Analysis of Redox Relationships in the Plant Cell Cycle: Determinations of Ascorbate, Glutathione and Poly (ADPribose)Polymerase (PARP) in Plant Cell Cultures

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Abstract
Reactive oxygen species (ROS) and low molecular weight antioxidants, such as glutathione and ascorbate, are powerful signaling molecules that participate in the control of plant growth and development, and modulate progression through the mitotic cell cycle. Enhanced reactive oxygen species accumulation or low levels of ascorbate or glutathione cause the cell cycle to arrest and halt progression especially through the G1 checkpoint. Plant cell suspension cultures have proved to be particularly useful tools for the study of cell cycle regulation. Here we provide effective and accurate methods for the measurement of changes in the cellular ascorbate and glutathione pools and the activities of related enzymes such poly (ADP-ribose) polymerase during mitosis and cell expansion, particularly in cell suspension cultures. These methods can be used in studies seeking to improve current understanding of the roles of redox controls on cell division and cell expansion.

Keywords: Antioxidants, plant cell suspension cultures, cell cycle, cellular redox state, oxidative stress, mitochondria, cell death, poly (ADPribose) polymerase.

1. Introduction

Plant cell cultures have been widely used to study cellular metabolism and gene expression. Such systems provide a homogenous cell source in which the uptake and processing of substrates, inhibitors, or effectors can be evaluated. However, it must be recognised that plant cell cultures represent a unique system, where cell-to-cell communication and signalling and other processes may differ from these occurring in whole tissues and organs. The suspension media contain all the required nutrients and elements that allow optimal growth and division while maintaining the cells in an undifferentiated state. It is important that the culture consists largely of single cells that are equally distributed throughout the liquid media and, hence, the suspension has to be shaken during growth to disperse cell aggregates and prevent the formation clumped cells. The cells will then continuously grow until either one of the factors becomes limiting, causing cell growth to slow or division stops due to endogenous genetic controls that arrest the cell cycle.

The mitotic cell cycle can be readily synchronized in cell suspension cultures by the addition of drugs blocking specific cell cycle steps, such as aphidicolin (1). The addition of the pro-oxidant, menadione to synchronized tobacco BY-2 cells causes specific arrests of the G1/S and the G2/M transitions in the cell cycle and, hence, slows DNA replication and mitosis (2). These findings consistently support the concept of the redox control of cell cycle progression (3). Reduction-oxidation (redox) reactions are central to cellular energy metabolism (4, 5). They are important regulators of plant growth, development, and defence (4, 5, 6). Reactive oxygen species (ROS) are formed during the reduction of molecular oxygen or water oxidation during metabolism (7, 8). The accumulation of ROS in any tissue or cellular compartment is tightly controlled by the endogenous redox systems, in which ascorbate (ASC) and glutathione (GSH) are central components (9). A well-established heterotrophic Arabidopsis cell suspension culture has been the subject of several investigations into the responses of plant cells to oxidative stress, as well as of the study of the synthesis and roles of GSH and ASC (10). Cell suspension cultures can be easily supplied with a range of potential biosynthetic precursors, analogues and inhibitors. This approach was used to investigate the pathway of ASC synthesis in Arabidopsis cultured cells (11). The data obtained from this study not only provided evidence in support of the L-galactose pathway of ASC biosynthetic described by Wheeler et al. (12), but also showed that the Arabidopsis cells were also able to use two other routes of ASC production (11). Arabidopsis cell cultures have also used to investigate the regulation of reduced GSH synthesis and provided evidence for the stimulatory role of cellular oxidation in increasing the abundance of this antioxidant (10). Studies on cell suspension cultures have provided evidence that depletion of cellular antioxidants GSH and ASC cause inhibition of mitosis at G1/S transition. On the other hand high level of GSH accumulated in the nuclei of tobacco TBY-2 cells blocked at S/G2 phase (13).

Studies on cell suspension cultures have demonstrate that oxidation of ASC (to dehydroascorbate) inhibits the progression through the cell cycle (14 – 17). Large changes in ASC contents are observed during the different growth stages of cells in culture suggesting that extensive turnover of the ASC pool occurs from the onset of the logarithmic growth phase to the point where cell growth ceases (17, 18) Moreover, the observation that depletion

of GSH together with addition of dehydroascorbate caused additive effects on cell division in synchronized tobacco BY-2 suspension cultures led to the concept that these redox metabolites act in independent pathways to regulate the cell cycle (19, 20, 9). The addition of GSH to the BY-2 suspension cultures caused a slight inhibition rather than a stimulation of the cell cycle suggesting that the redox-mediated regulated progression through the cell cycle by ROS, ASC, and GSH is complex with each metabolite differentially affecting specific processes (20). Studies on the regulation of the cell cycle in suspension cultures have been pivotal in advancing current concepts of the respective roles for ROS, ASC, and GSH in plant growth and development (6; 21). ROS production and GSH level, for example, are important in the orchestration of root growth and architecture (22; 23) and the presence of high levels of dehydroascorbate has been linked to the formation of the population of slowly dividing cells in the root meristem called the quiescent center (QC), which is controlled and maintained by auxin (24) and modulation of mitochondrial function (25). The relative amounts of reduced ascorbate and GSH pools are depleted in the QC such that the QC cells are maintained in a much more oxidized state than those of surrounding cells (24). When the root cap is removed and the auxin signal is decreased the ascorbate and glutathione pools in the QC become more reduced and the cells start to divide (24).

The aforementioned studies indicate the complexities of the redox signaling network that is involved in the orchestration of plant growth and development, as each ROS form (superoxide, hydrogen peroxide, singlet oxygen) and each low molecular weight antioxidant (ascorbate, glutathione, tocopherol) can have discrete effects on plant cell division and growth, and can regulate discrete processes and sets of genes.

Specific oxidation of target proteins and other signal molecules may be an intrinsic mechanism underpinning the perception and responses to environmental and developmental triggers. The recognition of damaged DNA by the enzyme poly(ADPribose) polymerase (PARP) has been linked to cell signalling events, initiating structural changes and isoform replacement, to protection against a range of abiotic stresses and to the regulation of cell cycle-related genes (26; 27).

Studies on the roles of redox regulation on the mitotic cell cycle and other key processes in plant cell cultures requires not only accurate and effective methods for ROS detection but also similar methods for the assay of antioxidant pools and related enzymes. In such studies samples usually consisting of mass of cells are harvested throughout the growth cycle for analysis. Cellular antioxidants are very susceptible to oxidation and degradation during extraction and hence metabolism has to be rapidly arrested in order to preserve the in planta redox state of metabolites and proteins. The series of procedures and methods that are described below are central to the analysis of the cellular redox state and the impact of changes in redox metabolism on metabolic integration and oxidative signalling in plant cell cultures. They are also important in any consideration of how ASC or GSH influences cell division and controls the plant cell cycle in cells in suspension culture.

2. Materials

1. 1X MS media with vitamins containing 3% sucrose, 0.5 mg/L NAA and 0.05 mg/L kinetin, pH 5.8.
2. Trypan Blue Stain 0.4% (Gibco, cat. no. 15250-061).
3. 14-mL Falcon tubes.
4. 1X phosphate-buffered saline (PBS).
5. Nylon membrane filters (0.45-μm pore size; Whatman, cat.no. 7404004).
6. Perchloric acid ($HClO_4$).
7. 120 mM sodium phosphate buffer (pH 5.6).
8. 2.5 M K_2CO_3.
9. pH paper.
10. 120 mM sodium phosphate buffer (pH 7.6).
11. Ascorbate oxidase.
12. 20 mM dithiothreitol (DTT).
13. 2x reaction buffer (240 mM sodium phosphate buffer with 12 mM ethylene diamine tetraacetic acid [EDTA] at pH 7.5).
14. 12 mM 5, 5′-dithiobis-(2-nitrobenzoic acid), (DTNB).
15. Glutathione reductase.
16. 10 mM NADPH.
17. 2-vinyl pyridine.
18. 1 mM EDTA (pH 7.6).
19. 0.15% (v/v) Triton X-100.
20. Extraction buffer (30 mM 3-(N-Morpholino)-propanesulphonicacid [MOPS], 1 mM phenylmethanesulphonylfluoride [PMSF], 2 mM EDTA, pH 7.5).
21. 50 mM TRIS-HCl (pH 8.0), 60 μM cytochrome c, 3 mM galactono lactone, 1 mM KCN.
22. 35 mM ascorbate.

23. Protoplast extraction buffer (0.4 M mannitol, 20 mM 2-Morpholinoethanesulfonic acid, monohydrate (MES), pH 5.5 with HCl, with 0.25% (w/v) cellulase, 0.05% (w/v) pectolyase, 0.1% (v/v) pectinase; added on the day).
24. CelLytic P Kit (Sigma C2360) containing 4X NIB (Nuclear Isolation Buffer), 2.3 M sucrose stock, and Nuclei Extraction Buffer.
25. Protease inhibitor cocktail (Sigma P9599).
26. Dounce homogenizer.
27. Vortex with tube attachment.
28. 1X TBS buffer (Tris-buffered saline: Tris-HCl 20 mM, pH 7.5, containing 150 mM NaCl).
29. Dried skimmed milk (see **Note 6**).
30. 0.1% (v/v) Tween 20.
31. Anti-rabbit IgG alkaline phosphatase conjugate.
32. Desktop scanner and BioRad QuantityOne program.

3. Methods

3.1. Cell Cultures

3.1.1. Growth of Suspension Cultures

Arabidopsis thaliana Landsberg erecta cultures MM1 and MM2d can be maintained as described previously (10).

1. Weekly dilute 3.5 mL of saturated culture in 100 mL of fresh 1X MS media with vitamins containing 3% sucrose, 0.5 mg/L NAA and 0.05 mg/L kinetin, pH 5.8 (see **Note 1**).
2. Shake cultures are in 250-mL Erlenmeyer flasks at a rotation of 120 rpm. MM1 is cultured for 16-h light (~10 μmol/m 2 PAR)/8 h dark at 22°C. MM2d is cultured constantly in the dark at 25°C.
3. Follow the growth of the culture by measuring the optical density at 595 nm for at least six replicate aliquots of 250 μL using a plate reader spectrophotometer. For high concentrations, the culture should be diluted one in four in 1x PBS buffer prior to measurement.

3.1.2. Cell Viability

The viability of the cells is tested using Trypan Blue Staining.

1. Gently mix aliquots of the cultures with equal volume of Trypan Blue Stain 0.4%.
2. Observe under a light microscope. Dead cells show up blue due to the penetration of the dye through disrupted membranes.

3.1.3. Harvest

1. Using a pipet place cells into 2-mL microfuge tubes with an insertion in the bottom (see **Note 2**).
2. Centrifugate in 14-mL Falcon tubes at 250 g for 2 min with no brake applied.
3. Then snap freeze the pellet of packed cells in the microfuge tube in liquid nitrogen and stored at −80°C.
4. Alternatively, cells can be immediately re-suspended in the appropriate buffer for analysis.

This method can be also used to determine the cell fresh and dry weights of known culture volumes. The medium that is allowed to escape through the microfuge insertion tube (see **Note 2**) can be used to analyze the media as well as to estimate the packed cell volume by subtraction of the recovered media volume to the cell suspension volume.

5. Otherwise, larger volumes cells are harvested by vacuum aspiration on nylon membrane filters (0.45-μm pore size).

3.2. Metabolite Analysis

3.2.1. Extraction of Metabolites

1. Resuspend the harvested samples (from Sect. 3.1.3.) in a minimum of 550 μL of 1 M perchloric acid ($HClO_4$).
2. Freeze in liquid nitrogen.
3. Thaw and vortex the frozen samples.
4. Clarify the homogenate in a cooled microfuge (at 4°C) for 10 min at maximum speed.

3.2.2. Adjustment of pH Before Assay

1. Transfer the supernatant (500 μL) to fresh microtubes containing 120 mM sodium phosphate buffer (100 μL; pH 7.6).

2. Adjust the pH of each sample carefully to pH values between pH 5.0 and 6.0 for ascorbate assays by the addition of 2.5 M K_2CO_3. Attention: do not overshoot the pH as ascorbate is rapidly oxidized at alkaline pH values! For glutathione determinations alone, the pH can be adjusted to pH 7.0 and remain relatively stable. Make a note of the added volume and check the pH with pH paper. Care! The reaction is volatile!
3. Remove the insoluble $KClO_4$ produced during adjustment of the pH is by centrifugation at 4°C and maximum speed for 10 min. The supernatant is used for the assay of metabolites such as ascorbate and glutathione.

3.2.3. Determination of Reduced Ascorbate and Dehydroascorbate (Adapted from ref. 28)

Reduced ascorbate is measured by the change in absorption at 265 nm (OD_{265}) as ascorbate is depleted from the reaction mixtures by oxidation to dehydroascorbate.

1. Established a stable background reading for the extract (200 μL at pH 5.6) in 120 mM sodium phosphate buffer (800 μL at pH 5.6).
2. Add ascorbate oxidase (0.25 U in 5 μL of reaction buffer [see **Note 3**]).
3. Follow the reaction is until a minimum is reached (usually less than 1 min).

To measure total ascorbate, dehydroascorbate is first reduced to ascorbate in the extracts.

4. Incubate each sample of extract (100 μL at pH 5.6) with Dithiothreitol (DTT; 10 μL 20 mM) and 120 mM sodium phosphate buffer (pH 7.6; 140 μL 120 mM) for up to 30 min.
5. Stop this reaction by adding 120 mM sodium phosphate buffer (750 μL; pH 5.6).
6. Measure the total ascorbate by the addition of ascorbate oxidase as previously described (see **step 2**).

Ascorbate concentrations are calculated from the OD 265 change and the extinction coefficient for ascorbate at this wavelength (12.8/mM/cm). The amount of oxidized ascorbate is equivalent to the difference between the total and reduced ascorbate. Ascorbate concentrations can be confirmed by production of standard curves with known amounts of pure ascorbate. Recovery experiments involving mixtures of extract and known amounts of ascorbate should be performed at the outset for each new cell type to be studied.

3.2.4. Determination of Reduced GSH and Glutathione Disulfide (Adapted from ref. 29)

Total glutathione (GSH plus GSSG) can be analyzed with a microplate-based cycling assay procedure. In this method, reduced GSH is measured by the interaction with dithio-bis- 2-nitrobenzoic acid (DTNB) forming 2-nitro-5-thiobenzoic acid with an absorbance peak at 412 nm. Glutathione reductase (GR) is used to reduce oxidized glutathione (GSSG) to GSH in the presence of NADPH.

1. Per reaction, mix 100 μL of the 2x reaction buffer (240 mM sodium phosphate buffer with 12 mM EDTA at pH 7.5) with 10 μL of 12 mM DTNB and 0.25 U GR (see **Note 3**).
2. Make up to a volume of 180 μL with ddH_2O.
3. Add 10 μL of 10 mM NADPH and 10 μL of the extract (pH 6.0–7.0) to the flat-based microplates.
4. With a multichannel pipet, add 180 μL of the reaction mixture.
5. Monitored the OD_{412} change over 10 min.

The initial rate of this reaction correlates to the total glutathione content and is compared to standard curves for up to 0.3 nmol GSH (see **ref.** 29). GSSG is quantified in a similar manner but only following prior removal of GSH from the extracts with 2-vinyl pyridine. GSH forms an insoluble complex with 2-vinyl pyridine and can therefore be removed easily from the extract.

1. To the neutralized metabolite extract (200 μL), add 5 μL of 2-vinyl pyridine.
2. Gently mixed the sample and incubate at room temperature for 30 min.
3. Then, transfer to ice for 30 min.
4. Subject the mixture to centrifugation for 10 min at 4°C with maximum speed.
5. Avoid removing any 2-vinyl pyridine with the sample for assay as far as possible, as this compound will inhibit the reaction. The supernatant is then used for GSSG determination in the cycling assay, as described above.
6. GSH and GSSG concentrations should be confirmed by production of standard curves with known amounts of pure GSH or GSSG. Please note that standard curves for GSSG should include the 2-vinyl pyridine extraction step. As with ascorbate, recovery experiments, as illustrated in **Fig. 14.1**, involving mixtures of extract and known amounts of GSH and/or GSSG should be performed at the outset for each new cell type to be studied.

3.2.5. Extraction and Assay of Total Protein in Acid Precipitates

1. Solubilize proteins precipitated in the pellet after acid extraction using 500 µL of 120 mM sodium phosphate buffer with 1 mM EDTA (pH 7.6).
2. Extract the pellet on ice using a microfuge pestle.
3. Centrifuge the sample (10 min; 4°C; max. speed).
4. Determine the soluble proteins using a standard Bradford assay.

3.3. Enzyme Activity Measurements

3.3.1. Extraction Procedures

1. Homogenize samples to a fine powder in liquid nitrogen.
2. For the extraction of l -galactono-1,4-lactone dehydrogenase (GLDH), add 0.15% (v/v) Triton X-100 to the extraction buffer (30 mM MOPS, 1 mM PMSF, 2 mM EDTA, pH 7.5).
3. Homogenized samples on ice until fully thawed.

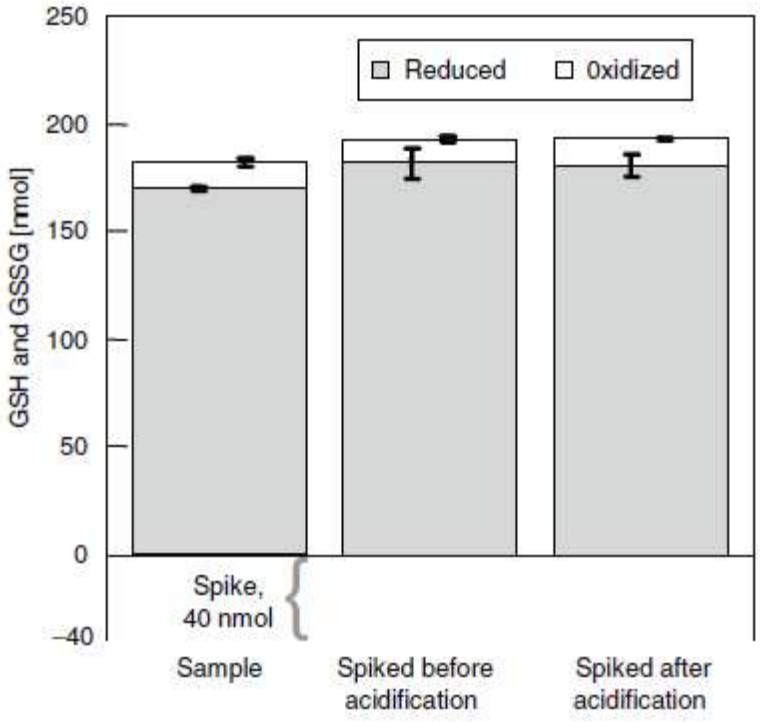

Fig. 14.1. Recovery of glutathione (GSH). Replicate samples from a stationary cell culture were harvested as described in the Methods. A known amount of pure reduced glutathione was added to subsamples (spike) either to the cell pellet before or after the addition of perchloric acid and all samples analysed in parallel. Amounts of standard pure GSH recovered ranged from 90% to 110%. Values less than 80% and greater than 130% are unacceptable. The amounts of oxidized GSH are very similar in all experiments showing that GSH is not oxidized during the extraction procedures. Oxidation of GSH during extraction must be minimal.

4. Subject the extract to centrifugation (15 min, 4°C, 18,000 g), and the supernatant fractions are used for the following assay procedures.
5. Determine the protein content of each supernatant fraction using a standard Bradford assay.

GLDH is bound to the inner mitochondrial membrane and Triton X-100 can be added if required to liberate the enzyme into the soluble supernatant fraction. Its inclusion is not necessary when ascorbate oxidase is assayed, even though this enzyme is bound to the cell wall. Triton X-100 also interferes with the following assays and should be avoided. High salt can be used to liberate cell wall-bond ascorbate oxidase (30).

3.3.2. Galactono-1, 4-Lactone Dehydrogenase Activity

GLDH (EC 1.3.2.3) activity is assayed by the coupled reduction of cytochrome c, which leads to a specific change in OD_{550} as described by Bartoli et al. (31).

1. Make the reaction buffer up fresh daily with 50 mM TRIS-HCl (pH 8.0), 60 µM cytochrome c, 3 mM galactono lactone (see **Note** 4), and 1 mM KCN to inhibit the reoxidation of the reduced cytochrome c (**Fig. 14.2**).
2. Initiate the reaction by the addition of supernatant sample.

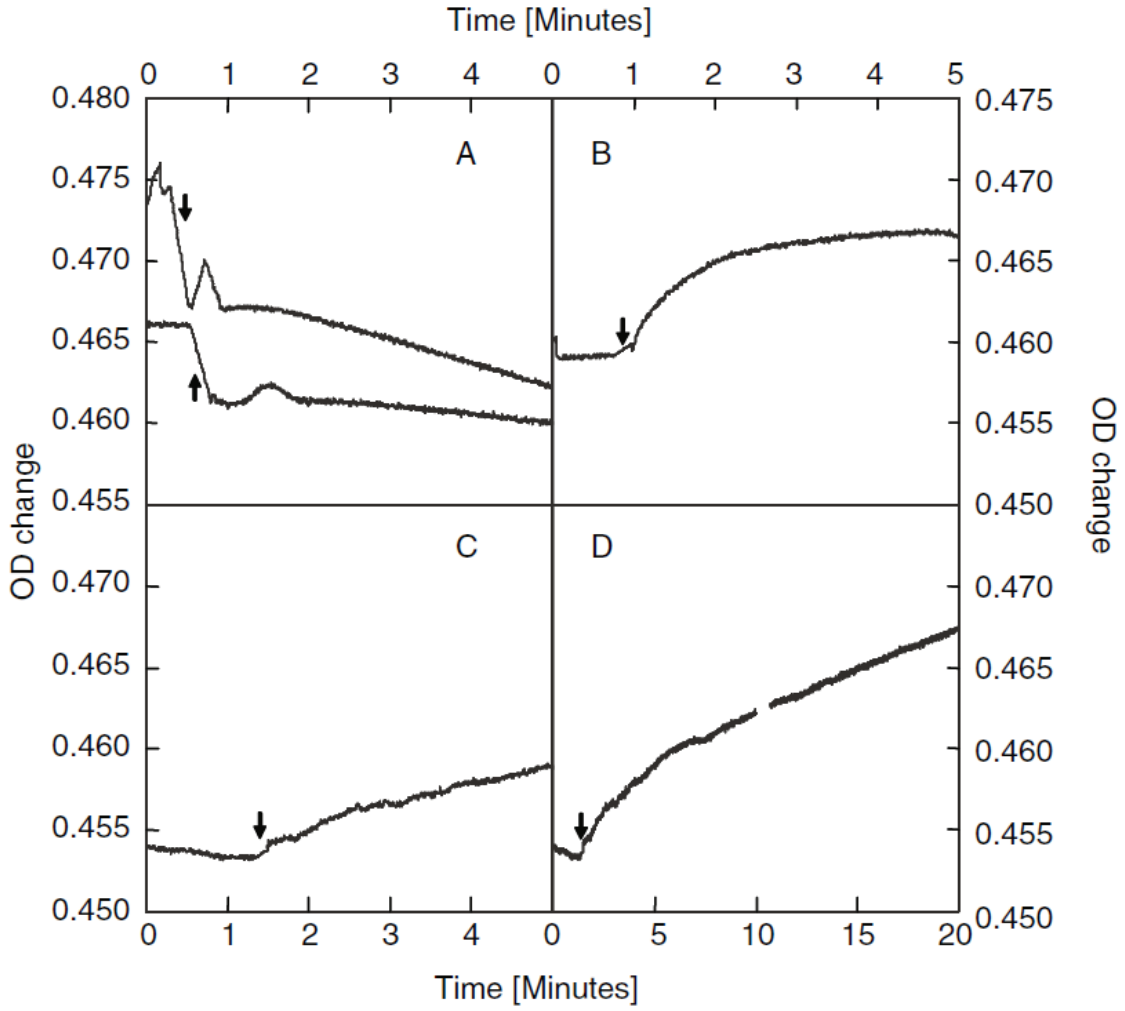

Fig. 14.2. Optimisation of GLDH assay. The presence of Triton X100 in the enzyme reaction mixtures causes artefacts. Although some literature methods use extraction procedures with Triton X100, we do not recommend its use in this assay. The presence of Triton X100 in the reaction mixtures leads to changes in optical density **(A)**, which are not observed when Triton X100 is omitted from the reaction **(B, C)**. The addition of KCN, which inhibits the reoxidation of the reduced cytochrome c, enables a steady-state reaction rate to be reached **(B)**. In the presence of KCN the reaction is stable for a long period **(C, D)**. The reaction is stable for a long period **(C)**. We recommend that the reaction rate is measured over a 10-min period **(D)**. The arrow indicates the addition of the extract, (equivalent to 50 mg protein).

3. Measured the enzyme activity by the change in absorption at 550 nm (OD_{550}).
4. The reaction rate should be followed for at least 10 min to ensure linearity.
5. GLDH activity is then calculated from that change in reduced cytochrome c content with time. The extinction coefficient of reduced cytochrome c is 21/mM/cm under these conditions.

3.3.3. Ascorbate Oxidase Activity

Ascorbate oxidase (EC 1.10.3.3) is determined using a modification of the procedures employed for measuring ascorbate.

1. Add an aliquot of supernatant to 120 mM phosphate buffer (pH 5.6) containing 6 mM EDTA in a final volume of 1 mL.
2. When a stable reading has been obtained, initiate the reaction by the addition of 8.5 μL of 35 mM ascorbate.
3. Measured the enzyme activity by the change in absorption at 290 nm (OD 290). The decrease in absorbance at OD_{290} is followed as ascorbate is oxidized.
4. Calculate the rate of ascorbate oxidation with time using the extinction coefficient of ascorbate (2.7/mM/cm at 290 nm).

3.3.4. Poly(ADPribose) Polymerase

3.3.4.1. Preparation of Protoplasts

To isolate nuclei, protoplasts are obtained from Arabidopsis thaliana cell culture.

1. Resuspend circa 500 mg (FW) Arabidopsis cells in 2 mL of protoplast extraction buffer (0.4 M mannitol, 20 mM MES, pH 5.5 with HCl with 0.25% (w/v) cellulase, 0.05% (w/v) pectolyase, 0.1% (v/v) pectinase; added on the day).
2. Incubated the cells in the dark with gentle agitation for about 1 h to obtain protoplasts.
3. Monitor progress by observing sub-samples of the cells under the light microscope.
4. Recover protoplasts by centrifugation at 1,000 g for 3 min at room temperature without the brake.
5. Wash the pellet twice with the protoplast extraction buffer without enzymes (3 mL) using the same centrifugation conditions.

3.3.4.2. Isolation of Nuclei

The isolation of nuclei is based on the CelLytic P Kit. Each operation should be performed on ice.

1. Resuspend protoplasts in 2 mL of NIBA (1XNuclear Isolation buffer with 1% (v/v) Protease Inhibitor Cocktail).
2. Disrupt using a Dounce homogenizer, with ten turns with the loose fitting pestle followed by ten turns with the tight fitting pestle.
3. Subsequently lyse the cells by incubation on ice after the addition of 0.3% (v/v) Triton X-100.
4. Monitor progress under the light microscope.

3.3.4.3. Purification of Nuclei

1. Carefully apply aliquots of the lysate (500 μL) to the top of 800 μL of a 1.5 M sucrose solution (obtained by diluting a 2.3 M sucrose stock with 1X NIB buffer).
2. Centrifuge the tubes for 10 min at 4°C, 12,000 g without the brake.
3. Wash the pellets twice in 1 mL of NIBA and then combined in 100 μL of Extraction Buffer for Nuclei containing 1% (v/v) Protease Inhibitor Cocktail and 5 mM DTT.

3.3.4.4. Extraction of Nuclear Proteins

1. Mix the nuclear suspension at medium-high speed for 30 min at 4°C in a vortex with tube attachment.
2. Centrifuge for 10 min at 4°C; 12,000 g.
3. Recover the supernatant.
4. Freeze in liquid nitrogen and store at −80°C.

3.3.4.5. Immuno-Dot Blot Assay for Poly (ADPribose)Polymerase Activity

The activity of PARP is measured by the immuno-detection of the Poly (ADP)ribose chain as described by De Block et al. (26).

1. Determine the total concentration of the extracted nuclear proteins with a standard Bradford assay.
2. Normalize the samples to a concentration of 0.5 mg protein in a volume of 15 μL with 1X TBS buffer.
3. Spot the samples are on to a Hybond C membrane, which has been presoaked in TBS buffer (see **Note** 5), and air-dried.
4. Float the membrane is on TBS buffer until evenly wet followed by submergence.
5. Follow this by incubation in TBS with 5% (w/v) dried skimmed milk (see **Note** 6) and 0.1% (v/v) Tween 20 with gentle agitation for 1 h.

6. Dilute the anti-PAR primary antibody 1:2,500 in TBS with 1% (w/v) dried skimmed milk and 0.1% (v/v) Tween 20.
7. Incubate the membrane in this for 1 h at room temperature with gentle agitation.
8. Then wash the membrane five times for 5 min in TBS with 1% (w/v) dried skimmed milk and 0.1% (v/v) Tween 20.
9. Follow by incubation with the anti-rabbit IgG alkaline phosphatase conjugate (diluted 1:5,000 in 1% blocking solution) for 1 h at room temperature with gentle agitation.
10. Wash five times for 5 min in TBS with 0.1% (v/v) Tween 20, followed by two rinses with TBS.
11. Develop the membrane by incubation in BCIP-NBT for 10–30 min until a clear signal is obtained.
12. Stop the reaction is by washing the membrane repeatedly with ddH_2O.
13. Capture the image as a TIFF document, using a desktop scanner, and analyse spot intensities with the BioRad Quantity-One program.

4. Notes

1. Dissolve the phytohormones in 1 N NaOH (approx 1 mL for 10 mg) and make up 1 mg/mL stock by slowly adding ddH_2O. Add phytohormones before autoclaving and adjust the pH to 5.6 with KOH.
2. Use a 2-mL microfuge tube slit with the very top of hypodermic needle. Do not pierce it fully as this will create a hole through which cells will escape.
3. Ascorbate oxidase from Cucurbita sp. (Sigma A0157) lyophilized powder. Make up a 50 U/mL stock solution in 120 mM sodium phosphate buffer with 1 mM EDTA (pH 5.6). Either store at 4°C and use within 1 wk of preparation, or store in aliquots at −20°C. The enzyme is inactivated by freeze-thawing so do not freeze and thaw repeatedly. Glutathione reductase from baker's yeast (S. cerevisiae) (Sigma; G3664) is diluted in reaction buffer to a concentration of 10 U/mL and stored in aliquots at −20°C.
4. It is difficult to obtain stocks of pure L(+) Galactono γ lactone commercially at present. Sigma no longer stocks this substrate. Hence, we currently rely on existing stocks of the pure metabolite.
5. Avoid using phosphate-buffered saline buffer in conjunction with alkaline phosphatase detection.
6. The anti-PAR antibody shows cross reactions with bovine serum albumin, do not use it as a blocking agent.

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