Imaging of photo-oxidative stress responses in leaves

Michael J. Fryer\textsuperscript{1}, Kevin Oxborough\textsuperscript{1}, Phillip M. Mullineaux\textsuperscript{2} and Neil R. Baker\textsuperscript{1,3}

\textsuperscript{1}Department of Biological Sciences, University of Essex, Colchester, Essex CO4 3SQ, UK
\textsuperscript{2}John Innes Centre, Norwich Research Park, Colney, Norwich, Norfolk NR4 7UH, UK

Received 10 July 2001; Accepted 7 December 2001

Abstract

High resolution digital imaging was used to identify sites of photo-oxidative stress responses in Arabidopsis leaves non-invasively, and to demonstrate the potential of using a suite of imaging techniques for the study of oxidative metabolism in planta. Tissue-specific photoinhibition of photosynthesis in individual chloroplasts in leaves was imaged by chlorophyll fluorescence microscopy. Singlet oxygen production was assessed by imaging the quenching of the fluorescence of dansyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole (DanePy) that results from its reaction with singlet oxygen. Superoxide and hydrogen peroxide accumulation were visualized by the reduction of nitroblue tetrazolium (NBT) to formazan deposits and by polymerization with 3,3'-diaminobenzidine (DAB), respectively. Stress-induced expression of a gene involved with antioxidant metabolism was imaged from the bioluminescence from leaves of an Arabidopsis APX2-LUC transformant, which co-expresses an ascorbate peroxidase (APX2) with firefly luciferase. Singlet oxygen and superoxide production were found to be primarily located in mesophyll tissues whereas hydrogen peroxide accumulation and APX2 gene expression were primarily localized in the vascular tissues.

Key words: Ascorbate peroxidase, chlorophyll fluorescence, hydrogen peroxide, singlet oxygen, superoxide.

Introduction

Singlet oxygen, superoxide radical and hydrogen peroxide are reactive oxygen species (ROS) that are generated when plant tissues are exposed to a variety of environmental stresses (Halliwell, 1984; Asada, 1996; Fryer et al., 1998). The extremely short life-times of ROS makes the study of their production in planta very difficult. Damage to proteins, lipids and DNA has often been used as an index of oxidative stress (Halliwell and Gutteridge, 1985; Rice-Evans et al., 1991), although this is a rather indirect measure of the production of ROS. Spin labels have been applied with some success for the secondary detection by electron paramagnetic resonance (EPR) of oxygen free radicals and organic radicals from their semi-stable adducts (Hodgson and Raison, 1991; Heber et al., 1996; Van Doorslaer et al., 1999), but this does not provide information on the specific sites of ROS production in tissues. Cell fractionation techniques have been used in attempts to identify sites of ROS generation and the location of ROS detoxification systems (Doulis et al., 1997; Kingston-Smith et al., 1999), however, these involve extensive tissue disruption which can lead to the generation of ROS. Consequently, data produced using such destructive methods does not always result in an accurate picture of the distribution of ROS and their detoxification systems. Also, such in vitro studies on macerated material cannot resolve the differences in predisposition to oxidative stress that undoubtedly exist between various tissues and cell types within leaves. In this paper it is demonstrated how the use of ROS-specific tracer dyes in conjunction with high resolution imaging now provides the opportunity to identify sites of photo-oxidative stress and ROS accumulation in leaves.

Abbreviations: CCD, charge-coupled device; DAB, 3,3'-diaminobenzidine; DanePy, dansyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole; \( F' \), fluorescence level at any point between \( F_{o} \) and \( F_{m} \); \( F_{o} \), minimal fluorescence level of leaves in light; \( F_{m} \), maximal fluorescence level from leaves in light; \( F_{q} \), difference in fluorescence between \( F_{m} \) and \( F' \); NBT, nitroblue tetrazolium; \( ^{1}O_{2} \), singlet oxygen; PPFD, photosynthetically-active photon flux density; ROS, reactive oxygen species; SOD, superoxide dismutase.

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Manipulation of genes encoding components of antioxidant systems has facilitated the non-invasive study of the expression of genes coding for enzymes involved in ROS metabolism. The example presented in this paper is the transformation of Arabidopsis with an APX2-LUC transgene, a cytosolic ascorbate peroxidase promoter fused to a firefly luciferase reporter gene (Karpinski et al., 1999). APX2-LUC is silent under non-stressed conditions, but its mRNA is detectable within 15 min of the onset of a high light stress. Induction of APX2-LUC allows for the non-invasive bioluminescent imaging of APX2 gene expression and the analysis of factors that influence it.

The combination of advanced molecular genetics techniques with non-invasive digital imaging of the generation of ROS illustrated in this paper provide extremely powerful tools for future investigations of the development of oxidative stress in leaves and the regulation of ROS metabolism.

Materials and methods

Plant material, probe application and stress conditions

Arabidopsis thaliana plants were germinated and raised to mature rosette stage under controlled environmental conditions (PPFD, 200 μmol m⁻² s⁻¹ during a 16 h photoperiod at 25 °C in a relative humidity of c. 80%). Leaves were excised at the petiole with a razor blade and allowed transpirationally to imbibe aqueous solutions of fluorescent probes and dyes for 90 min at the growth PPFD. Solutions were replaced with water during the following stress period. For the majority of the treatments, tip regions of the leaf were placed inside the chamber of a computerized infrared gas analysis system (CIRAS, PP Systems, Hitchin, Hertfordshire) and exposed to an air flow rate of 347 cm³ min⁻¹. Photo-oxidative stress conditions (PPFD 650 μmol m⁻² s⁻¹ at 30 °C and water vapour pressure > 0.3 kPa) were imposed for 90 min.

The development of the Arabidopsis thaliana transformant with an APX2-LUC transgene has been previously reported (Karpinski et al., 1999). This transformant was grown under the conditions described above.

High resolution, chlorophyll fluorescence imaging

High resolution imaging of chlorophyll fluorescence from chloroplasts in intact leaves was carried out essentially as described previously (Oxborough and Baker, 1997). F₀ and Fₘ define the minimal and maximal fluorescence levels from leaves in the light, respectively. F is the fluorescence level at any point between F₀ and Fₘ. For the construction of parametized images, the specific term F₀ was recently introduced (Oxborough and Baker, 1997; Baker et al., 2001) which denotes the difference between Fₘ and F measured immediately before the application of a saturating pulse to measure F₀. Under these conditions, F₀/Fₘ equates to the operating quantum efficiency of PSII photochemistry. A Peltier-cooled charge-coupled device (CCD) camera, as described earlier (Oxborough and Baker, 1997), was used for non-invasive imaging of ROS-fluorogenic compounds and bioluminescent expression of APX2-LUC.

Detection of reactive oxygen species

Singlet oxygen (¹O₂) activity was detected by infiltrating leaves with 40 mM DanePy (dansyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrrole), a dual fluorescent and spin probe. Reaction of highly fluorescent DanePy with ¹O₂ yields a non-fluorescent nitroxide radical (DanePyO) (Hideg et al., 1998, 2000, 2001). DanePy fluorescence in leaves was excited with 345 nm radiation produced from a UV source (ULT1004 Black Ray LW, Scientific Laboratory Supplies, Wilford, UK) through a 345 nm band pass filter (E46-084, Edmund Scientific, York, UK) and imaged using the CCD camera (described above) protected with a 532 nm band pass filter (E43-122, Edmund Scientific, York, UK).

Infiltration of leaves with 6 mM nitroblue tetrazolium (NBT) allowed the detection of superoxide. When the pale yellow NBT reacts with superoxide a dark blue insoluble formazan compound is produced (Flohé and Otting, 1984; Beyer and Fridovich, 1987). Superoxide is thought to be the major oxidant species responsible for reducing NBT to formazan (Maly et al., 1989). Chlorophyll was removed from the leaves prior to imaging by infiltrating them with lacto-glycerol-ethanol (1:1:4 by vol) and boiling in water 5 min. The location of formazan deposits was visualized by subtracting background (non-formazan) pixels from the leaf image.

Infiltration of leaves with 5 mM 3,3′-diaminobenzidine (DAB) at pH 3.8 allows the detection of hydrogen peroxide in leaves (Thordal-Christensen et al., 1997; Orozoco-Cardenas and Ryan, 1999). DAB forms a deep brown polymerization product upon reaction with H₂O₂ in the presence of peroxidase (Thordal-Christensen et al., 1997), which can be imaged after removal of chlorophyll from the leaf, as described above.

Imaging of gene expression

Expression of APX2-LUC was monitored in leaves after spraying with 1 mM luciferin (Promega). The treated leaves were then imaged for 60 min with the Peltier-cooled CCD camera (described above) in order to generate an image of the bioluminescence produced.

Results and discussion

Photo-inhibition of photosynthesis results in changes in the quantum efficiency of photosynthetic electron transport, which can be estimated from the fluorescence parameter, F₉ₐ/Fₘ. Examples of images of the chlorophyll fluorescence parameter F from chloroplasts in the bundle sheath (Fig. 1A) and mesophyll (Fig. 1D) cells of an intact leaf were used to generate images of F₉ₐ/Fₘ (Fig. 1B, E) from which the individual chloroplasts can be isolated and their F₉ₐ/Fₘ values determined (Fig. 1C, F). The chloroplasts in the bundle sheath and mesophyll cells of the leaf prior to the photoinhibitory treatment of 60 min at a PPFD of 600 μmol m⁻² s⁻¹ had F₉ₐ/Fₘ values of 0.67 and 0.65, respectively (data not shown), which decreased to 0.25 and 0.39, respectively during the photo-oxidative stress. During the operating efficiency of non-cyclic photosynthetic electron transport.

The accumulation of ¹O₂ during this photo-oxidative stress treatment is shown in Fig. 2. An Arabidopsis leaf
was infiltrated with DanePy and then a region of the leaf tip was irradiated with a PPFD of 600 μmol m⁻² s⁻¹. Images of the fluorescence of the DanePy show that there is a marked decrease in the fluorescence emission from the area of the leaf exposed to the high light treatment (Fig. 2), indicating the production of the nitroxide radical DanePyO produced from the reaction of DanePy with ¹⁰₂. It has been previously demonstrated that DanePy...
penetrates leaf tissues well and enters cells and chloroplasts (Hideg et al., 2001). It appears that $^1\text{O}_2$ is formed primarily in mesophyll tissue (Fig. 2). This would be expected since the majority of the leaf’s photosynthetic apparatus is contained in mesophyll chloroplasts and $^1\text{O}_2$ will be produced by photosensitized energy transfer reactions between excited triplet state chlorophyll and ground state molecular oxygen whenever excess excitation energy cannot be efficiently dissipated as photochemistry or heat from chlorophylls (Halliwell, 1984; Wise and Naylor, 1987). $^1\text{O}_2$ is suspected to be involved in damage to the D$_1$ reaction centre protein of PSII (Vass et al., 1992; Aro et al., 1993), has also been implicated in lipid peroxidation by direct reaction with polyunsaturated fatty acyl residues in membranes and in general oxidative degradation of proteins (Halliwell, 1984).

Imaging of purple formazan deposits, which result from the reaction of NBT with superoxide, identifies the regions of superoxide formation in a leaf. The tip region of the leaf that has been exposed to the high light treatment clearly shows more intense staining than the non-stressed area, with the majority of the staining being associated with mesophyll tissue (Fig. 3). Numerous small, localized ‘hotspots’ were observed at the distal points of the microvasculature (Fig. 3). The petiole exhibited very heavy staining which, presumably, is associated with superoxide accumulation resulting from NADPH oxidase activity, a major effector of the oxidative burst in the wound response (Mehdy et al., 1996; Wojtaszek, 1997). Staining in the petiole was most intense in veinal tissue close to where the leaf was excised from the plant and decreased with distance away from the wound; presumably this demonstrates that a systemic wounding signal that stimulates production of superoxide has been propagated along the veins from the wound. Although NBT also reacts with ascorbate, large changes in the ascorbate pool would not be expected in leaf tissues during the period of photo-oxidative stress used and, consequently, imaging of formazan production can be considered to indicate superoxide accumulation; large increases in the rate of superoxide production would be expected during the photoinhibitory stress. Areas of formazan deposits indicate cells in which the rate of superoxide production has become significantly greater than the rate of detoxification.

The production of $\text{H}_2\text{O}_2$ was imaged in leaves infiltrated with DAB, which reacts with $\text{H}_2\text{O}_2$ in the presence of peroxidases to produce a brown polymerization product. Considerably more $\text{H}_2\text{O}_2$ was detected in the tip region of the leaf that had been exposed to the high light treatment, with it being primarily associated with the vascular tissue (Fig. 4). The ascorbate–glutathione cycle has been proposed for the removal of $\text{H}_2\text{O}_2$ in order to prevent its conversion to the extremely reactive and oxidizing hydroxyl radical within leaves (Halliwell, 1984). Ascorbate peroxidase (APX) converts $\text{H}_2\text{O}_2$ to monodehydroascorbate. Using the APX2-LUC Arabidopsis transgenote (Karpinski et al., 1999) it is possible to image the expression of the gene for a cytosolic form of ascorbate peroxidase when leaves are exposed to the high light treatment. Clearly there is widespread APX2 gene expression in the light-treated area of the leaf, however,

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**Fig. 3.** Image of an Arabidopsis leaf infiltrated with NBT. The top half of the leaf was exposed to a high light treatment of 600 μmol m$^{-2}$ s$^{-1}$ for 60 min. The purple coloration indicates the formation of insoluble formazan deposits that are produced when NBT reacts with superoxide.
some expression was also observed around the vascular tissue in the regions of the leaf that were kept in the dark (Fig. 5) demonstrating the systemic signalling system which induces APX2 expression (Karpinski et al., 1999).

Imaging the accumulation of \(^1\)O\(_2\), superoxide and H\(_2\)O\(_2\) in intact leaves in parallel with fluorescence imaging of the operating efficiencies of electron transport in specific tissues can clearly provide some interesting and novel insights into the spatial organization of oxidant biochemistry occurring in response to photo-inhibitory stresses. In the leaves examined in this study accumulation of \(^1\)O\(_2\) (Fig. 2) and superoxide (Fig. 3) appeared to be principally located in mesophyll tissues during photo-oxidative stress, whereas H\(_2\)O\(_2\) accumulation was more associated with the vascular tissue (Fig. 4). These observations suggest that excess light results in a larger increase in superoxide production in the mesophyll chloroplasts compared to that in the chroplasts of the vascular tissue, while hydrogen peroxide preferentially accumulates in the vascular tissues. A similar specific localization of H\(_2\)O\(_2\) within vascular tissue has been described in wounded leaves and is thought to be associated with the systemic octadecanoid signalling pathway (Orozoco-Cardenas and Ryan, 1999). Presumably the accumulation of ROS in photosynthetic tissues is likely to be involved in the signalling process associated with the switching on of APX2 gene expression in response to the high light stress.

Clearly, imaging of photoinhibition of photosynthesis, ROS production and the expression of genes coding for enzymes involved in detoxification of ROS in intact leaves has great future potential for resolving the heterogeneity and nature of the responses of leaf tissues to photo-oxidative stress.

Acknowledgements

These studies were supported by a grant from the Biotechnology and Biological Sciences Research Council to NRB and PMM. The authors are grateful to Christine Edwards for synthesizing the DanePy.

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