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BRIEF COMMUNICATION

Diurnal changes of subcellular glutathione content in *Arabidopsis thaliana*

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Abstract

The aim of this study was to investigate diurnal changes of subcellular glutathione content in leaves of *Arabidopsis thaliana* by immunohistochemistry and quantitative transmission electron microscopy. The results revealed major diurnal changes in glutathione content. The highest content was found within 2 - 3 h of exposure to light (increase of 489 % in mitochondria, 318 % in plastids, 857 % in nuclei, 511 % in peroxisomes, and 900 % in the cytosol when compared to that during darkness), followed by a strong drop (49 % in mitochondria and the cytosol, 53 % in plastids, 68 % in nuclei, 38 % in peroxisomes) within the next 1 - 2 h. Glutathione content was 67 % lower in mitochondria, 76 % in plastids, 73 % in nuclei, 50 % in peroxisomes, and 68 % in the cytosol at the end of the light period which lasted for 8 h when compared to highest content. Lowest glutathione content was found in most cell compartments at the very end of the dark period. In context with previous studies it could be concluded that low glutathione content at the end of the dark period was caused by lack of glycine and cysteine due to lack of photorespiration and decreased sulfur uptake and assimilation during darkness. The rapid increase of glutathione content observed within 3 h of light was most probably caused by a strong increase in glutathione synthesis triggered by increased glycine and cysteine content. The strong drop of glutathione after that period was most probably due to elevated glutathione degradation rate or increased use of glutathione for phytochelatin synthesis.

Additional key words: antioxidants, chloroplasts, cytosol, mitochondria, peroxisomes, transmission electron microscopy.

Antioxidants such as glutathione protect plants against reactive oxygen and nitrogen species (ROS/RNS) that are produced and accumulated within cells and organelles at different concentrations depending on the environmental circumstances and their developmental stages (Kocsy *et al.* 2013, Zechmann 2014, Foyer and Noctor 2016). Abiotic and biotic stresses can lead to changes in glutathione content and its redox state which is aimed to protect plants against possible negative effects induced by these environmental stresses (Kocsy *et al.* 2013, Zechmann 2014). Additionally, glutathione content varies greatly during different stages of plant growth and development as it is part of a regulatory network that influences and controls plant metabolism and involves hormones, ROS, RNS, and other antioxidants (Kocsy *et al.* 2013). As total glutathione content and its redox state react very sensitive to environmental conditions it is not surprising that glutathione content shows a diurnal rhythm (Schupp and Rennenberg 1988, Noctor *et al.* 1997), *e.g.*, in needles of spruce tree total glutathione content was found to be lowest during the night, climbed steadily until reached the highest value about 6 h after day light started, and then declined (Schupp and Rennenberg 1988). Similar results were found in poplar plants where day light induced increases of foliar reduced glutathione (GSH) content by 29 - 55 % (Noctor *et al.* 1997). In *Arabidopsis thaliana* plants a significant increase (about 2-fold) of glutathione content was observed within the first 4 h of exposure to day light (Huseby *et al.* 2013). A drop in glutathione content started during day and continued during night until it reached lowest values at the end of the dark period. In contrast, a diurnal rhythm for GSH was not found in shoots and roots of *Zea may*s seedlings (Massi *et al.* 2002). Similar results were found in *Nicotiana sylvestris* where total glutathione content and its redox state in leaves did not show any significant variation during the day/night cycle (Dutilleaul *et al.* 2003).

All of the above mentioned studies have investigated glutathione content in whole leaves or organs based on

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Abbreviations: GSH - reduced glutathione; GSSG - oxidized glutathione; RNS - reactive nitrogen species; ROS - reactive oxygen species; TEM - transmission electron microscopy.
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biochemical methods. Nevertheless, these investigations do not reflect the situation in single cells and organelles which would be crucial to understand how differences in the environment between day (*e.g*. day light, higher temperature) and night (*e.g*. lack of light, drop in temperature) effect glutathione content on the subcellular level. Recently, changes of glutathione content in chloroplasts have been simulated under light/dark conditions showing only very small fluctuations of a GSH/oxidised glutathione (GSSG) ratio and unchanged total glutathione content between night and day (Valero *et al.* 2009, 2016). As in this study total glutathione content in chloroplasts was set at a constant value of 4 000 µM for both day and night, based on data collected previously (Polle 2001, Valero *et al.* 2009), the obtained data does not necessarily represent the actual situation in chloroplasts. Recently, we have demonstrated a strong drop of glutathione content in all cell compartments of *Arabidopsis thaliana* during dark induced senescence. When keeping plants in complete darkness for 24 h glutathione content dropped between 53 % in peroxisomes and 84 % in mitochondria (Luschin-Ebengreuth and Zechmann 2016). From this study it becomes obvious that glutathione content is strongly influenced by day/light and night/dark conditions.

Considering the lack of data concerning the subcellular distribution of glutathione during the diurnal cycles, the aim of this study was to investigate detailed diurnal changes of the glutathione content in all cell organelles simultaneously on a high level of resolution by computer supported transmission electron microscopy (TEM) after immunocytochemical detection of glutathione in leaves of *Arabidopsis thaliana*.

Arabidopsis thaliana [L.] Heynh. ecotype Columbia (Col-0) was raised in growth chambers with 8-h photoperiod, an irradiance of 150 µmol m^{-2} s^{-1}, day/night temperatures of 22/18 °C, and a relative humidity of 60 %. Six weeks after stratification fully developed leaves from the 3rd rosette of the plants were harvested at the beginning of every hour over a period of 24 h and prepared for cytohistochemical detection of glutathione as described previously (Zechmann *et al.* 2008a). Briefly, sections of leaves were fixed in 0.06 M phosphate buffer (pH 7.2) containing 2.5 % (m/v) paraformaldehyde and 0.5 % (m/v) glutaraldehyde for 90 min, rinsed 4 times (15 min each) in buffer, dehydrated in increasing concentrations (50, 70, 90, and 100 %, v/v) of acetone for 2 times (10 min each), and then gradually infiltrated with increasing concentrations of *LR-White* (*London Resin Company*, Berkshire, UK) resin. Specimens were finally polymerized at 50 °C for 48 h. Ultra-thin sections were blocked with 2 % (m/v) bovine serum albumine and then treated with the primary antibody against glutathione (*EMD Millipore Corp.*, Billerica, MA, USA) diluted 1:50 for 120 min. As shown in previous studies the antibody against glutathione does not distinguish between the reduced and oxidized forms (Zechmann *et al.* 2008, Queval *et al*. 2011). After rinsing the sections in buffer, they were incubated with the secondary gold conjugated antibody (*BBI Solutions*, Cardiff, UK) diluted 1:50. Labeled grids were washed in distilled water, post stained with uranyl-acetate for 15 s and investigated with a *JEM1010* transmission electron microscope (*JEOL*, Tokyo, Japan). Gold particles were counted using the software package *Cell F*. A minimum of 20 (peroxisomes and vacuoles) to 60 (other cell structures) sectioned cell structures of at least 15 different cells were analyzed. The obtained data were statistically evaluated with *SPSS* Statistics (*IBM Corp*., New York, USA) by applying the Kruskal-Wallis test followed by a *post hoc* comparison according to Conover. $P < 0.05$ was regarded as significant. The specificity and accuracy of the immunogold localization method has been demonstrated in detail in previous experiments (Zechmann *et al.* 2008, Zechmann and Müller 2010).

The results of this study clearly demonstrate that subcellular glutathione content in leaves of *Arabidopsis thaliana* followed a diurnal rhythm in all cell compartments except vacuoles (Figs. 1, 2*A*-*D*). Generally, the highest glutathione content of 508, 26.5, 119, 59.7, and 73.5 gold particles per μm^2 was found in mitochondria, plastids, nuclei, peroxisomes and the cytosol, respectively, after the exposure to light between 2 - 3 h (Figs. 1, 2*A*). The strong increase of glutathione (489 % in mitochondria, 318 % in plastids, 857 % in nuclei, 511 % in peroxisomes, 900 % in the cytosol when compared to the lowest content in the respective cell compartment during darkness) found in this study supports the current opinion that glutathione synthesis is light dependent. Glutathione synthesis and subsequently its content in plants depend on the availability of its precursors cysteine, glycine, and glutamate. While glutamate content remained relatively constant during the diurnal cycle (reviewed by Forde and Lea 2007), fluctuations of glycine and cysteine content are thought to regulate glutathione metabolism in plants. It has been demonstrated that the shortage of glycine as a consequence of lacking photorespiration limits glutathione synthesis in leaves during darkness (Buwalda *et al.* 1990, Noctor *et al.* 1997, Noctor *et al.* 1999). Feeding glycine to these leaves as well as exposing them to day light resulted in glutathione synthesis to be fully restored. The artificial elevation of cysteine led to the accumulation of glutathione which led to the conclusion that cysteine is the main limiting factor of glutathione synthesis in plants exposed to light (Zechmann *et al.* 2007, 2008b, Höller *et al.* 2011, Király *et al.* 2012). When compared to daylight conditions lower cysteine content was found in shoots of *Zea mays* plants during darkness (Masi *et al.* 2002). Additionally, sulfur uptake and incorporation into thiols (cysteine and glutathione), and proteins was strongly reduced during darkness in *Arabidopsis* plants when compared to day light conditions (Huseby *et al.* 2013). These results indicate that cysteine regulates diurnal glutathione content in plants as well. In this respect cysteine seems to play a key role for diurnal glutathione metabolism since reversible cysteine modifications of proteins triggered by oxidation of

cysteine thiols through ROS provide a mechanism for signaling events that activate downstream stress responses. As the circadian hormones jasmonic and salicylic acid influence glutathione pools and its redox state as well, cysteine could provide a link between these clock gated hormones and the activation of stress responses through regulating diurnal glutathione metabolism (Spoel and Loake 2011, Van Ooijen and Millar 2012, Spoel and Van Ooijen 2014).

As glutathione synthesis in *Arabidopsis* plants takes

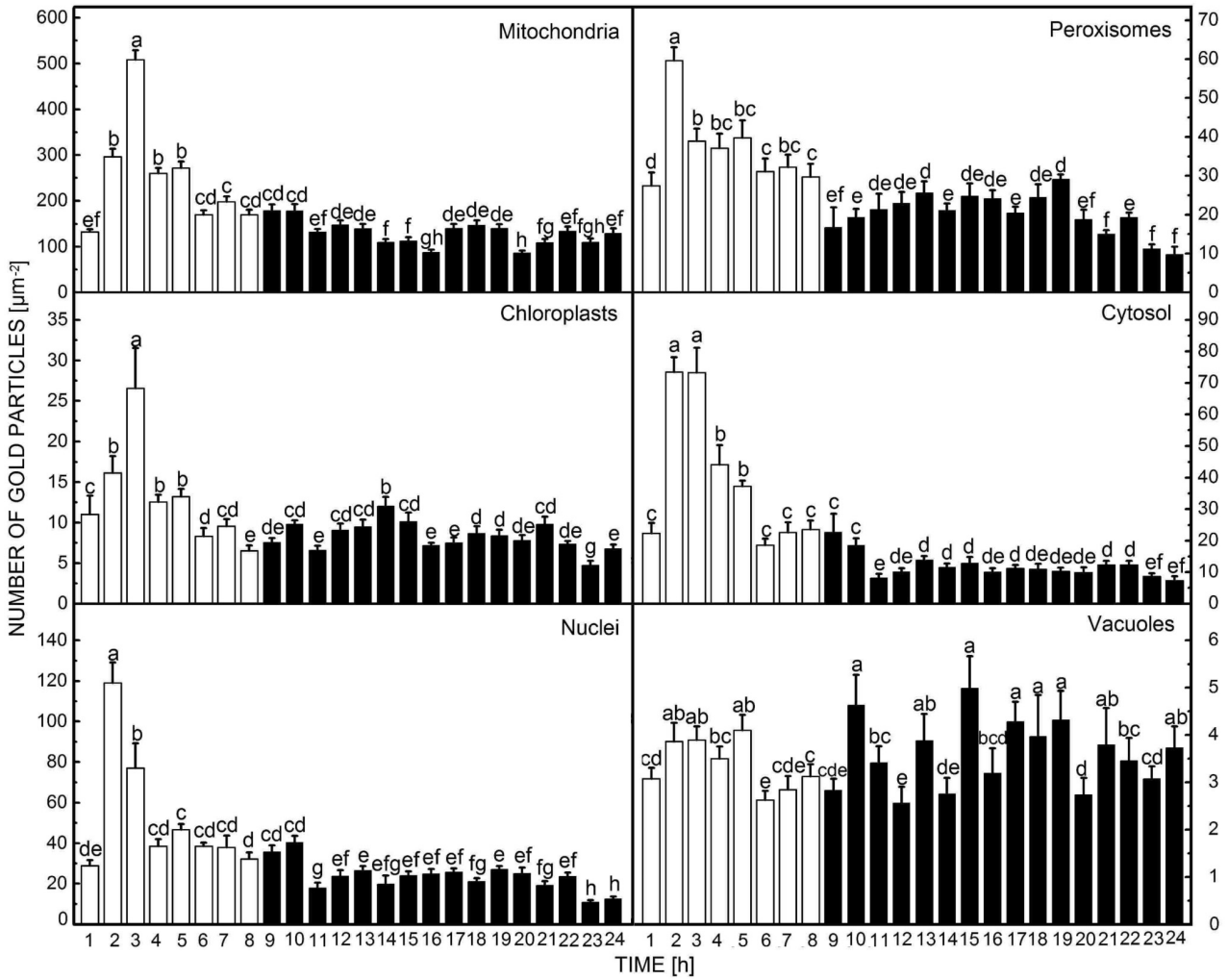

Fig. 1. Diurnal changes of compartment specific glutathione content. Graphs show the numbers of gold particles bound to glutathione per µm^2 in different cell compartments of mesophyll cells of *Arabidopsis thaliana* Col-0 plants over a period of 24 h. The data have been extracted from TEM-micrographs. Different light regimes are indicated by *white* or *black* bars (light or darkness, respectively). Means ± SEs, $n > 20$ for peroxisomes and vacuoles and $n > 60$ for all other cell structures. Significant differences ($P < 0.05$) are indicated by different lowercase letters (Kruskal-Wallis test followed by *post hoc* comparison according to Conover).

place exclusively in chloroplasts and the cytosol (Wachter *et al.* 2005) these results indicate that large amounts of glutathione must be transported from the chloroplasts and the cytosol into the other cell compartments within 2 - 3 h of exposure to light. The transport of glutathione through the chloroplast envelope is facilitated by protein homologs of the *Plasmodium falciparum* chloroquine resistance transporter, CLT1, CLT2, and CLT3 (Maughan *et al.* 2010). Nevertheless, glutathione transporters have not yet been characterized for the other cell compartments and therefore it remains unclear if glutathione gets actively transported into all of these cell compartments or distributed equally by diffusion (*e.g.* into nuclei through nuclear pores). The latter seems very unlikely as strong compartment specific differences between all cell compartments (*e.g.* cytosol always contained between 94 and 83 % lower glutathione content than mitochondria; in all but 2 time points the cytosol contained between 20 to 62 % less glutathione content than nuclei) were found throughout this experiment (Fig. 1) which indicates a selective transport and accumulation of glutathione in all cell compartments. Surprisingly glutathione content dropped rapidly (49 % in mitochondria and the cytosol, 53 % in plastids, 68 % in

nuclei, 38 % in peroxisomes) in all cell compartments within 1 - 2 h after reaching the highest values which was about 4 - 5 h after day light started (Fig. 1). Glutathione content continued to drop and was 67 % lower in mitochondria, 76 % lower in plastids, 73 % lower in nuclei, 50 % lower in peroxisomes, and 68 % in the cytosol at the end of the light period when compared to the highest level (Figs. 1 and 2*B*). These results indicate that glutathione content in plants is not only influenced by light conditions but also follows a general daily rhythm independent of light exposure. Similar results have also been observed in needles of spruce and in *Arabidopsis*, which both showed highest glutathione content (about 2-fold higher than during darkness) within the first 4 - 6 h of exposure to day light and a decrease in glutathione content during day light conditions afterwards

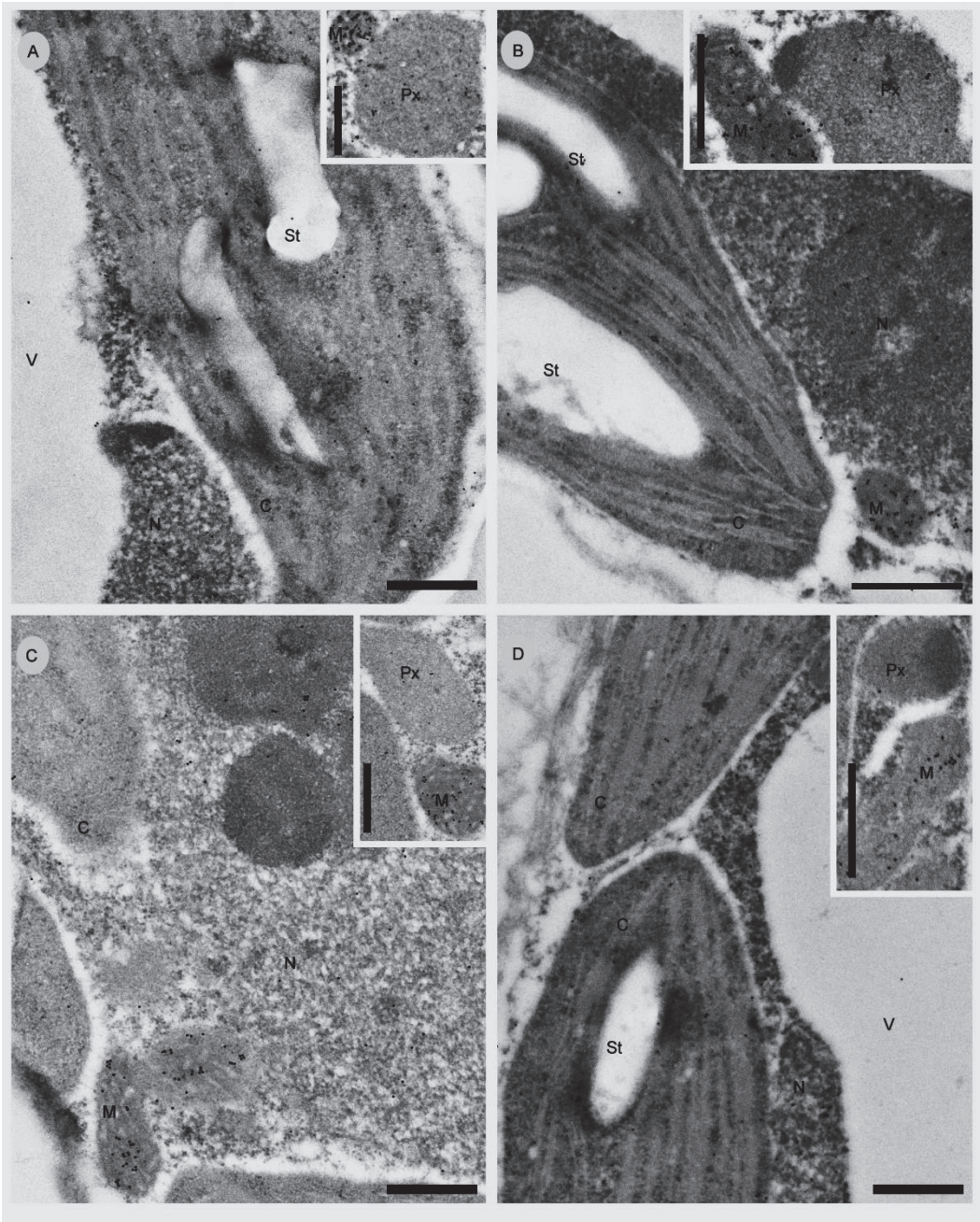

Fig. 2. TEM-micrographs showing diurnal changes in the subcellular distribution of glutathione. Representative transmission electron micrographs of leaf cells from *Arabidopsis thaliana* Col-0 showing the compartment specific distribution of gold particles (*dark dots*) indicate the presence of glutathione in chloroplasts (C) with and without starch grains (St), mitochondria (M), nuclei (N), peroxisomes (Px), and vacuoles (V). TEM-micrographs represent cells exposed to 3 h of light (corresponding to 3 h in Fig. 1; *A*), exposed to 8 h of light (*B*), at the end of the dark period (*C*), and after 8 h of darkness (*D*). Insets show peroxisomes (Px) and mitochondria (M) as these organelles are not always displayed in the larger micrograph. *Bars* = 0.5 μm.

(Schupp and Rennenberg 1988, Huseby *et al.* 2013). Based on sulphate uptake/corporation rate and gene expression studies the latter study which was performed in *Arabidopsis thaliana* demonstrates that highest glutathione content is reached long before the period of highest synthesis rate. The authors argued that metabolite content is not only dependent on the biosynthesis rate but also on the breakdown of the compound (Huseby *et al.* 2013). In this respect one could argue that the steep increase in glutathione content within the first few hours of exposure to day light found in this study is due to high glutathione synthesis rate and low rate of glutathione degradation whereas at later time points rate of glutathione degradation starts to exceed rate of glutathione synthesis. As glutathione also serves as a direct precursor of phytochelatins (Seth *et al.* 2012) the depletion of glutathione during these situations might not only be related to glutathione degradation but could also be correlated to increased production of phytochelatins.

In this study, the lowest glutathione content was found in most cell compartments (86,5, 4.7, 12.5, 9.8, and 7.4 gold particles per μm^2 in mitochondria, plastids, nuclei, peroxisomes and the cytosol, respectively cytosol) at the very end of the dark period (Figs. 1 and 2*C*). A drop of glutathione in leaves due to lack of day light has also been observed in poplar, spruce, and *A. thaliana* (Schupp and Rennenberg 1988, Noctor *et al.* 1997, Huseby *et al.* 2013). As mentioned above the drop of glutathione during darkness is the result of lacking photorespiration and subsequently lack of glycine (Buwalda *et al.* 1990, Noctor *et al.* 1997, 1999). In a previous study we demonstrated that the complete absence of day light for 24 h led to a drop of glutathione content in leaves of *A. thaliana* Col-0 in all cell compartments between 53 % in peroxisomes and 84 % in mitochondria while ascorbate content remained largely unchanged (Luschin-Ebengreuth and Zechmann 2016). Within 48 h of darkness glutathione content dropped by 90 % in all cell compartment and within one week glutathione content was completely diminished. These results clearly demonstrate that extended periods of darkness completely diminish glutathione content in plants which is accompanied by loss of photosynthetic pigments and a decrease of the activity of important enzymes involved in the ascorbate-glutathione cycle such as glutathione reductase and dehydroascorbate reductase. Subsequently, extended darkness led to the accumulation of hydrogen peroxide and eventually to senescence and cell dead (Luschin-Ebengreuth and Zechmann 2016).

It can be concluded that low glutathione content at the end of the dark period is caused by lack of its precursors glycine and cysteine induced by lack of photorespiration and low sulfur uptake and incorporation, respectively. The rapid increase of glutathione content observed within 3 h of day light is most probably caused by a strong increase in glutathione synthesis induced by day light when glycine and cysteine production is restored. The strong drop of glutathione after that period is most probably due to increased glutathione degradation and increased use for phytochelatin synthesis.

References

Buwalda, F., Stulen, I., De Kok, L.J., Kuiper, P.J.C.: Cysteine, c-glutamylcysteine and glutathione contents of spinach leaves as affected by darkness and application of excess sulfur. II. Glutathione accumulation in detached leaves exposed to H_2S in the absence of light is stimulated by the supply of glycine to the petiole. - Physiol. Plant. **80**: 196-204, 1990.

Dutilleul, C., Garmier, M., Noctor, G., Mathieu, C., Chetrit, P., Foyer, C.H., Paepe, R.: Leaf mitochondria modulate whole cell redox homeostasis, set antioxidant capacity, and determine stress resistance through altered signaling and diurnal regulation. - Plant Cell **15**: 1212-1226, 2003.

Forde, B.G., Lea, P.J.: Glutamate in plants: metabolism, regulation, and signaling. - J. exp. Bot. **58**: 2339-2358, 2007.

Foyer, C.H., Noctor, G.: Stress-triggered redox signalling: what's in pROSpect? - Plant Cell Environ. **39**: 951-964, 2016.

Höller, K., Király, L., Künstler, A., Müller, M., Gullner, G., Fattinger, M., Zechmann, B.: Enhanced glutathione metabolism is correlated with sulfur induced resistance in *Tobacco mosaic virus*-infected genetically susceptible *Nicotiana tabacum* plants. - Mol. Plant-Microbe Interact. **23**: 1448-1459, 2011.

Huseby, S., Koprivova, A., Lee, B.R., Shaha, S., Mithen, R., Wold, A.B., Bengtsson, G.B., Kopriva, S.: Diurnal and light regulation of sulphur assimilation and glucosinolate biosynthesis in *Arabidopsis*. - J. exp. Bot. **64**: 1039-1048, 2013.

Király, L., Künstler, A., Höller, K., Fattinger, M., Juhász, C., Müller, M., Gullner, G., Zechmann B.: Sulfate supply influences compartment specific glutathione metabolism and confers enhanced resistance to *Tobacco mosaic virus* during a hypersensitive response. - Plant Physiol. Biochem. **59**: 44-54, 2012.

Kocsy, G., Tari, I., Vankova, R., Zechmann, B., Gulyas, Z., Poor, P., Galiba, G.: Redox control of plant growth and development. - Plant Sci. **211**: 77-91, 2013.

Luschin-Ebengreuth, N., Zechmann, B.: Compartment specific investigations of antioxidants and hydrogen peroxide in leaves of *Arabidopsis thaliana* during dark induced senescence. - Acta Physiol. Plant. **38**: 133, 2016.

Massi, A., Ghisi, R., Ferretti, M.: Measuring low-molecular-weight thiols by detecting the fluorescence of their SBD-derivatives: application to studies of diurnal and UV-B induced changes in *Zea mays* L. - J. Plant Physiol. **159**: 499-507, 2002.

Maughan, S.C., Pasternak, M., Cairns, N., Kiddle, G., Brach, T., Jarvis, R., Haas, F., Nieuwlanda, J., Lim, B., Mueller, C., Salcedo-Sorae, E., Kruse, C., Orsel, M., Hell, R., Miller, A.J., Bray, P., Foyer, C.H., Murray, A.H., Meyer, A., Cobbett, C.S.: Plant homologs of the *Plasmodium falciparum* chloroquine resistance transporter PfCRT are required for glutathione homeostasis and stress responses. - Proc. nat. Acad. Sci. USA **107**: 2331-2336, 2010.

Noctor, G., Arisi, A.C.M., Jouanin, L., Foyer, C.H.:

Photorespiratory glycine enhances glutathione accumulation in both the chloroplastic and cytosolic compartments. - J. exp. Bot. **50**: 1157-1167, 1999.

Noctor, G., Arisi, A.C., Jouanin, L., Valadier, M.H., Rouc, Y., Foyer C. H.: Light-dependent modulation of foliar glutathione synthesis and associated amino acid metabolism in poplar overexpressing c-glutamylcysteine synthetase. - Planta **202**: 357-369, 1997.

Polle, A.: Dissecting the superoxide dismutase-ascorbate-glutathione pathway in chloroplasts by metabolic modeling: computer simulations as a step towards flux analysis. - Plant Physiol. **126**: 445-462, 2001.

Queval, G., Jaillard, D., Zechmann, B., Noctor G.: Increased intracellular H_2O_2 availability preferentially drives glutathione accumulation in vacuoles and chloroplasts. - Plant Cell Environ. **34**: 21-32, 2011.

Schupp, R., Rennenberg, H.: Diurnal changes in the glutathione content of spruce needles (*Picea abies* L.). - Plant Sci. **57**: 113-117, 1988.

Seth, C.S., Remans, T., Keunen, E., Jozefczak, M., Gielen, H., Opdenakker, K., Weyens, N., Vangronsveld, J., Cuypers, A.: Phytoextraction of toxic metals: a central role for glutathione. - Plant Cell Environ. **35**: 334-346, 2012.

Spoel, S.H., Loake, G.J.: Redox-based protein modifications: the missing link in plant immune signalling. - Curr. Opin. Plant Biol. **14**: 358-364, 2011.

Spoel, S.H., Van Ooijen, G.: Circadian redox signaling in plant immunity and abiotic stress. - Antioxid. Redox Signal. **20**: 3024-3039, 2014.

Valero, E., González-Sánchez, M.I., Macià, H., García-Carmona, F.: Computer simulation of the dynamic behavior of the glutathione-ascorbate redox cycle in chloroplasts. - Plant Physiol. **149**: 1958-1969, 2009.

Valero, E., Macià, H., Fuente, I., Hernandez, J.A., Gonzales-Sanchez, M.I., Garcia-Carmona, F.: Modeling the ascorbate-glutathione cycle in chloroplasts under light/dark conditions. - BMC Systems Biol. **10**: 11, 2016.

Van Ooijen, G., Millar, A.J.: Non-transcriptional oscillators in circadian timekeeping. - Trends Biochem. Sci. **37**: 484-492, 2012.

Wachter, A., Wolf, S., Steininger, H., Bogs, J., Rausch, T.: Differential targeting of GSH1 and GSH2 is achieved by multiple transcription initiation: implications for the compartmentation of glutathione biosynthesis in the *Brassicaceae*. - Plant J. **41**: 15-30, 2005.

Zechmann, B.: Compartment specific importance of glutathione during abiotic and biotic stress. - Front. Plant Sci. **5**: 566, 2014.

Zechmann, B., Mauch, F., Sticher, L., Müller, M.: Subcellular immunocytochemical analysis detects the highest concentrations of glutathione in mitochondria and not in plastids. - J. exp. Bot. **59**: 4017-4027, 2008a.

Zechmann. B., Müller, M.: Subcellular compartmentation of glutathione in dicotyledonous plants. - Protoplasma **246**: 15-24, 2010.

Zechmann, B., Müller, M., Zellnig, G.: Modified levels of cysteine affect glutathione metabolism in plant cells. - In Khan, N.A., Singh S., Umar S. (ed.): Sulfur Assimilation and Abiotic Stress in Plants. Pp. 193-206. Springer-Verlag, Berlin - Heidelberg 2008b.

Zechmann, B., Zellnig, G., Urbanek-Krajnc, A., Müller, M.: Artificial elevation of glutathione affects symptom development in ZYMV-infected *Cucurbita pepo* L. plants. - Arch. Virol. **152**: 747-762, 2007.