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High resolution imaging of subcellular glutathione concentrations by quantitative immunoelectron microscopy in different leaf areas of *Arabidopsis*

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ABSTRACT

Glutathione is an important antioxidant and redox buffer in plants. It fulfills many important roles during plant development, defense and is essential for plant metabolism. Even though the compartment specific roles of glutathione during abiotic and biotic stress situations have been studied in detail there is still great lack of knowledge about subcellular glutathione concentrations within the different leaf areas at different stages of development.

In this study a method is described that allows the calculation of compartment specific glutathione concentrations in all cell compartments simultaneously in one experiment by using quantitative immunogold electron microscopy combined with biochemical methods in different leaf areas of *Arabidopsis thaliana* Col-0 (center of the leaf, leaf apex, leaf base and leaf edge). The volume of subcellular compartments in the mesophyll of *Arabidopsis* was found to be similar to other plants. Vacuoles covered the largest volume within a mesophyll cell and increased with leaf age (up to 80% in the leaf apex of older leaves). Behind vacuoles, chloroplasts covered the second largest volume (up to 20% in the leaf edge of the younger leaves) followed by nuclei (up to 2.3% in the leaf edge of the younger leaves), mitochondria (up to 1.6% in the leaf apex of the younger leaves), and peroxisomes (up to 0.3% in the leaf apex of the younger leaves). These values together with volumes of the mesophyll determined by stereological methods from light and electron micrographs and global glutathione contents measured with biochemical methods enabled the determination of subcellular glutathione contents in mM.

Even though biochemical investigations did not reveal differences in global glutathione contents, compartment specific differences could be observed in some cell compartments within the different leaf areas. Highest concentrations of glutathione were always found in mitochondria, where values in a range between 8.7 mM (in the apex of younger leaves) and 15.1 mM (in the apex of older leaves) were found. The second highest amount of glutathione was found in nuclei (between 5.5 mM and 9.7 mM in the base and the center of younger leaves, respectively) followed by peroxisomes (between 2.6 mM in the edge of younger leaves and 4.8 mM in the base of older leaves, respectively) and the cytosol (2.8 mM in the edge of younger and 4.5 mM in the center of older leaves, respectively). Chloroplasts contained rather low amounts of glutathione (between 1 mM and 1.4 mM). Vacuoles had the lowest concentrations of glutathione (0.01 mM and 0.14 mM) but showed large differences between the different leaf areas. Clear differences in glutathione contents between the different leaf areas could only be found in vacuoles and mitochondria revealing that glutathione in the later cell organelle accumulated with leaf age to concentrations of up to 15 mM and that concentrations of glutathione in vacuoles are quite low in comparison to the other cell compartments.

1. Introduction

The tripeptide glutathione (γ-glutamyl-cysteinyl-glycine) is the major non-protein thiol in plant cells and is present in many cellular compartments at millimolar concentrations (Diaz Vivancos et al., 2010a,b). As an antioxidant it is involved in detoxifying reactive oxygen species (ROS) through the ascorbate-glutathione cycle (Foyer and Noctor, 2009, 2011; Noctor et al., 2012). Glutathione is also involved in the detoxification of xenobiotics, herbicides (Cummins et al., 2011; DeRidder and Goldsbrough, 2006; Edwards et al., 2005; Mohsenzadeh et al., 2011) and heavy metals (Ammar et al., 2008; DalCorso et al., 2008; Jozefczak et al., 2012; Nocito et al., 2008; Seth et al., 2012; Tan et al., 2010; Zawoznik et al., 2007) and protects proteins from oxidation through glutathionylation

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(Zaffagnini et al., 2012a,b). Additionally, glutathione is a key regulator of redox signaling which controls gene expression and contributes to cell survival (Foyer et al., 2001; Maughan and Foyer, 2006). Glutathione is also essential for proper plant development. The glutathione deficient *Arabidopsis* mutant *rml1* which contains up to 97% less glutathione than the wildtype lacks a root meristem and develops severe growth defects (Cairns et al., 2006; Vernoux et al., 2000; Zechmann and Müller, 2010). Additionally, it has been shown recently that pollen grains with inhibited glutathione synthesis resulting in low glutathione contents during germination show a strong decrease in germination rates (Zechmann et al., 2011a,b). High and stable levels of glutathione in mitochondria have been found to be of significant importance in *Arabidopsis* as the glutathione deficient *pad2-1* mutant which shows a phenotype similar to the wildtype (Zechmann et al., 2008) contains control levels of glutathione in mitochondria despite a drop of glutathione contents in all other cell compartments of up to 90%.

Generally, the compartmentation of metabolites in different compartments, tissues and organs is of great importance for many physiological processes and metabolic regulation (Bowsher and Tobin, 2001; Hartmann et al., 2003). To get a greater understanding of the function of metabolites, such as glutathione, it is important to analyze the subcellular localization and measure compartment specific concentrations. There are different approaches which have given more detailed insight about the subcellular distribution of glutathione. It has been measured with biochemical methods (high performance liquid chromatography; HPLC) in different compartments (like mitochondria, chloroplasts, peroxisomes, apoplast, and vacuoles) after isolation and fractionation (Jiménez et al., 1997, 1998; Krueger et al., 2009; Kuźniak and Sklodowska, 2001, 2004, 2005; Ohkama-Ohtsu et al., 2007; Vanacker et al., 1998a,b,c). By using these methods glutathione has been detected at a concentration between 0.5 and 5 mM in chloroplasts (Foyer and Halliwell, 1976; Noctor et al., 2002; Krueger et al., 2009), between 1 and 3.52 mM in the cytosol (Noctor et al., 2002; Krueger et al., 2009) and 0.73 mM in the vacuole (Krueger et al., 2009). Nevertheless, biochemical methods face the problems that large amounts of plant material are needed for the isolation of organelles, and that it is not possible to isolate all cell compartments simultaneously in one experiment. Additionally, as postulated by Krueger et al. (2009) the isolation procedure can also lead to a loss of glutathione from organelles and to contaminations of non-organelle specific substances. Thus, it remains unclear how well the obtained results reflect situations *in vivo* (Chew et al., 2003; Krueger et al., 2009; Noctor et al., 2002). Other methods for the detection of glutathione include light microscopy, and staining of glutathione with monochloro- or monobromobimane (Fricker et al., 2000; Meyer and Fricker, 2000; Müller et al., 2005). By using these methods glutathione could be detected in nuclei, cytosol and vacuoles (Meyer et al., 2007; Müller et al., 2005). The concentration of glutathione in the cytosol was estimated to be between 2.7 and 3.2 mM (Meyer et al., 2001). However, these studies were limited by a resolution of about 200 nm and the inability of the stains to infiltrate certain organelles. Thus, it was not possible to obtain more information about the localization of glutathione in plastids, peroxisomes, mitochondria, dictyosomes and endoplasmic reticulum (ER) and to quantify compartment specific differences with these methods. Ratiometric redox-sensitive green fluorescence protein (GFP) expressed in *Arabidopsis thaliana* (Gutscher et al., 2008; Meyer et al., 2007) has been used recently to detect glutathione in the cytosol and also in smaller organelles such as the ER as well as changes in glutathione contents in the cytosol during environmental stress situations (Jubany-Marí et al., 2010). Additionally, this method has been used to analyze the redox state of the cytosol and other cell compartments (Gutscher et al., 2008; Maughan et al., 2010; Maughan and Foyer, 2006; Meyer et al., 2007). Nevertheless, these methods are limited by the fact that it is difficult to investigate the situation in deeper cell layers (mesophyll) or tissues (*e.g.* vascular cells) due to background fluorescence and therefore it remains unclear how changes observed in epidermal cells, isolated cells or cell cultures reflect the situation in other cell layers. Additionally, it has not been possible so far and will remain technically challenging to measure changes in glutathione contents in all cell compartments simultaneously in one experiment and to translate the obtained signal into compartment specific glutathione concentrations. One powerful method that has been developed recently is the study of the subcellular distribution of glutathione based on immunogold-cytochemistry and computer-supported transmission electron microscopy which allows the simultaneous detection of glutathione in all cell compartments at a high level of resolution (Queval et al., 2011; Zechmann et al., 2006, 2008; Zechmann and Müller, 2010). This method has been applied on different plant species and revealed that the highest amount of glutathione was always detected in mitochondria, followed by nuclei, peroxisomes, the cytosol and plastids. None or very little glutathione was detected in vacuoles and the apoplast (Zechmann and Müller, 2010).

Many studies showed differences in glutathione contents either between organs or cell compartments, but the subcellular distribution in different leaf areas as well as compartment specific glutathione concentrations throughout the leaf remain unclear. Such information could be essential in order to study differences in the stress response between different leaf areas, during leaf development, and in the accumulation of glutathione in certain leaf areas. Thus, the aim of the present study was to develop a method that allows the determination of compartment specific glutathione concentrations in all cell compartments simultaneously in one experiment. This method was further used to determine differences in the subcellular distribution of glutathione in *A. thaliana* Col-0 leaves between different stages of development (older and youngest fully developed leaves) and different leaf areas (center of the leaf, leaf apex, leaf base, and leaf edge). To analyze the subcellular distribution of glutathione quantitative immunogold-cytochemistry was combined with biochemical measurements of global glutathione contents (LC/MS/MS) in order to measure compartment specific concentrations of glutathione. Together with the mesophyll volume, which was analyzed by light microscopy, fresh and dry weight of the particular leaf area, and the relative compounds of the compartments the concentration was calculated in mM. Some information about the mesophyll volume has already been determined in other studies with species such as barley (Winter et al., 1993), spinach (Winter et al., 1994) and wheat (Bowsher and Tobin, 2001; Hopkins, 1997) but data is still missing for *Arabidopsis* leaves. Thus, the described method gives compartment specific glutathione concentrations in mM on a high level of resolution by a combination of stereological, quantitative cytohistochemical and biochemical methods.

2. Materials and methods

2.1. Plant material and growth conditions

After stratification for 4 days at 4 °C seeds of *A. thaliana* accession Col-0 from the European *Arabidopsis* stock centre (NASC, Loughborough, UK) were grown on soil in growth chambers with a 9/15 h day/night photoperiod. The temperatures during the days were 22 °C and 18 °C during the night. While the relative humidity was 60% the plants were kept at 100% relative soil water content. Light intensity was 150 μmol m^{-2} s^{-1}. Six weeks after stratification samples of different leaf areas from older leaves (5 cm × 2.5 cm from the second rosette after cotyledons) and youngest fully developed