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ARTICLE

In vivo NAD(P)H fluorescence from the epidermal cells of onion (*Allium cepa*) bulb scale leaves

Csilla Slezak, Eva Herrhofer, Gabor Laskay*

Department of Plant Biology, University of Szeged, Szeged, Hungary

ABSTRACT *In vivo* fluorescence measurements were carried out on intact layers of onion (Allium cepa) bulb scale leaf epidermal cells in order to study if the blue autofluorescence can be used for monitoring the redox satate of NAD(P)H in these plant leaf cells and/or mitochondria *in vivo*. When recording the fluorescence spectra (excited at 340 nm) of the intact cell layers, a well-resolvable shoulder was always present at around 465-469 nm. An empirical formula ((F_{467}-F_{500})/F_{500}) was derived to normalize the differential fluorescence intensity of the shoulder at 467 nm (F_{467}-F_{500}) to that at 500 nm (F_{500}). Treatment of the cell layers with 1 mM KCN, an inhibitor of the mitochondrial electron transport chain at Complex IV led to an increase in the (F_{467}-F_{500})/F_{500} fluorescence emission ratio, leaving the fluorescence intensity of the untreated cell layers unchanged. Qualitatively similar results were obtained using 1 mM rotenone, an inhibitor of Complex I (NADH-oxidase) of the mitochondrial electron transport chain. These findings imply that the fluorescence at 467 nm derives dominantly from mitochondrial NADH in the epidermal cells of onion scale leaves. Moreover, it is concluded that the (F_{467}-F_{500})/F_{500} fluorescence emission ratio can be used to observe changes in the redox state of the NAD pool in these cells.
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KEY WORDS

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Pyridine nucleotides (NAD^+, $NADP^+$) are the main H-carriers in both plant and animal cells and play therefore important roles in cellular bioenergetic processes. They serve as co-enzymes for various enzymatic reactions and at the same time they constitute one of the key determinants of cellular redox regulation and provide reducing equivalents for various biosynthetic processes. In addition, the roles of pyridine nucleotides in various signaling processes (Berger et al. 2004) and their connection to stress tolerance (Noctor et al. 2006) have been receiving renewed attention. Being essential determinants of cellular redox reactions, there have been various attempts to quantify the absolute or relative amounts of the oxidized or reduced forms of the various pyridine nucleotides in living cells (Eggers et al. 1982; Eng et al. 1989; Leskovac et al. 1995; Queval and Noctor 2007). Although these methods are considered accurate *in vitro*, they suffer from the major drawback of having to disrupt the cells during the extraction procedure leading to the inevitable loss of cell viability. NAD(P)H fluorescence, however, may offer an easily conducible yet reliable means to monitor the status of these redox compounds *in vivo*. Both NADH and NADPH absorb ultraviolet light with a maximum at 340 nm and emit a blue fluorescence centered at 465-470 nm while their oxidised counterparts NAD^+ and $NADP^+$ neither absorb nor fluoresce significantly at these wavelengths (Chance 1954; Galeotti et al. 1970). Monitoring the fluorescence of individual cells or tissue slices at these wavelengths therefore offers a convenient and reliable way to assess the redox state of the cells and/or the metabolic state of the mitochondria *in vivo*. This method has been successfully used in a wide range of studies with animal cells, tissues and organs (Balaban and Blum 1982; Mayevsky and Rogatsky 2007).

Epidermal cells of onion bulb scale leaves form natural mono- or bicellular layers on both sides of every scale leaf. They are valuable experimental objects as they are easy to obtain and work with. In addition, they are devoid of chloroplasts and therefore offer a convenient and appropriate experimental means with which to study the interconnection between mitochondrial functions and the redox state of the cells. The objective of this communication is to report on the successful application of NAD(P)H fluorescence in epidermal cells of onion bulb scale leaves *in vivo*.

Materials and Methods

Plant material and treatment

Brown onion (*Allium cepa* L.) bulbs were obtained from a local supplier. The same batch of onions were used throughout the experiments. Before the measurements the outer brown leaf scales of the onions were removed and the upper epidermal layer from the inner (concave) surface of the tissue between the 4th and 5th fleshy subleaves was excised care-

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*Corresponding author. E-mail: glaskay@bio.u-szeged.hu

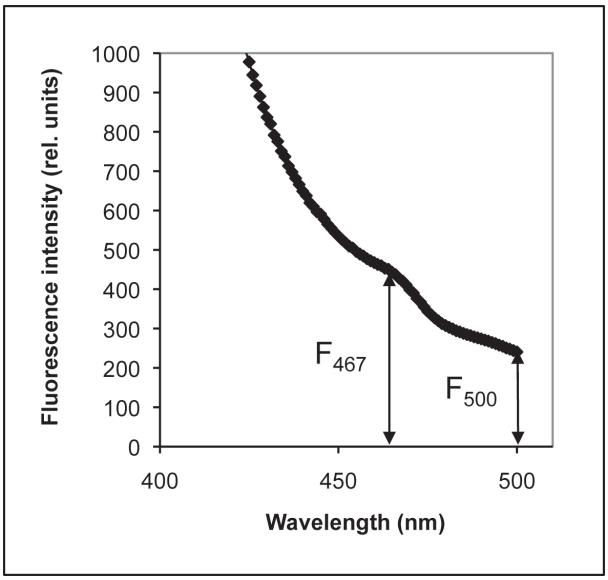

Figure 1. Representative fluorescence spectrum of an intact onion bulb epidermal cell layer excited at 340 nm. Fluorescence intensities at 467 and 500 nm (F_{467} and F_{500}, respectively) are indicated by arrows.

fully using a pair of forceps and placed immediately into an incubation solution containing 0.1 mM KCl, 0.05 mM $CaCl_2$, 5 mM HEPES, pH 6.5. Typically, a section of approximately 10-15 mm x 10-15 mm was prepared for analysis using a combination of razor and scalpel blades. The control and treated samples were examined in parallel experiments carried out on the same day, using leaf cell layers from the same bulb for each experiment. The experiments were repeated at least 3 times on the same day using different bulbs of the same batch for different parallel experiments. The samples were placed in the incubation solution in the presence or absence of 1 mM KCN, an inhibitor of Complex I (Cooper and Brown 2008) or rotenone, an inhibitor of Complex I (Soole and Menz 1995) of the mitochondrial electron transport chain.

Fluorescence measurements

For fluorescence measurements the cell layers were applied to a manually shaped plastic plate of 0.8 x 0.3 cm, which was then carefully immersed diagonally in a 1-cm quartz glass cuvette containing the incubation solution. The cuvette was placed in the sample holder of a Hitachi F-2500 recording spectrofluorimeter. The plate was oriented at 45° with respect to the excitation light beam and so the perpendicularly emitted fluorescence was directed towards the detector. All experiments were carried out at room temperature.

Data analysis and statistical treatments

For the quantitation of the fluorescence intensities at 467 and 500 nm, fluorescence spectra were recorded of the cell layers. Every registered spectrum was converted into an Excel file from which the fluorescence intensities were recorded. All experiments were repeated 9 times (3 parallel experiments on 3 different days) and the values are expressed as the mean fluorescence intensity ± SD (n=number of experiments). Statistically significant differences ($p<0.005$) were assessed by the use of Student's *t*-test.

Results and Discussion

A representative fluorescence spectrum (excited at 340 nm) of an intact onion bulb scale leaf epidermal layer *in vivo* is shown in Figure 1. A shoulder at 465-469 nm can be resolved in the spectrum. This shoulder lies exactly in the range of the NAD(P)H fluorescence emission maximum measured both *in vitro* and *in vivo* (Chance 1954; Galeotti et al. 1970). An

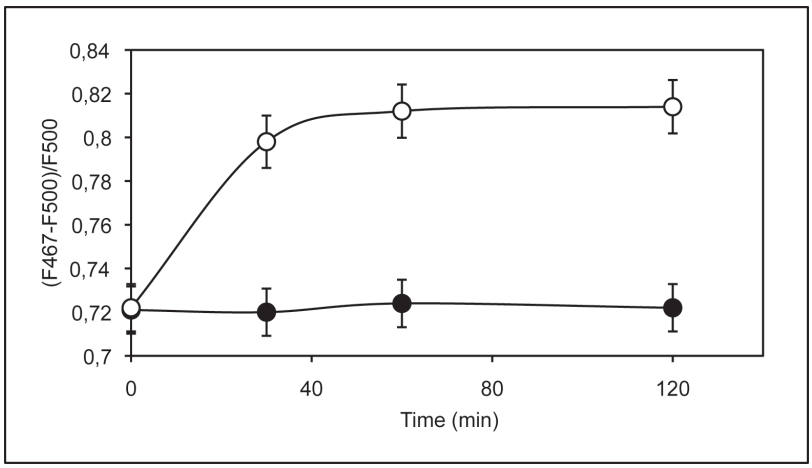

Figure 2. Effect of 1 mM KCN (open circles) on the $(F_{467}-F_{500})/F_{500}$ fluorescence emission ratio in the epidermal cells of onion bulb scale leaves in the function of time. Each point represents the mean ± S.D. from 9 independent experiments.

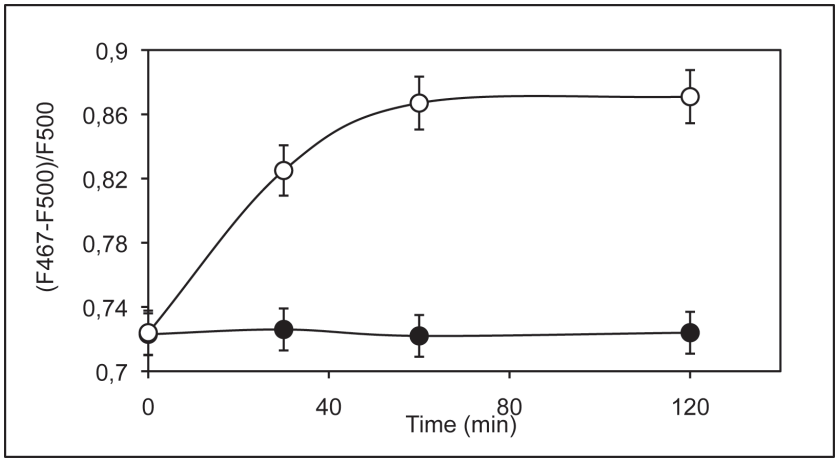

Figure 3. Effect of 1 mM rotenone (open circles) on the $(F_{467}-F_{500})/F_{500}$ fluorescence emission ratio in the epidermal cells of onion bulb scale leaves in the function of time. Each point represents the mean ± S.D. from 9 independent experiments.

empirical formula $(F_{467}-F_{500})/F_{500}$ was derived to normalize the differential fluorescence intensity at 467 nm $(F_{467}-F_{500})$ to that of 500 nm (Fig. 1).

KCN, a commonly used inhibitor of the mitochondrial electron transport chain at Complex IV (cytochrome c oxidase, Cooper and Brown 2008) was used at 1 mM concentration to study if the observed fluorescence at 467 nm was sensitive to the inhibition of the mitochondrial electron transport. Treatment of epidermal cells of onion bulb scale leaves with 1 mM KCN induced significant elevation in the fluorescence intensity at 467 nm during the examination period (0 to 2 hours), leaving the fluorescence intensity of the untreated samples unchanged (Fig. 2). This finding indicates that the *in vivo* fluorescence at 467 nm reflects the inability of the mitochondrial electron transport to reoxidise NADH and therefore it indicates that the fluorescence at 467 nm originates mainly from the NADH in the mitochondria in the epidermal cells of onion scale leaves.

Plant mitochondria exhibit an alternative electron transport route mediated by the alternative oxidase bypassing the KCN site (Maxwell et al. 1999). Therefore, the direct inhibition of NADH oxidation was also studied using 1 mM rotenone, a specific inhibitor of Complex I (NADH oxidase) of the mitochondria (Soole and Menz 1995). Rotenone treatment produced qualitatively similar results to those obtained by the use of KCN, it induced significant elevation in the fluorescence intensity at 467 nm during the examination period (0 to 2 hours) leaving the fluorescence intensity of the untreated samples unchanged (Fig. 2). This finding gives further support that the 467-nm fluorescence originates mainly from the NADH in the mitochondria in the epidermal cells of onion scale leaves. Moreover, it is concluded that monitoring the fluorescence intensity at 467 nm can be used to study the redox state of the NAD pool *in vivo.*

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