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A fluorometer-based method for monitoring oxidation of redox-sensitive GFP (roGFP) during development and extended dark stress

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Redox-sensitive GFP (roGFP) localized to different compartments has been shown to be suitable for determination of redox potentials in plants via imaging. Long-term measurements bring out the need for analyzing a large number of samples which are averaged over a large population of cells. Because this goal is too tedious to be achieved by confocal imaging, we have examined the possibility of using a fluorometer to monitor changes in roGFP localized to different subcellular compartments during development and dark-induced senescence. The degree of oxidations determined by a fluorometer for different probes was similar to values obtained by confocal image analysis. Comparison of young and old leaves indicated that in younger cells higher levels of H_2O_2 were required to achieve full roGFP oxidation, a parameter which is necessary for calculation of the degree of oxidation of the probe and the actual redox potential. Therefore, it is necessary to carefully determine the H_2O_2 concentration required to achieve full oxidation of the probe. In addition, there is an increase in autofluorescence during development and extended dark stress, which might interfere with the ability to detect changes in oxidation–reduction dependent fluorescence of roGFP. Nevertheless, it was possible to determine the full dynamic range between the oxidized and the reduced forms of the different probes in the various organelles until the third day of darkness and during plant development, thereby enabling further analysis of probe oxidation. Hence, fluorometer measurements of roGFP can be used for extended measurements enabling the processing of multiple samples. It is envisaged that this technology may be applicable to the analysis of redox changes in response to other stresses or to various mutants.

Introduction

Changes in the accumulation of reactive oxygen species (ROS) have been implicated in the execution of cell death program, as well as in signaling in the activation of cell death programs caused by different stresses (Foyer and Noctor 2005, Van Breusegem et al. 2008, Van Breusegem and Dat 2006). Localized ROS production

Abbreviations – ddw, double distilled water; DR, dynamic range; GFP, green fluorescent protein; GRX, glutaredoxin; roGFP, redox-sensitive GFP; ROS, reactive oxygen species; SKL, serine-lysine-leucine; WT, wild type.

and its amelioration is essential for cell responses to the environmental and developmental cues, and perturbation in ROS levels in specific organelles might be responsible for initiation of either a cell salvage or a cell death programs. Glutathione in conjunction with ascorbate is used to detoxify ROS via the glutathione–ascorbate cycle to avoid deleterious effects. However, glutathione also has long being suggested to transduce environmental signals to the nucleus (May et al. 1998). The thiol redox potential, which is reflected in the reduced to oxidized glutathione ratio (GSH/GSSG), is highly regulated within the various cellular compartments – and changes in the redox equilibrium activate various cellular processes and bring about a whole array of functions (Meyer 2008). Because electrons for ROS detoxification are at least in part drawn from the GSH pool, perturbation of ROS levels are manifested in concomitant changes in the thiol redox potential (Marty et al. 2009).

Imaging of ROS in live cells has been achieved by various fluorescent dyes detailed in recent reviews (Halliwell and Whiteman 2004, Van Breusegem et al. 2008). The most popular dye is H_2DCF-DA which was used to determine mitochondrial and chloroplastic ROS levels in protoplasts (Gao et al. 2008, Zhang et al. 2009), but so far these methods suffer various pitfalls (Halliwell and Whiteman 2004). An alternative option for imaging of thiol redox potential in independent compartments has emerged with the introduction of redox-sensitive GFP (roGFP) (Hanson et al. 2004, Jiang et al. 2006, Meyer et al. 2007, Schwarzlander et al. 2008). GFP, in which two surface exposed amino acids were substituted with cysteines to form reduction–oxidation dependent disulfide bonds, has been targeted to various compartments, and it was demonstrated that these probes can be used to detect redox changes in various compartments in mammalian as well as plant cells (Dooley et al. 2004, Hanson et al. 2004, Jiang et al. 2006, Meyer et al. 2007, Schwarzlander et al. 2008). Several roGFPs have been created with different midpoint potentials (Hanson et al. 2004). The change in fluorescence of these probes in plants is dependent on the redox potential (E_{GSH}) of the glutathione buffer based on specific interaction with glutaredoxins (GRXs) (Meyer et al. 2007). The roGFP probes are ratiometric by excitation which eliminates any errors resulting from probe concentration, photobleaching and variable thickness of the tissue (Hanson et al. 2004). It has been shown that these probes offer an advantage over other usually destructive methods for assessing the redox potential (Dooley et al. 2004, Jiang et al. 2006, Meyer et al. 2007).

Our study describes the application of roGFP technology to extended dark stress and to monitoring redox potential during development, by applying fluorometer measurements. In this study we also pinpoint the considerations that should be addressed when long-term measurements are being performed.

Materials and methods

Plant material and preparation of transgenic plants harboring roGFP

Several *Arabidopsis* lines were used in this study in addition to non-transformed WT (Col-0): WT plants expressing roGFP1 in cytoplasm (cyt-roGFP1), mitochondria (mit-roGFP1) and peroxisome (per-roGFP1); WT plants expressing roGFP2 in plastids (pla-roGFP2) (Jiang et al. 2006, Meyer et al. 2007, Schwarzlander et al. 2008, 2009); and WT and *gr1*-1 transgenic plants expressing roGFP2 conjugated to GRX1 in cytoplasm (cyt-GRX1-roGFP2) (Marty et al. 2009). Cytoplasmic roGFP1 was transformed to *Agrobacterium* strain GV3101. *Arabidopsis* plants were transformed by floral-dip (Clough and Bent 1998) to generate cyt-roGFP1 line. For targeting of roGFP1 into peroxisomes (per-roGFP) roGFP1 sequence was amplified with 5′-CTA CAA GGC GCG CCT AGC GCA TGG TGA GCA AGG GCG AGG A-3′ and 5′-TAC GTC TTA ATT AAC TTT TAT AGT TTC GAC TTG TAC AGC TCG TCC ATG CCG A-3′ to generate an *Asc*I restriction site at the N-terminus and the SKL peroxisomal targeting motif and a *Pac*I restriction site at the C-terminus. The fragment roGFP1-SKL was cloned to the vector pMDC32 with *Asc*I and *Pac*I downstream of the 2×CaMV 35S promoter creating a pMDC32-roGFP1-SKL. The transformed seeds were planted on 0.5 MS agar plates containing 20 μg ml^{-1} hygromycin to select transformants. These lines showed a very low fluorescence and were used only for localization studies.

Seedlings of WT and transgenic plants were planted in peat mix-containing 10 cm pots. Plants were grown at 21°C under a 12 h day/12 h night regime and light intensity was of 80 μmol m^{-2} s^{-1}. For most of the experiments five-week-old plants were used which were at stage 6.0 according to Boyes et al. (2001) after bolting. During early plant development the seventh leaf was marked using sewing thread and this leaf was used for most experiments. In some cases, however, plants of different stages of development or different leaves were used as indicated. For dark-induced senescence, roots and bolting stems were severed and rosettes were placed in dark at 21°C on a wet paper. Samples were taken from these rosettes during 1–4 days of darkness.

Measurements of roGFP fluorescence by a fluorometer

Leaf discs (0.5 cm diameter) were cut out from detached rosettes held in darkness for various lengths of time or from different age and floated on 200 µl double distilled water (ddw) in 96 well ELISA plates with their abaxial side up. Fluorescence measurements were performed on a fluorescence plate reader Synergy TM2 (BioTek Instruments Inc., Winooski, VT) from the upper side. Leaves were excited by using 400 ± 15 nm and 485 ± 10 nm filters and fluorescence values were measured using 528 ± 20 nm emission filter. The reader sensitivity was adjusted according to the probe properties. For roGFP1, sensitivity was adjusted to 110 for excitation of 400 nm and 485 nm, however for roGFP2, sensitivity was adjusted to 110 for 400 nm, and to 80 for 485 nm excitation. These sensitivity values gave similar magnitudes of intensity at 528 nm emission.

Time course measurements of fluorescence were read in the resting state and then the leaf discs were treated with 50 m*M* H_2O_2 in order to measure the fluorescence when roGFP is fully oxidized. Subsequently, the leaf discs were washed three times with ddw and then treated with 50 m*M* DTT for full reduction of roGFP. For background correction emission intensities was determined for 16 leaf discs of similar developmental status obtained from non-transformed WT plants which were exposed to same excitation wavelengths under the same conditions. These values were averaged and subtracted from the fluorescence values of roGFP.

The degrees of oxidation of the roGFP and estimation of redox potential were calculated according to Schwarzlander et al. (2008). Midpoint potentials of −288 mV for roGFP1 and −272 mV for roGFP2 (Hanson et al. 2004) were used for all calculations. pH values used for calculation of redox potential were 7.2 for the cytoplasm, 7.8 for the mitochondria and 8.0 for plastids (Schwarzlander et al. 2008).

Confocal laser scanning microscopy and image analysis

Leaf discs of *Arabidopsis* plants expressing roGFP directed to the various compartments were viewed on their abaxial side with a model Olympus IX 81 inverted laser scanning confocal microscope Fluoview 500 (Olympus, Tokyo, Japan). The microscope was equipped with lasers for 405 and 488 nm excitation. Images of fluorescence related to roGFP were acquired by using a BA 515–525 filter following excitation at 405 and 488 nm. According to the probe properties, the ratio of 405/488 nm laser power was 1:4 in roGFP1 lines and 3:1 in roGFP2 lines, and the energy of the excitation beams was kept constant during the experiment. Chlorophyll autofluorescence was detected by using a BA 660 IF emission filter, following excitation at 488 nm. Images of epidermal cytoplasm, mitochondria and plastids represent one confocal section, however, that of peroxisomes is composed by a merge of seven confocal sections of 1 µm each.

Anchored leaf discs were treated consecutively with 50 m*M* H_2O_2 and 50 m*M* DTT under the microscope in order to bring the protein to fully oxidized or reduced forms, respectively. Preliminary experiments revealed that these concentrations were sufficient in most of the experiments for obtaining the maximum dynamic range (DR), unless otherwise stated.

Fluorescence ratio analysis was performed using IMAGEJ software (http://rsb.info.nih.gov/ij/). Image analysis of roGFP in mit-roGFP2 and cyt-roGFP1 was performed on sections within images taken from 405 and 488 nm pictures and in pla-roGFP2 lines only on individual chloroplasts which exhibited chlorophyll autofluorescence. Intensities values were collected from each picture using ROI manager. When needed, ratiometric imaging of the chloroplasts was performed as described (Meyer et al. 2007).

Results

The use of a fluorometer for monitoring redox conditions in *Arabidopsis* leaves

The applicability of roGFP for measuring the redox potential in plant cells has been demonstrated by confocal imaging (Jiang et al. 2006, Meyer et al. 2007, Schwarzlander et al. 2008, 2009). The feasibility of using a fluorometer for measurements of redox potential in *Arabidopisis* leaves is demonstrated by using four *Arabidopsis* transgenic lines expressing roGFPs in the cytosol (cyt-roGFP1), mitochondria (mit-roGFP1, mit-roGFP2) and in the plastids (pla-roGFP2) (Fig. 1). Fluorescence intensities at 528 nm have been determined following excitation at 400 and 485 nm for the resting state and after addition of H_2O_2 and DTT (Fig. 1A). Fluorescence intensity for excitation at 400 nm excitation increased due to H_2O_2 and decreased due to DTT applications. A reciprocal pattern of relative changes in fluorescence was observed following excitation at 485 nm. The fluorescence ratio (R) of 400/485 nm increased after addition of H_2O_2 and decreased after addition of DTT (Fig. 1B). These results are consistent with the probe properties as described in plants and mammalian cells (Dooley et al. 2004, Hanson et al. 2004, Jiang et al. 2006). Because by fluorometry the total fluorescence intensity in the tissue was measured,

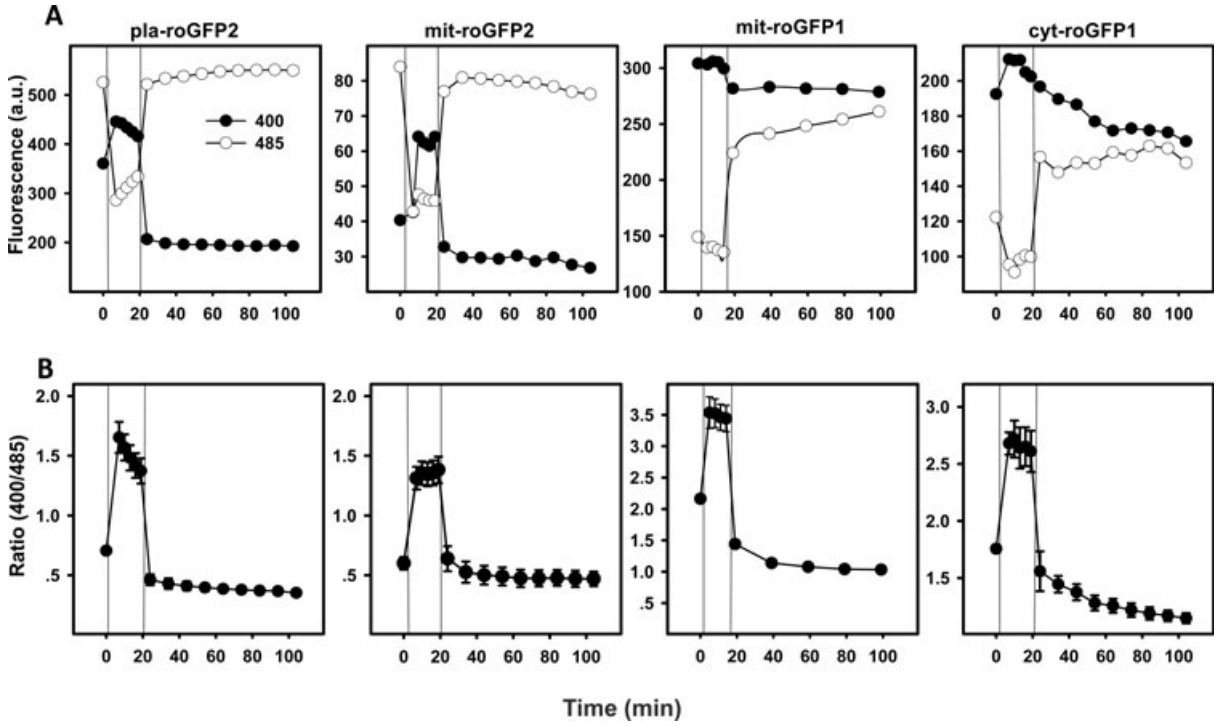

Fig. 1. Time course of fluorescence response to oxidizing and reducing conditions. Fluorescence read by a fluorometer was recorded from roGFP2 localized to plastids and to mitochondria and from roGFP1 localized to the cytoplasm and mitochondria in *Arabidopsis* transgenic plants (A). Leaf's discs were excited at 400 and 485 nm and emission was recorded at 528 nm and the ratio of 400/485 nm fluorescence is presented (B). The resting state is followed by treatment with 50 m*M* H_2O_2 (0–20 min) and 50 m*M* DTT (20–100 min). Vertical lines represent the addition of H_2O_2 followed by the addition of DTT. Average autofluorescence values were determined in WT plants exposed to the same excitation/emission regimes and subtracted from fluorescence of roGFP lines. The data in (A) were obtained from one representative disc and calculated as the mean of 16 leaf discs from 8 plants ± SE (B). au-arbitrary units.

there was a need to verify correct roGFP probe localization. Through complementary confocal analysis it was shown that all probes were indeed localized to their designated organelles (Fig. 5, day 0). Note that roGFP2 directed to plastids was localized to organelles containing chlorophyll autofluorescence as judged by chlorophyll fluorescence (Ex/Em = 488/680 nm), as well as to organelles devoid of it (arrows at Fig. 5, day 0), which are presumably proplastids. Fluorescence values generated from the per-roGFP1 line were too low and similar to that from WT plants; therefore, this line was not suitable for fluorometer analysis, however, other lines might be suitable.

The fluorescence ratios described in Fig. 1B were used to calculate the DRs which represents the ratio between the maximum value (most oxidized) and the minimum value (most reduced) (R_{ox}/R_{red}). A high DR is desirable to obtain high sensitivity and the ability to resolve small redox changes. The maximum DR determined was different for each of the used *Arabidopsis* lines. For both roGFP1 lines (cyt-roGFP1, mit-roGFP1) the DR was similar and reached 2.5, however, a higher DR was found for roGFP2 lines: 3.23 for mit-roGFP2 and 4.49 for pla-roGFP2 (Fig. 2A).

Measuring the fluorescence ratio (R) at resting state and after treatments with H_2O_2 and DTT enabled the calculation of the probes degree of oxidation at a resting state in each of the transgenic lines according to formulas described by Schwarzlander et al. (2008). Using roGFP2, the degree of oxidation of the protein was 48.7% in chloroplast and 28.2% in mitochondria, while when using roGFP1 the degree of oxidation was 75.5% in mitochondria and 62.1% in cytoplasm (Fig. 2B). Assuming that the probes are fully oxidized/reduced by H_2O_2/DTT, respectively, the redox potential was estimated in the chloroplast, mitochondria and cytoplasm at resting stage before any exposure to stress (Fig. 2C). The results show that the redox potential in the chloroplasts and mitochondria was very similar and about −330 mV, while that of the cytoplasm was more oxidized and reached −292 mV.

The degree of oxidation of roGFP determined by the fluorometer measurement has been compared with that obtained by confocal imaging, using the pla-roGFP2,

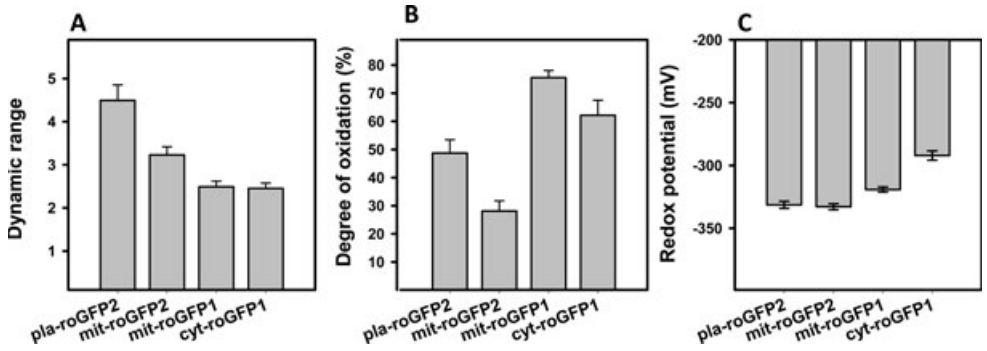

Fig. 2. Fluorescence measurements by fluorometer enabled the determination of the DR (A), degree of oxidation (B), and redox potential (C) of roGFP2 expressed in plastids and mitochondria and roGFP1 expressed in cytoplasm and mitochondria. DR (A) was calculated by division of 400/485 nm ratio after full oxidation by the ratio values after full reduction (see Fig. 1B for data of full oxidation and full reduction for the various transgenic lines). The degree of oxidation and redox potential was calculated as described in Materials and Methods. The presented data are average of 16 leaf discs ± SE.

mit-roGFP2 and cyt-roGFP1 *Arabidopsis* lines (Fig. 3A). No significant difference in degree of oxidation of the roGFPs was found in plastids, and mitochondria. In the cytoplasm the fluorometer measurement showed a higher degree of oxidation in comparison to confocal analysis (Fig. 3A).

The applicability of a fluorometer for monitoring roGFP fluorescence in leaves was further tested in *gr1-1* plants lacking cytosolic glutathione reductase (Marty et al. 2009). We were able to determine that cytosolic GRX1-roGFP2 in *gr1-1* plants was 74% oxidized while in WT plants it was 15% oxidized (Fig. 3B). The increase in oxidation in *gr1-1* plants determined on fluorometer is similar to that obtained by confocal imaging (Marty et al. 2009).

Oxidation of roGFP by hydrogen peroxide during leaf development

Addition of H_2O_2 and DTT to leaf sections is necessary for determination of the actual probe oxidation. Maximum oxidation or reduction of the probe has to be demonstrated to enable the calculations of the degree of oxidation at a specific time and hence the respective redox potential.

Probe calibrations for mit-roGFP1 and mit-roGFP2 showed that 50 m*M* DTT was sufficient for maximum reduction in young and older leaves (data not shown). Also full oxidation of these probes was achieved by 50 m*M* H_2O_2 in 32 days old plants. This conclusion is because higher H_2O_2 concentration (100 m*M*) exhibited the same fluorescence ratio (Fig. 4A, B). However, treatment with 50 m*M* H_2O_2 was not sufficient to oxidize the probe in the seventh leaf of 25 days old plants (Fig. 4A, B). Moreover, different oxidation responses have been found among leaves of different emergence

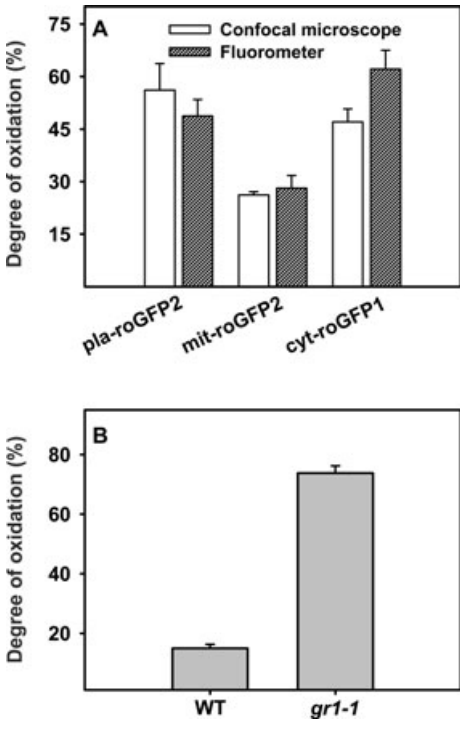

Fig. 3. Comparison between the degrees of oxidation of the probes obtained by confocal image analysis and fluorometer measurements (A) and measurement of the degree of probe oxidation in *gr1-1*(B). For confocal microscopy, raw images were obtained by excitation at 405 nm and subsequently at 488 nm collecting fluorescence emission at 515–525 nm for both wavelengths. Fluorescence of 528 nm following excitation in 400 and 485 nm excitation was measured by a fluorometer. Oxidation of roGFPs in both instruments were achieved by treatment with 50 m*M* H_2O_2 (oxidized state) followed by 50 m*M* DTT (reduced state). Calculations of the degree of oxidation are described in Materials and Methods. Degree of oxidation in WT and *gr1-1* was determined for GRX1-roGFP2 localized to the cytoplasm (B).

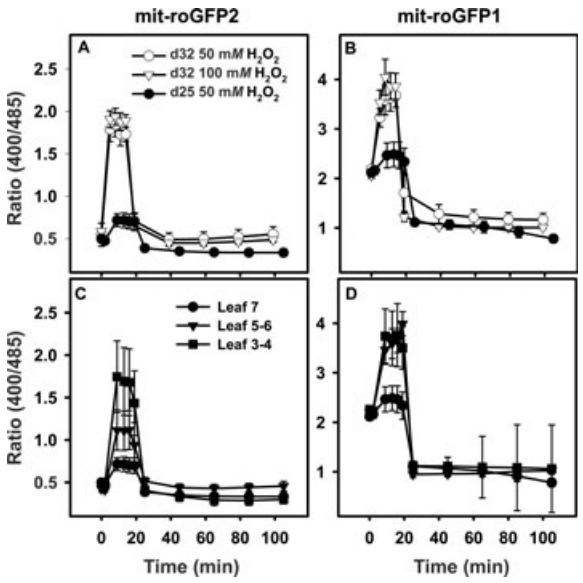

Fig. 4. Effect of leaf age on the oxidation response of roGFP by hydrogen peroxide. Response of mit-roGFP2 (A, C) and mit-roGFP1 (B, D) to oxidation. The seventh leaf at different ages from germination (A, B), or leaves of different emergence order (C, D) on 25 days old plants, were subjected either to various concentrations of H_2O_2 as indicated (A, B) or to 50 m*M* H_2O_2 (C, D). Data was obtained from fluorometer measurements and an average of 10 leaf discs ± SE is presented.

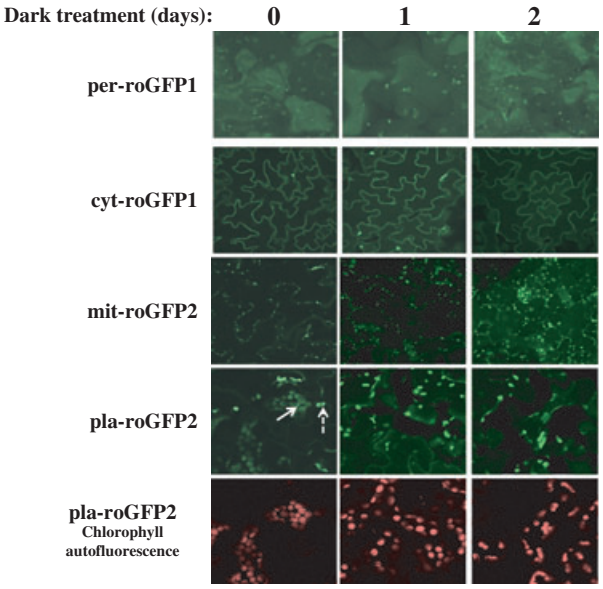

Fig. 5. Localization of roGFP1 in peroxisomes (per-roGFP1) and cytoplasm (cyt-roGFP1), and roGFP2 in mitochondria (mit-roGFP2) and in plastids (pla-roGFP2) during dark treatment. Images were acquired at 515–525 nm following 405 nm excitation, using a confocal microscope of non-darkened tissue and after one and two days under darkness. One optical section was used for images of all lines except for images obtained from per-roGFP1 which was composed of seven optical sections. Dashed arrow in the figure of day 0 shows 405/525 nm fluorescence from plastids devoid of chlorophyll autofluorescence and the full line arrow shows this fluorescence from plastids containing chlorophyll autofluorescence.

times in the same 25 days old plant; while 50 m*M* H_2O_2 was sufficient to bring the probe to fully oxidized state in the third and fourth leaf, it was insufficient in younger leaves (Fig. 4C, D). These results indicate that the buffering capacity of young leaves may be higher than in older leaves. Nonetheless, no differences in resting state fluorescence ratios (400/485 nm) of mitochondrial roGFP have been found between young and old leaves (data not shown), indicating that the mitochondrial redox potential does not change during leaf development. Additionally, roGFP1 was found to be oxidized more easily than roGFP2, because 50 m*M* was sufficient to turn the probe to its fully oxidized state in the fifth and sixth leaf of the mit-roGFP1 line, but not in the mit-roGFP2 line (Fig. 4C, B).

Monitoring the dynamic range of roGFP probes over an extended period of leaf development and dark-induced stress

Determination of redox potential in the various compartments over an extended period using the fluorometer requires the demonstration that the localization of the probe did not change during that period. In addition, autofluorescence at the indicated wavelengths needs to be low enough to avoid background artifacts and to enable the determination of a sufficient DR between the oxidized and the reduced forms of roGFP.

Localization of the various probes to their designated compartments during the extended dark stress is presented in Fig. 5. Localization of roGFP2 to mitochondria at resting stage has been demonstrated (Schwarzlander et al. 2008), and it is seen that during extended darkness the pattern is very similar. Also the localization of roGFP to the cytoplasm and to peroxisomes was not affected by the extended dark stress. On the other hand, GFP-dependent fluorescence was reduced in plastids exhibiting chlorophyll autofluorescence after the second day of darkness. Nevertheless, roGFP-dependent fluorescence was obtained from plastids devoid of autofluorescence (Table 1).

The autofluorescence due to excitation either at 400 or 485 nm of WT cells has been monitored during extended darkness (Fig. 6A) and during leaf development (Fig. 6B) using the fluorometer. No autofluorescence following excitation at 485 nm of the seventh leaf during development or darkness has been detected. However, due to excitation at 400 nm autofluorescence increased gradually during development and during extended darkness

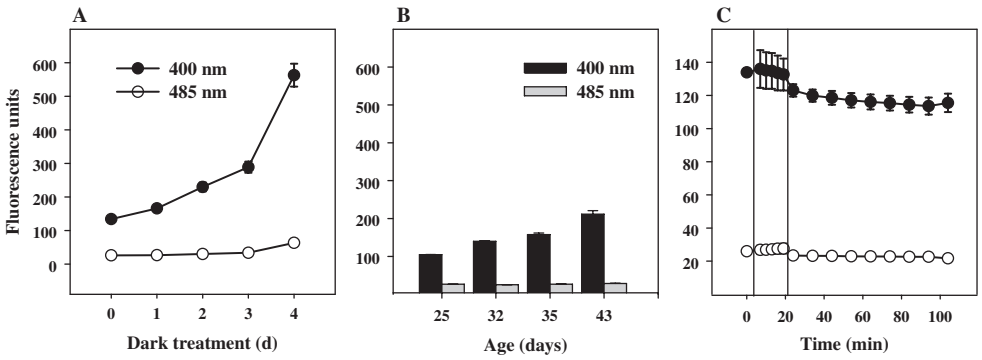

Fig. 6. Determination and characterization of autofluorescence using fluorometer detection. Autofluorescence was measured in WT plants at 528 nm following excitation at 400 and 485 nm. Autofluorescence intensities have been determined during extended dark treatment (A) and leaf development (B). WT leaf discs were exposed to either H_2O_2 or DTT to determine their effects on autofluorescence (C). Vertical lines represent the addition of H_2O_2 followed by the addition of DTT. The presented data are averages of eight leaf discs ± SE.

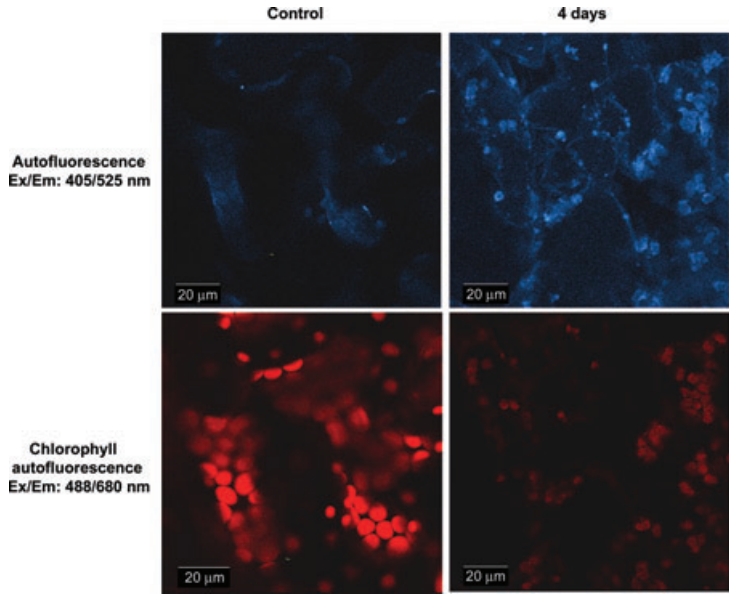

Fig. 7. Detection of autofluorescence by a confocal microscope following 4 days of dark period. Images show fluorescence due to excitation of WT cells at 405 nm and emission at 525 nm and chlorophyll autofluorescence due to excitation at 488 nm and emission at above 680 nm. Images were taken before the dark and following 4 days of darkness; intensities of excitation were kept the same.

until the third day, but on the fourth day the levels increased drastically. Autofluorescence due to excitation at 405 nm is also demonstrated in WT cells at the fourth day of darkness in confocal images of mesophyll cells, and the images reveal that the autofluorescence emanate mainly from cytoplasm and plastids (Fig. 7). The autofluorescence during probe calibration was not affected by treatment of H_2O_2 or DTT (Fig. 6C), indicating that short exposure to a stress-inducing compound like H_2O_2 has no effect on autofluorescence and hence facilitating the measurements.

Fluorescence emanating from WT plants after excitation at 400 nm was highest at 528 nm in comparison to 508 and 590 nm, and when comparing excitation at 340, 400 and 485 nm, the excitation at 400 nm yielded the highest fluorescence intensity at 528 nm (data not shown).

Despite the increase in autofluorescence excited at 400 nm, observed during the extended dark stress and during leaf age, examination of the DR revealed sufficient values until the third day in darkness and also in the seventh leaf late during development (43 days)

Table 1. RoGFP DR within the various lines following dark treatment and in different developmental stages. The DR was calculated based on data obtained by the fluorometer, as described in Fig. 2. The DR for the third day of darkness and for each of the indicated ages has been determined on the seventh leaf. Averages of autofluorescence values were determined for WT plants of similar developmental stage which were exposed to similar excitation conditions and these values were subtracted from the fluorescence obtained from roGFP in the indicated lines. The presented data are averages ± SE for 16 leaf discs.

Treatment	*Arabidopsis* line	DR
Darkness		
3 days	cyt-roGFP1	3.56 ± 0.40
	mit-roGFP1	2.00 ± 0.20
	mit-roGFP2	3.90 ± 0.36
	pla-roGFP2	3.55 ± 0.79
Development		
35 days	mit-roGFP1	3.23 ± 0.19
	mit-roGFP2	3.57 ± 0.33
43 days	mit-roGFP1	3.95 ± 0.19
	mit-roGFP2	4.50 ± 0.41

(Table 1). In contrast, subtraction of autofluorescence values obtained in WT tissue at the fourth day of darkness gave very low or negative fluorescence values which prevented the determination of the DR at this point.

Calculations of the degree of oxidation at different time points during darkness will be presented elsewhere.

Discussion

Previous studies on the feasibility of using roGFP technology to measure redox potentials in plants have used confocal image analysis and it was concluded that both roGFP1 and roGFP2 are suitable as reporters for the glutathione redox potential in plants (Meyer et al. 2007, Schwarzlander et al. 2008, 2009). Although, this is an excellent method for determination of the glutathione redox potential in tissue within different subcellular compartments at steady state, it is difficult to implement in a large-scale experiment, because it is time consuming. Moreover, results from microscopic image analysis usually represent an average of only a few cells and this may be a disadvantage when the effect of a stress has to be studied. In this study, we encountered the need for analyzing a large number of samples and because this task was too tedious to be achieved by image analysis, we developed a fluorometer-based assay. The utilization of a fluorometer for these measurements enables the processing of many samples, and it averages the whole tissue rather than only few cells within a tissue, as in the case of confocal imaging. It should be noted, that when there is a need for a measurement from specific cells, the confocal approach has an advantage over the fluorometer method. Using a fluorometer to detect redox-related changes of roGFPs has been demonstrated for isolated roGFP (Dooley et al. 2004) and for HeLa cells (Hanson et al. 2004), but not for plants. In this study we have adapted fluorometer reading to this technology (Figs. 1 and 2) and have compared this assay with confocal imaging (Fig. 3A). The fluorometer readings enabled us to determine the DR of each of the employed probes. Hence, DR values for roGFP1 and roGFP2 were similar to those that have been measured using confocal imaging (Schwarzlander et al. 2008). This meant that the values obtained for the oxidized and reduced forms of the various roGFPs enabled us to calculate the degree of oxidation of the probes and the redox potential in the indicated compartments, and we have established that similar values of probe oxidation were obtained by fluorometer and confocal measurements (Fig. 3A). This definitely validates the use of a fluorometer for roGFP-based in situ measurements. Further validation for the use of a fluorometer was obtained by the ability to determine that the degree of oxidation of GRX1-roGFP2 in *gr1-1* is more oxidized than in WT, similarly to the data obtained by Marty et al. (2009) by confocal analysis. Our data show higher oxidizing condition in all compartments compared with those described by Schwarzlander et al. (2008). We assume that the differences are due to different leaf development stages, because Schwarzlander et al. (2008) used young rosette leaves of 2–3 weeks old plant, while in our study we have used leaf 7 in 4–5 weeks old plant. Indeed, at least for the plastid and cytoplasmic lines, we have shown that plastids of older leaves are more oxidized than those of younger leaves (data not shown).

Recently, the roGFP technology has been applied to examine the effects of various mitochondrial drugs and short stresses of cold, salt and heat which required the measurement of single time points (Schwarzlander et al. 2009). However, in some cases it is necessary to examine the exposure to stress over a longer-time period which requires several sampling times. When applying the technology to follow changes during development or during extended dark stress, several obstacles have been identified, and these obstacles might be encountered in other experimental systems as well. First, it is necessary to establish in situ that full oxidation or reduction of the roGFP is achieved under all conditions examined. Verification of full oxidation is necessary in order to calculate the degree of oxidation. In this study we have demonstrated that younger leaves need higher levels of H_2O_2 than older leaves for full oxidation of mitochondrial roGFP (Fig. 4). Inability to fully oxidize the probe in younger leaves with the same H_2O_2 concentration required for full oxidation in old leaves, is probably because older leaves contain lower levels of

antioxidants than younger ones, as has been suggested earlier (Abarca et al. 2001, Dertinger et al. 2003). It is interesting to note, that in mammalian HeLa cells lower concentration of H_2O_2 (200 μ*M*) were required to achieve full oxidation of roGFP2 (Dooley et al. 2004), suggesting even lower buffering capacity.

When monitoring the redox potential over a long period, it is also necessary to confirm that the probe localization has not been changed during the experiment. Changes in probe localization can result from changes in cell organization. Deterioration of specific organelle can lead to probe migration to other locations or can lead to probe degradation. In this work we have shown that localization of the probes to their destinations in the cytoplasm, mitochondria and peroxisomes did not change during extended darkness (Fig. 5). The roGFP2 directed to the plastids, accumulated in mature chloroplasts containing chlorophyll autofluorescence as judged by chlorophyll fluorescence (Ex/Em = 488/680 nm), as well as in plastids devoid of it (Fig. 5). It was impossible to detect changes in redox-dependent roGFP2 fluorescence from chlorophyll fluorescence-containing plastids following the first day of darkness using confocal image analysis due to the reduction in roGFP fluorescence (data not shown). However, detection of redox-dependent fluorescence manifested in the DR was possible using the fluorometer (Table 1), but this signal probably emanated from plastids devoid of chlorophyll fluorescence which are also apparent during extended darkness (Fig. 5). The measurements of roGFP fluorescence using the fluorometer undoubtedly also include fluorescence emanating from plastids in the epidermis. Hence, when there is a need to differentiate between roGFP fluorescence emitted from chlorophyll fluorescence-containing chloroplast and from those that do not, the analysis should be done by confocal imaging.

Another important issue is to determine autofluorescence at the relevant wavelengths, which might change during a process. High levels of autofluorescence might interfere with the determination of the DR and might hamper any further calculations. In this study we have determined that autofluorescence following excitation at 400 nm steadily increased during dark treatment until the fourth day when it reached such high levels that prevented ratiometric analysis of roGFP fluorescence. Although autofluorescence increased also during development (Fig. 6B), it did not hamper the use of the technology, as determined by the ability to obtain a suitable DR value at different plant age (Table 1). The increase of autofluorescence during extended darkness occurred in the cytoplasm as well as in the plastids (Fig. 7). Several candidate metabolites that contribute to this autofluorescence have been considered, however none of those have the reported characteristics of the autofluorescence which was detected in this work. Primary fluorescence of chlorophyll catabolite-1 (pFCC) was detected at 450 nm following excitation at 320 nm (Pruzinska et al. 2005). Mg-protoporphyrin IX or its derivative which accumulates during stress in chloroplasts has an emission between 580 and 640 nm (Ankele et al. 2007). Another fluorescent metabolite that was found to increase in parsley during leaf senescence is lipofuscin-like compound which originates in vivo from reaction of malondialdehyde with amino acids. However, its excitation is in the range of 300–370 nm while no excitation occurs at 400 nm and its emission is in 450–500 nm range (Meir et al. 1992). In another study, several sources of fluorescence without detailed identification have been detected in stressed tissue; one of 470–500 nm (blue) excitation and 690 nm emission, and two others of UV excitation and emission at 440 and 550 nm (Lenk et al. 2007). Additional leaf autofluorescence was reported due to excitation at 370 nm and emission maxima at 450 and 530 nm most likely due to phenolic or alkaloids compounds (Lang et al. 1991).

Taken together, it was demonstrated that a fluorometer can be used for analysis of redox potentials by roGFP, provided that the proper considerations outlined in this study are taken into account.

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