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Monitoring intra- and extracellular redox capacity of intact barley aleurone layers responding to phytohormones

Christina Mark [a, 1], Kinga Zór [b, 1], Arto Heiskanen [b], Martin Dufva [c], Jenny Emnéus [b], Christine Finnie [a, d, *]

[a] Agricultural and Environmental Proteomics, Department of Systems Biology, Technical University of Denmark, DK-2800 Kgs.Lyngby, Denmark
[b] Bioanalytics, Department of Micro- and Nanotechnology, Technical University of Denmark, DK-2800 Kgs.Lyngby, Denmark
[c] Fluidic Array Systems and Technology, Department of Micro- and Nanotechnology, Technical University of Denmark, DK-2800 Kgs.Lyngby, Denmark
[d] Carlsberg Research Laboratory, J.C. Jacobsens Gade 4, DK-1799 Copenhagen V, Denmark

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ABSTRACT

Redox regulation is important for numerous processes in plant cells including abiotic stress, pathogen defence, tissue development, seed germination and programmed cell death. However, there are few methods allowing redox homeostasis to be addressed in whole plant cells, providing insight into the intact *in vivo* environment. An electrochemical redox assay that applies the menadione-ferricyanide double mediator is used to assess changes in the intracellular and extracellular redox environment in living aleurone layers of barley (*Hordeum vulgare* cv. Himalaya) grains, which respond to the phytohormones gibberellic acid and abscisic acid. Gibberellic acid is shown to elicit a mobilisation of electrons as detected by an increase in the reducing capacity of the aleurone layers. By taking advantage of the membrane-permeable menadione/menadiol redox pair to probe the membrane-impermeable ferricyanide/ferrocyanide redox pair, the mobilisation of electrons was dissected into an intracellular and an extracellular, plasma membrane-associated component. The intracellular and extracellular increases in reducing capacity were both suppressed when the aleurone layers were incubated with abscisic acid. By probing redox levels in intact plant tissue, the method provides a complementary approach to assays of reactive oxygen species and redox-related enzyme activities in tissue extracts.

1. Introduction

Redox activity in plant cells plays a major role in a number of processes including abiotic stress, pathogen defence, tissue development, seed germination and programmed cell death (PCD) [1–3]. Plant cells contain five to ten times more genes encoding enzymes involved in regulation of redox homeostasis and protection of cells against oxidative stress than bacteria or mammalian cells [2,3]. Regulation of the cellular redox environment is an important and dynamic process, since elevated levels of reactive oxygen species (ROS) can cause oxidative stress leading to PCD [2–5]. PCD, however, is an integral part of plant growth and survival, as it is involved in a range of central processes including seed germination, tissue development and defence against pathogens [6–9]. In the barley (*Hordeum vulgare*) aleurone layer, the phytohormone gibberellic acid (GA) induces germination-associated metabolism, enzyme secretion and subsequent PCD [1,10]. Abscisic acid (ABA) counteracts GA, and in the presence of both phytohormones germination-related events of the aleurone layer are delayed, while ABA suppresses PCD entirely in the absence of GA [1,10]. The barley aleurone layer is an excellent model system for investigations of phytohormone signalling, enzyme secretion and PCD during seed germination [11–13] due to this well-known and widely studied tight hormonal regulation process. Production of ROS is observed in aleurone layers after incubation with GA [14], which in combination with decreased redox enzyme activities [15] has been predicted to shift the overall redox balance towards a state of oxidative stress, ultimately leading to PCD.

The most important metabolic pathways in cells involve redox

Abbreviations: ABA, abscisic acid; FiC, ferricyanide; FoC, ferrocyanide; GA, gibberellic acid; M, menadione; PCD, programmed cell death; ROS, reactive oxygen species.

* Corresponding author. Carlsberg Research Laboratory, J.C. Jacobsens Gade 4, DK-1799 Copenhagen V, Denmark.
E-mail address: Christine.Finnie@carlsberg.com (C. Finnie).

[1] These authors contributed equally to this work.

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couples that shuttle between oxidised and reduced states. Concentrations of the different redox couples affect metabolic flux and are in turn responsive to environmental stimuli [16]. Current knowledge about the redox environment in aleurone layers is largely based on colorimetric quantification of H_2O_2 [12,13], measurement of selected enzyme activities, gene expression and protein appearance profiles [14,15,17], each representing a single component of cellular redox homeostasis measured after tissue homogenisation. Plant plasma membrane redox reactions [18,19] and extracellular H_2O_2 concentrations [20] have been monitored electrochemically in living plant cells, but there is a lack of data addressing the redox environment in living plant tissue, and no established method has allowed measurement of separate intracellular, extracellular and membrane-associated redox activities in intact plant tissues. Monitoring of the redox changes occurring in living tissues could provide information closer to *in vivo* conditions than the more invasive, traditional methods, and would represent a complementary approach towards understanding redox regulation in plant cells.

Electrochemical techniques have been used successfully as non-invasive methods to evaluate intracellular redox activity in living mammalian [21–23], yeast [24–27] and bacterial [28,29] cells. As a measure of intracellular redox activity, NAD(P)H availability can be probed in whole cells using an amperometric detection method based on the menadione-ferricyanide (M-FiC) double mediator system [30]. Mediator molecules are able to transfer electrons between an electrode and enzymes. The principle of intracellular probing is based on the lipid-soluble quinone M that can pass through cell membranes (Fig. 1), and can be reduced by intracellular menadione reducing enzymes (MREs), such as quinone reductases (QRs), to menadiol (MH_2) [24,30,31] or possibly semiquinone radicals ($M^{\bullet -}$, not shown) [21]. The quantification of MH_2, which is also lipid-soluble, is enabled via its return to the extracellular environment where it rapidly reduces the hydrophilic and membrane impermeable FiC to ferrocyanide (FoC), and is in turn in re-oxidised to M (Fig. 1). The menadione/menadiol couple has been shown to exchange electrons rapidly both with the intracellular environment and extracellular FiC/FoC redox couple [21]. The amount of accumulated FoC is therefore related to the overall reducing capacity of the cells and can be quantified electrochemically by oxidation on an electrode [21,24,30,31].

Menadione reduction in mammalian cells requires the supply of glucose [21] reflecting the production of NAD(P)H through glycolysis and the pentose phosphate pathway. The M-FiC double-mediator system has been used to dissect mitochondrial and cytoplasmic redox activity [31] and to evaluate fermentation efficiency in yeast [32]. The amperometric detection method proposed by Heiskanen et al. [30] enables real-time redox activity measurements from living cells immobilized in alginate [30], or on self assembled monolayers of thiols [26], wired by a redox polymer [28] as well as from cell culture suspension by combining the amperometric detection with flow injection [31].

In the present work, the amperometirc detection coupled with flow injection analysis proved to be a suitable and efficient approach for measuring the redox activity from non-immobilized aleurone layers in relatively large sample batches. The double mediator M-FiC system adapted to barley aleurone layers enabled both intra- and extracellular reducing capacity monitoring over time in intact tissue, as well as in response to phytohormones.

2. Materials and methods

2.1. Chemicals

Ethanol, ampicillin, nystatin, succinic acid, calcium chloride, tris(hydroxymethyl)aminomethane, GA (gibberellin A_3), phosphate buffer saline (PBS) powder (10 mM phosphate buffer, 2.7 m M potassium chloride and 137 mM sodium chloride), ABA, fluorescein diacetate, M, and FiC were obtained from Sigma-Aldrich® Co. (St. Louis, MO, USA), while MM 4–64 (N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phe-nyl)hexatrienyl)pyridinium dibromide) was obtained from Biomol GmbH (Hamburg, Germany). All other

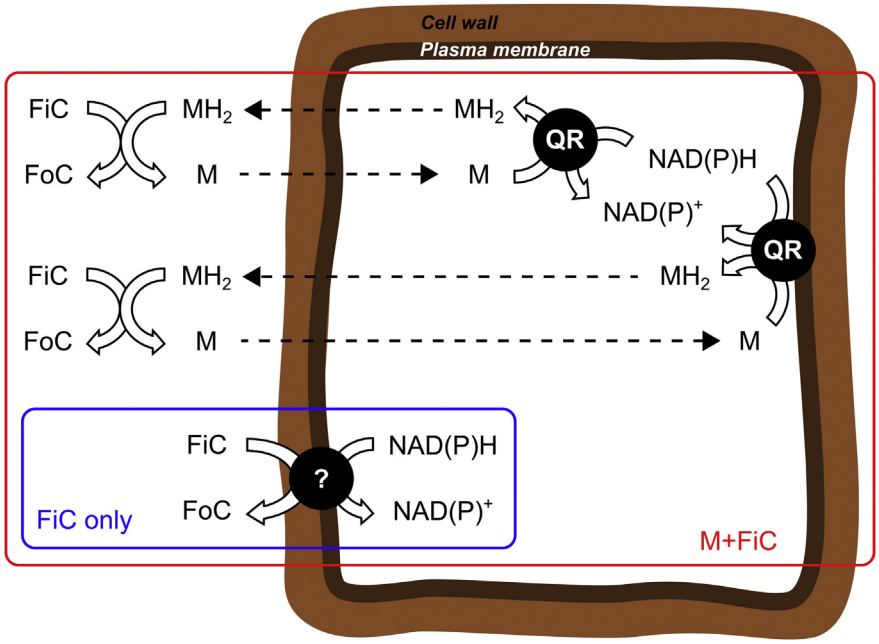

Fig. 1. The M-FiC double mediator system. FiC cannot pass the plasma membrane and electrochemical measurements based on this mediator alone (small box) will therefore only probe extracellularly. Analyses based on both mediators (large box) will assess the intracellular as well as extracellular redox activity. M: Menadione. MH_2: Menadiol. FiC: Ferricyanide. FoC: Ferrocyanide. QR: Quinone reductase.