated for the roGFP2 plants (Fig. 13).
Fig. 1. Immunolocalization of GSH by antibody-associated fluorescence microscopy in Arabidopsis anthers at stages 6–7 of development. (A) Negative control (meiocytes); (B) WT (meiocytes); (C) roGFP2 (tetrads); (D) cad2-1/roGFP2 (meiocytes). E, epidermis; En, endothecium; M, meiosis; P, petal; Se, sepal; Sm, stamen; T, tapetal layer; Te, tetrad.

Fig. 2. Immunolocalization of GSH by antibody-associated fluorescence microscopy in Arabidopsis anthers at stage 13. (A) Negative control; (B) WT; (C) roGFP2; (D) cad2-1/roGFP2. A, anther; MSp, microspores; N, nucleus; O, ovary; P, petal; Se, sepal.
Fig. 3. Immunolocalization of GSH by antibody-associated fluorescence microscopy in Arabidopsis anthers at stage 14. (A) Negative control; (B) WT; (C) roGFP2; (D) cad2-1/roGFP2. A, anther; BiPG, bicellular pollen grain; E, epidermis; En, endothecium; N, nucleus; P, petal; PG, pollen grain; Se, sepal; Sm, stamen; T, tapetal layer; TrPG, tricellular pollen grain.

Fig. 4. Immunolocalization of GSH by antibody-associated fluorescence microscopy in Arabidopsis flowers (pistils) at stage 11–12. (A) Negative control; (B) WT; (C) roGFP2; (D) cad2-1/roGFP2. A, anther; O, ovary; Ov, ovum; P, petal; Se, sepal; Sm, stamen.
Fig. 5. Immunolocalization of GSH by antibody-associated fluorescence microscopy in Arabidopsis pistils at stage 13. (A) Negative control; (B) WT; (C) roGFP2; (D) cad2-1/roGFP2. N, nucleus; O, ovary; Ov, ovum.

Fig. 6. Immunolocalization of GSH by antibody-associated fluorescence microscopy in Arabidopsis pistils at stage 14. (A) Negative control; (B) WT; (C) roGFP2; (D) cad2-1/roGFP2. P, petal; PG, pollen grain; S, stigma; SP, stigmatic papillae; St, style.
Germination was significantly slower in cad2-1/roGFP2 pollen than in roGFP2 pollen (Fig. 14). The germination percentage was also lower in the cad2-1/roGFP2 pollen. Pollen tube growth arrested earlier in cad2-1/roGFP2 pollen compared with roGFP2 pollen (Fig. 14). Viability experiments demonstrated that the non-germinated pollen grains were alive (data not shown).

**Discussion**

The redox state of cells is a crucial regulator of metabolism, signalling, and function. While very few tools are available to measure the redox state of living cells, the roGFP probes have proved to be reliable and widely applicable (Meyer et al., 2007; Schwarzländer et al., 2008, 2009; Aller et al., 2013), especially when they incorporate a glutaredoxin (Fernandes et al., 2004; Meyer et al., 2007; Gutschker et al., 2008). The data obtained from roGFP probes therefore provide reliable information concerning the abundance and oxidation state of the glutathione pool in situ and in vivo. Moreover, roGFP probes have been expressed in a wide variety of cells and organisms, where they can be targeted to specific organelles (Hanson et al., 2004; Birk et al., 2013). The effectiveness of the roGFP2 probes in measuring the redox status of the glutathione pool has previously been demonstrated in studies on other species, such as Drosophila (Liu et al., 2012; Aller et al., 2013). The data presented here show that all the tissues of the cad2-1/roGFP2 flowers had a lower GSH/GSSG ratio (Fig. 8) and a higher degree of oxidation than the roGFP2 flowers (i.e. in the WT background; Figs 12 and 13). These findings contrast with data from leaves: the level of total glutathione was decreased in the leaves of cad2-1 mutants but the GSH/GSSG ratios were similar to the WT (Schnaubelt et al., 2015). Taken together, these findings suggest that the flowers of cad2-1 mutants have a lower capacity to keep the cellular glutathione pool reduced and maintain high GSH/GSSG ratios than the leaves.

A strong association between the extent of glutathione accumulation and flowering time has been demonstrated (Ogawa et al., 2001; Yanagida et al., 2004). Flowering was accelerated in the cad2-1/roGFP2 mutants compared to the WT (Kocsy et al., 2013). Moreover, glutathione is required for the initiation of the floral meristem (Bashandy et al., 2010, Kocsy et al., 2013) and also pollen tube germination (Zechmann et al., 2011b). The data presented here builds on this firm foundation and reveals the presence of a low degree of oxidation in all the flower parts (stigma, style, anther, germinated pollen grains and pollen tubes) except the ungerminated pollen. Ungerminated pollen exists in a highly oxidized state that is similar to that found in other quiescent cells (Schippers et al., 2016). In contrast to
ungerminated pollen, germinated pollen grains and pollen tubes have a similar degree of oxidation to the other flower parts. These findings suggest that the glutathione pool in pollen is either increased or becomes more reduced (or both) once metabolism is triggered by germination. This process appears to be essential for germination and pollen tube growth because...
these processes are significantly impaired in the cad2-1/roGFP2 pollen, which maintained a high degree of oxidation compared with the roGFP2 controls.

The pollen germination rates measured in the WT in the present study were comparable to values in the literature (~71%; Boavida et al., 2007). Germination rates were decreased to ~5–20% in the pollen grains of the cad2-1/roGFP2 mutants, which have a restricted capacity for glutathione synthesis (Cobbett et al., 1998; Meyer et al., 2007; Maughan et al., 2010; Zechmann et al., 2011, a, b; Aller et al., 2013). In addition, the growth rates were much lower in the pollen grains of the mutants. Calculations of glutathione redox potentials based on the roGFP2 measurements confirmed that all the flower parts apart from ungerminated pollen are maintained in a highly reduced state, as is the case for the germinated pollen and pollen tubes. The highly reduced states measured in the germinated pollen show that the endogenous antioxidant systems have sufficient capacity to deal with the large amounts of ROS and reactive nitrogen species produced by the mitochondria (Traverso et al., 2013; Jiménez-Quesada et al., 2016, 2017; Zafra et al., 2018). Interestingly, the mitochondria in leaf cells were found to have the highest concentrations of glutathione of any of the intracellular compartments, even more than the chloroplasts (Zechmann et al., 2008). A roGFP probe targeted specifically to the mitochondria may be useful in future studies of the role of the glutathione pool in pollen tube mitochondria (Rosenwasser et al., 2010).
The finding that the glutathione levels were highest in mature flowers, with a gradual increase in glutathione accumulation during flower development, is interesting. Increasing accumulation of ROS during flower development may lead to the oxidative activation of γ-ECS in the plastids, resulting in increased glutathione synthesis and accumulation (Gromes et al., 2008). ROS accumulate during leaf senescence and during the orchestration of programmed cell death because of alterations in the expression of enzymes involved in the antioxidant system and changes in mitochondrial metabolism (Mari et al., 2009; Cui et al., 2012). However, the concept that ROS are pro-life signals generated by aerobic metabolism is widely accepted (Foyer et al., 2018). Open flowers contain mature pollen, which will have already interacted with the pistil to trigger the signalling cascade that results in the germination of the pollen tube. A flow cytometry approach to determine the viability of mature tomato and Arabidopsis pollen related to ROS accumulation revealed that two types of pollen populations could be identified, namely ‘low-ROS’ and ‘high-ROS’ populations, representing low or high metabolic activity, respectively (Luria et al., 2019). The high-ROS pollen germinated with a much higher frequency than the low-ROS pollen (Luria et al., 2019). The metabolically active high-ROS pollen would require high levels of antioxidant activity, particularly high activities of enzymes such as glutathione reductase in order to maintain high GSH/GSSG ratios. Exposure to high temperatures resulted in accumulation of GSSG that was observed in the cad2-1/roGFP2 flowers (Luria et al., 2019). Such stress-induced increases in ROS pollen germination and tube growth (Zechmann et al., 2008). The transition from the oxidized quiescent state to the metabolically active germinated state that facilitates pollen growth therefore requires a large change in cellular redox homeostasis that involves both increased glutathione accumulation and extensive reduction of the glutathione pool. The data presented here show that the capacity to regenerate GSH from GSSG is restricted in cad2-1/roGFP2 flowers, as is the ability to synthesize glutathione. These findings demonstrate the importance of cellular redox control in pollen germination and tube growth. Further work is required to demonstrate the nature of the mechanisms involved.

Future studies using combinations of techniques such as roGFP2, fluorochromatic methods (Heslop-Harrison and Heslop-Harrison, 1970), and propidium iodide staining (Jimenez-Quesada et al., 2017) are required to investigate the mechanisms involved in oxidation-induced losses of viability and function. However, current methods that are able to determine the viability of individual pollen grains are difficult to use simultaneously with roGFP2 analysis of redox properties. For example, fluorochromatic detection interferes with the roGFP2 signal because both methods use similar excitation and emission wavelengths.

Pollen grains require a robust antioxidant system to germinate and penetrate through the stigma (Creissen et al., 1999, Zechmann, 2008); further studies are required to determine how much of this defence is constitutive and how much is induced upon alleviation of the quiescent state. The data presented here demonstrate that glutathione is an essential component of this antioxidant defence and perhaps also pollen–stigma signalling through thiol-disulfide exchange mechanisms, protein glutathionylation, and S-nitrosogluthathione-mediated processes (Corpas et al., 2013; Zafra et al., 2016). Future work using a range of other mutants and oxidative stress markers is required.
to elucidate the redox differences between ungerminated and germinated pollen and to provide a more complete picture of the involvement of GSH metabolism in pollen germination.

Acknowledgements

We thank the Scientific Instrumentation Service (Estación Experimental del Zaidín, CSIC, Granada) for assistance in GC-MS analysis. We also thank the Microscopy Service (Estación Experimental del Zaidín, CSIC, Granada) for help during the confocal microscopy. This work was supported by European Regional Development Fund-co-funded projects BFU2016-77243-P, P2011-CVT487, RTC2016-4824-2, and RTC2017-6654-2. EGQ thanks the Spanish Ministry of Economy and Competitiveness for “Formación del Personal Investigador” program grant funding.

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