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ARTICLE

In vivo and *in vitro* studies on fluorophore-specificity

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ABSTRACT *In vivo* and *in situ* microscopy is a selective and easy method for detecting reactive oxygen (ROS)- and nitrogen species (RNS). Of the several fluorescent indicators developed in the last 30 years, the specificity and sensitivity of 4-amino-5-methylamino-2'-7'-difluorofluorescein diacetate (DAF-FM DA) as a nitric oxide (NO) indicator was tested by spectrofluorimetry and fluorescence microscopy. The peroxynitrite ($ONOO^-$)-dependence of aminophenyl fluorescein (APF) and the hydrogen peroxide (H_2O_2)-sensitivity of 2'-7'-dichlorodihydrofluorescein diacetate (H_2DCF DA) and 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) was also determined. The results show that DAF-FM is a suitable fluorophore for detecting NO in plant tissues and aminophenyl fluorescein can be used as a $ONOO^-$ -responsive dye. It was also found that DCF does not detect NO in solutions, but its fluorescence emission is strongly sensitive to H_2O_2. Moreover, the DCF fluorescence was found to be $ONOO^-$-sensitive, as well. *In vivo* studies revealed that Amplex Red can be applied as a H_2O_2-sensitive and -selective fluorophore in plant tissues.
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KEY WORDS

fluorophore specificity
fluorescence miscroscopy
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In vivo and *in situ* staining methods proved to be very popular in plant and animal research because of their high specificity and simplicity. These methods offer the possibility to visualize and localize physiological events within the cells, tissues and organs. Beside the spatial information, with the help of these techniques we are able to carry out real time imaging. The measurement of reactive oxygen- (ROS) and nitrogen species (RNS) levels in plant and animal tissues is often difficult because of the high reactivity of these molecules. *In vivo* staining techniques offer a reliable and easy way for ROS and RNS detection.

The major RNS, nitric oxide (NO) is proved to be a multiactive signal molecule in animal and plant cells; however its detection in plant tissues is complicated (Mur et al. 2011). The most sensitive direct methods for NO detection are gas-phase chemiluminescence, gas chromatography-mass spectrometry (Archer 1993; Magalhaes et al. 2000) or laser-photoacoustic spectroscopy (Leshem and Pinchasov 2000); however these techniques are complicated and usually require expensive instrumentation.

In 1998, Kojima and co-workers developed a family of NO-sensitive fluorescence dyes, the diaminofluoresceins (DAFs) (Kojima et al. 1998). The most general molecule among these fluorophores is 4,5-diaminofluorescein diacetate (DAF-2DA), which was first applied in plant tissues by Pedroso et al. (2000). The 4-amino-5-methylamino-2'-7'-difluorofluorescein diacetate (DAF-FM DA) is a pH-stable derivative of DAF-2DA, which is also photostable. In diacetate form, the dye is membrane-permeant, and the acetyl groups are cleaved by intracellular esterases. The resulting molecule can react with N_2O_3, an oxidation product of NO yielding a fluorescent triazole molecule ($\lambda_{excitation}$ = 495 nm; $\lambda_{emission}$ = 515 nm). The fluorescence intensity, which can be measured by fluorescence spectrophotometric or microscopy methods, is proportional to the NO content of the tissue.

Peroxynitrite ($ONOO^-$) is a highly reactive nitrogen species, which can be produced in a reaction between NO and superoxide radical. It is able to modify enzyme or transcription factor activity via tyrosine nitration reactions (Arasimowicz-Jelonek and Floryszak-Wieczorek 2011). Several fluorescent probes, such as 3'-(p-hydroxyphenyl) fluorescein (HPF) 3'-(p-aminophenyl) fluorescein (APF, $\lambda_{excitation}$ = 490 nm; $\lambda_{emission}$ = 515 nm) developed in animal and plant tissues are capable of ONOO- detection, however, they were shown to react with hydroxyl radical ($OH^\cdot$), hypochlorite anion (^-OCl), or peroxyl radicals (Cohn et al. 2009). Recently, a new indicator of $ONOO^-$, HongKong Green-2 (Sun et al. 2009) was applied in plant systems to selectively detect $ONOO^-$ (Gaupels et al. 2011).

For detecting total intracellular reactive oxygen species (ROS), 2'-7'-dichlorodihydrofluorescein diacetate (H_2DCF DA) is a suitable fluorophore, since it can react with H_2O_2, $ONOO^-$ or $O_2^{\cdot-}$, as well (Gomes et al. 2005). It is a cell-permeant indicator that is nonfluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell. The fluorescent form has an absorption maximum at 498 nm and an emission maximum at 522 nm.

The Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) reacts with H_2O_2 in the presence of horseradish

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peroxidase with a 1:1 stoichiometry to form resorufin, the fluorescent product ($\lambda_{excitation}$ = 563 nm; $\lambda_{emission}$ = 587 nm). This indicator is sensitive, stable, and exhibits low background fluorescence (Gomes et al. 2005).

In this work, we wanted to demonstrate the specificity and sensitivity of several fluorescent probes in *Arabidopsis* root tissues with the help of fluorescence microscopy and also in solutions using spectrofluorimetry.

Materials and Methods

Verification of the fluorophore specificity *in vivo*

The seeds of wild type (Col-0) *Arabidopsis thaliana* L. were surface sterilized with 5% (v/v) sodium hypochlorite for 20 minutes and rinsed with sterile distilled water before being transferred to half-strength MS (Murashige and Skoog 1962) medium [1% (w/v) sucrose, 0.8% (w/v) agar]. The Petri dishes were placed in greenhouse at photon flux density of 150 µmol m^{-2} s^{-1} (12/12 day/night period) at a relative humidity of 55-60% and 25 ± 2°C. Seven-day-old *Arabidopsis* seedlings were incubated in NO donor and/or scavenger solutions (100 µM sodium nitroprusside, SNP and/or 100 µM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-l-oxyl-3-oxide, cPTIO) at 150 µmol m^{-2} s^{-1} light intensity for 2 hours then they were dyed with 10 µM 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM DA in Tris-HCl buffer, pH 7.2) for 30 min at room temperature in darkness. Samples were washed with the buffer solution 2 times within 30 min and were placed on microscope slides (Pető et al. 2011). In the case of Amplex Red, the seedlings were pre-incubated with 10 mM H_2O_2 and/or 200 U catalase for 10 min and were dyed with 50 µM fluorophore solution (prepared in 50 mM sodium phosphate buffer, pH 7.5) for 30 min in darkness and washed once with the buffer solution. All chemicals were purchased from Sigma-Aldrich.

Fluorescence microscopy and image analysis

The roots of *Arabidopsis* seedlings labelled with DAF-FM DA or Amplex Red were investigated under Zeiss Axiowert 200M invert microscope (Carl Zeiss, Jena, Germany) equipped with a high resolution digital camera (Axiocam MR, HQ CCD, Carl Zeiss, Jena, Germany) and filter set 10 (exc.: 450-490 nm, em.: 515-565 nm) or filter set 20HE (exc.: 546/12 nm, em.: 607/80 nm). The intensities of fluorescence were measured on digital images within an area of circles with 60 µm radii with the help of Axiovision Rel. 4.8 software. The radii of circles were not modified during the experiments.

Verification of the fluorophore specificity *in vitro*

For the *in vitro* experiments the fluorescent DAF-FM and DCF molecules were obtained by alkaline hydrolysis of DAF-FM DA and DCF-DA, respectively, according to Balcerczyk et al. (2005), respectively. Fluorescence intensities were measured by a fluorescent spectrophotometer (Hitachi F-4500, Hitachi Ltd., Tokyo, Japan). Different concentrations of nitric oxide donor (50, 500, 1000 µM SNP), scavenger (200 µM cPTIO) and hydrogen peroxide (5, 50, 100 µM H_2O_2) and 100 U/ml catalase (CAT) solutions were prepared and 2 µM DAF-FM, DCF or APF were added. As a peroxynitrite donor, SIN-1 was used at 0,5, 1, 1,5 mM concentrations. In all cases the excitation wavelength was set to 490 nm and the intensity of DAF-FM or APF fluorescence emission was measured at 515 nm. In the case of DCF, the fluorescence emission was recorded at 525 nm.

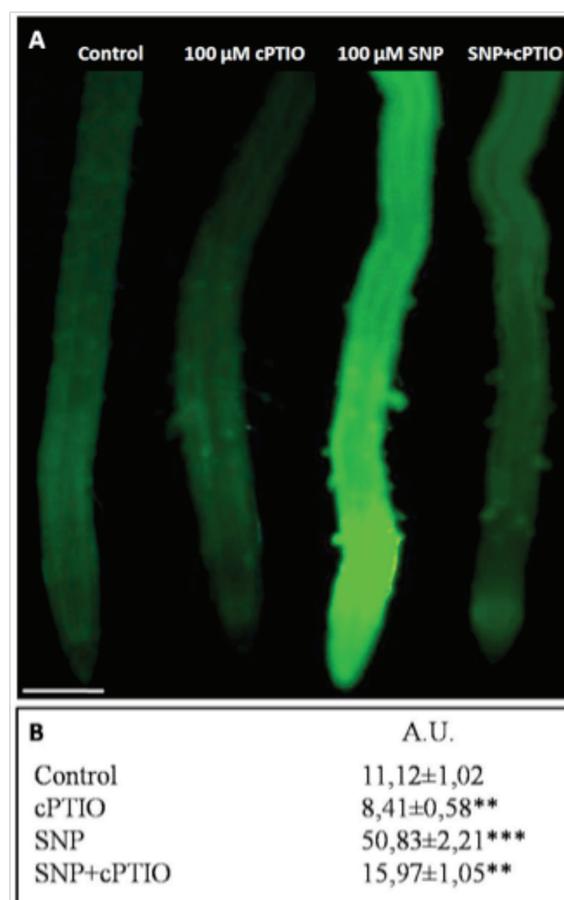


Figure 1. (A) Fluorescence microscopic visualization of control and NO donor/scavenger-treated Arabidopsis primary roots stained with DAF-FM. Bar=1 mm. (B) Values of fluorescence intensities measured on digital images (n=20, ±SD, **P≤0.01, ***P≤0.001).

Statistical analysis

Significant differences were determined using the Student's test applying Microsoft Excel 2007 software. All the experiments were carried out two times. Statistically sig-

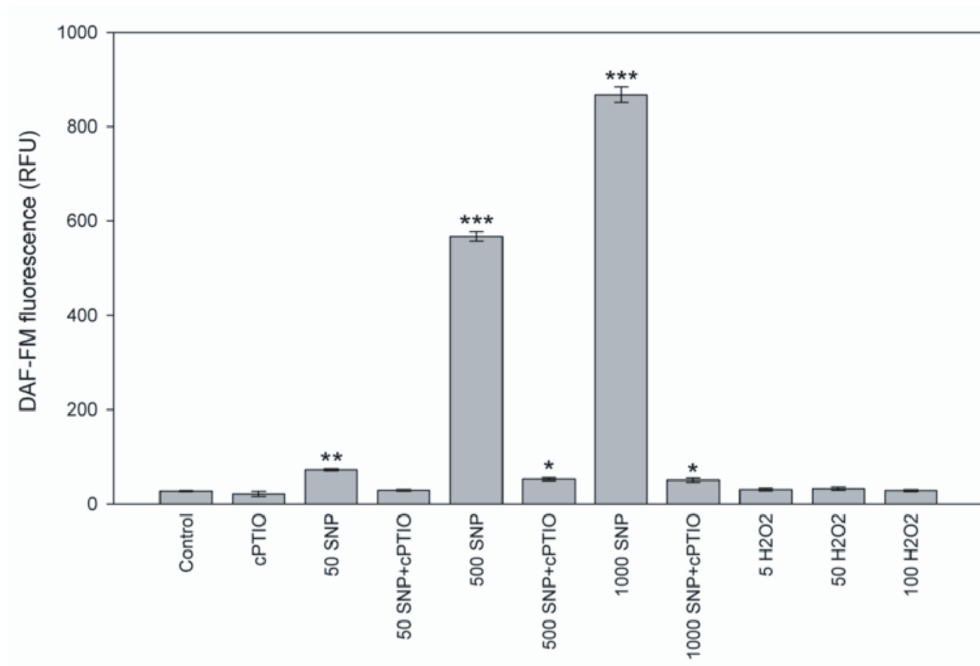


Figure 2. Relative fluorescence of control and SNP-, cPTIO- or H_2O_2- treated DAF-FM solution measured by spectrofluorimetry (n=6, *P≤0.05, **P≤0.01, ***P≤0.001).

nificant differences among means (n=6 or 20) are indicated by one (*P≤0.05), two (**P≤0.01) or three (***P≤0.001) asterisk(s).

Results and discussion

DAF-FM is a suitable indicator of changes in NO levels

In the primary roots of NO donor-treated (SNP) seedlings significantly higher DAF fluorescence was measured as compared to the control. The addition of NO scavenger decreased the fluorescence in both the control and the SNP-treated roots (Fig. 1). Similar results were obtained by *in vitro* measurements, where SNP significantly increased the DAF fluorescence and its effect was concentration-dependent, while cPTIO caused a significant inhibition of SNP-induced fluorescence emission. Hydrogen peroxide (5, 50, 100 μM) caused no increase in fluorescent intensities (Fig. 2). Similarly to the results of Kojima et al. (1998) our data suggest that the fluorescence intensity of DAF-FM changes according to the endogenous NO content of the *Arabidopsis* primary root and the fluorescence emission is independent of the presence of H_2O_2. Based on these findings, DAF-FM is considered to be a suitable, NO-specific fluorophore.

Aminophenyl fluorescein is a $ONOO^-$-sensitive fluorophore

During *in vitro* studies, a significant increase in APF fluorescence emission in response to SIN-1 was observed. Moreover, the NO-donor SNP and the H_2O_2 application had no effect on fluorescence (Fig. 3). These results suggest the $ONOO^-$-sensitivity of APF, and that the dye does not react with NO or H_2O_2.

DCF and Amplex Red detects H_2O_2

During *in vitro* measurements, hydrogen peroxide increased the fluorescence emission of DCF in a concentration-dependent manner, while the addition of catalase resulted in decreased fluorescence intensities. Application of NO donor and/or scavenger solutions had no significant effects on the fluorescence, which suggests that DCF fluorescence is independent of NO. The peroxynitrite donor, SIN-1, slightly increased the fluorescence emission of DCF, although its effect was not concentration-dependent (Fig. 4). The oxidation of DCF in the presence of peroxynitrite was published by Glebska and Koppenol (2003).

The *Arabidopsis* seedlings were treated with 10 mM H_2O_2 with or without 200 U/ml catalase, and stained with Amplex Red. Hydrogen peroxide leads to an enhanced AR fluorescence in the primary roots, while catalase strongly decreased it (Fig. 5). Based on this observation, it can be stated that Amplex Red is a H_2O_2-responsive fluorophore in the root tissues of *Arabidopsis*.

Taken together, these *in vivo* and *in vitro* results clearly show that DAF-FM is a suitable fluorophore for detecting NO in plant tissues and aminophenyl fluorescein can be used as

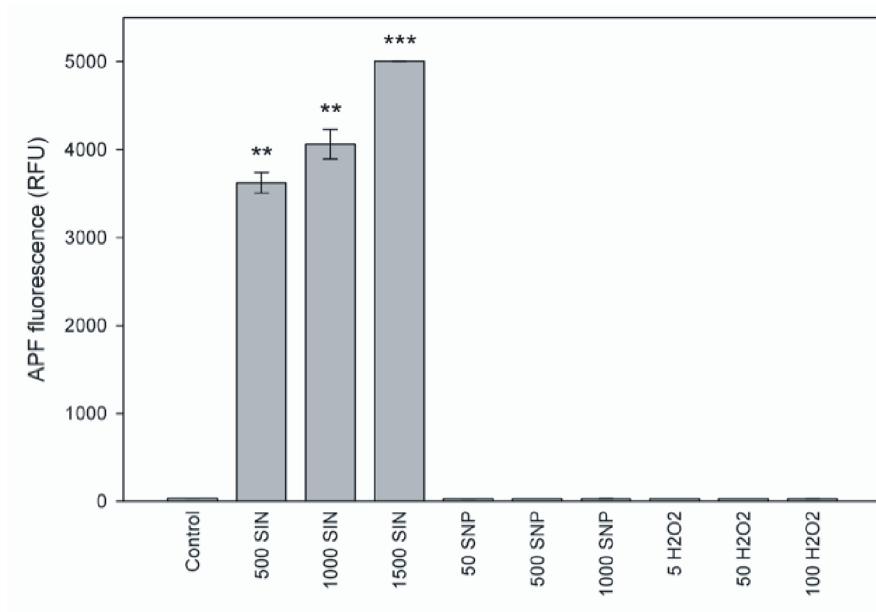


Figure 3. Relative fluorescence of control and SIN-, SNP- or H_2O_2- treated aminophenyl fluorescein solution measured by spectrofluorimetry (n=6, **P≤0.01, ***P≤0.001).

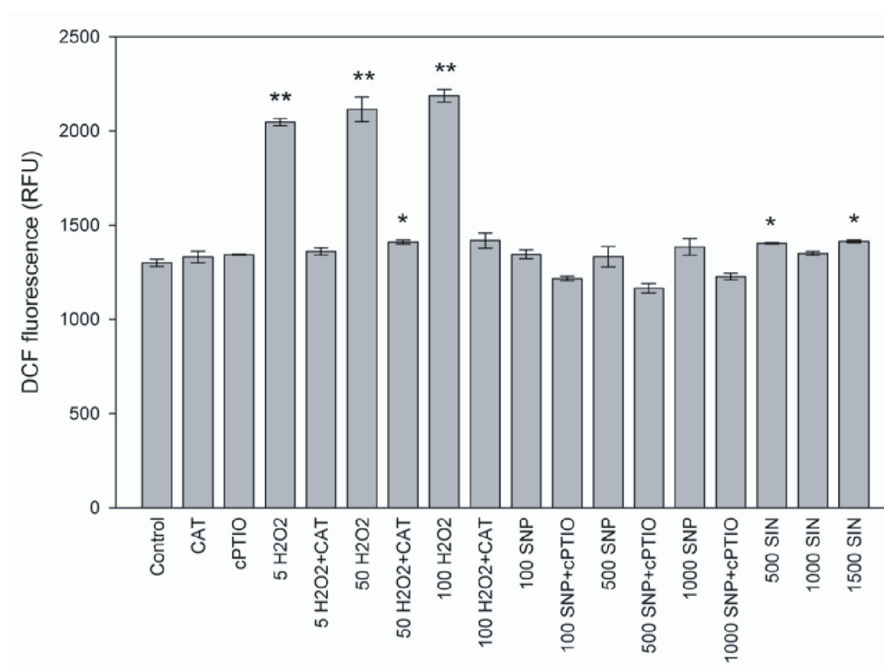
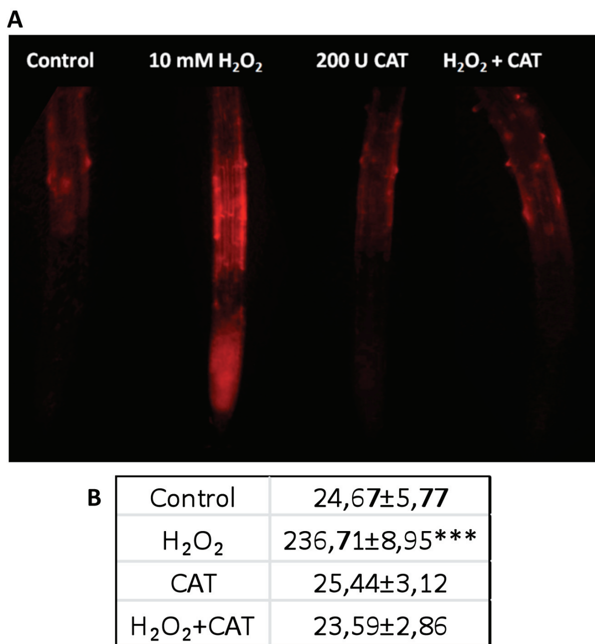


Figure 4. Relative fluorescence of control and H_2O_2 (with or without catalase)-, SNP (with or without cPTIO)-, or SIN- treated dichlorofluorescein solution measured by spectrofluorimetry (n=6, *P≤0.05, **P≤0.01).

$ONOO^-$ -responsive dye. However, it must be taken into consideration that APF is able to react with other molecules too, such as hydroxyl radical and hypochloric radical. It was also shown, that DCF does not detect NO in solutions, but its fluorescence emission is strongly sensitive to H_2O_2. Moreover, the DCF fluorescence was found to be SIN-1 ($ONOO^-$)-sensitive as well, therefore, this dye cannot be used as a H_2O_2-selective fluorophore. *In vivo* studies showed that Amplex Red can be



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Treatment	Value
Control	24,67±5,77
H_2O_2	236,71±8,95***
CAT	25,44±3,12
H_2O_2+CAT	23,59±2,86

Figure 5. (A) Fluorescence microscopic visualization of control and H_2O_2- and/or catalase-treated *Arabidopsis* primary roots stained with Amplex Red. Bar=1 mm. (B) Values of fluorescence intensities measured on digital images (n=20, ±SD, ***P≤0.001).

applied as a H_2O_2-sensitive fluorophore in plant tissues.

Acknowledgements

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References

Arasimowicz-Jelonek M, Floryszak-Wieczorek J (2011) Understanding the fate of peroxynitrite in plant cells – From physiology to pathophysiology. Phytochem 72(8):681-688

Archer S (1993) Measurement of nitric oxide in biological models. The FASEB Journal 7(2):349-360.

Balcerczyk A, Soszynskia M, Bartosz G (2005) On the specificity of 4-amino-5-methylamino-2′,7′-difluorofluorescein as a probe for nitric oxide. Free Rad Biol Med 39(3):327-335.

Cohn CA, Pedigo CE, Hylton SN, Simon SR, Schoonen MAA (2009) Evaluating the use of 3’-(p-Aminophenyl) fluorescein for determining the formation of highly reactive oxygen species in particle suspensions. Geochem Transact 10:8.

Gaupels F, Spiazzi-Vandelle E, Yang D, Delledonne M (2011) Detection of peroxynitrite accumulation in *Arabidopsis thaliana* during the hypersensitive defense response. Nitric oxide 25(2):222-228.

Glebska J, Koppenol W (2003) Peroxynitrite -mediated oxidation of dichlorodihydrofluorescein and dihydrorhodamine. Free Rad Biol Med 35(6):676-682.

Gomes A, Fernandes E, Lima JLFC (2005) Fluorescence probes used for detection of reactive oxygen species. J Biochem Biophys Met 65(2-3-):45-80.

Kojima H, Nakatsubo N, Kikuchi K, Kawahara S, Kirino Y, Nagoshi H, Hirata Y, Nagano T (1998) Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. Anal Chem 70:2446-2453.

Leshem YY, Pincasov Y (2000) Non-invasive photoacoustic spectroscopic determination of relative endogenous nitric oxide and ethylene content stoichiometry during the ripening of strawberries *Frageria anannasa* (Duch.) and avocados *Persea americana* (Mill.). J Exp Bot 51:1471-1473.

Magalhaes JR, Monte DC, Durzan D (2000) Nitric oxide and ethylene emission in Arabidopsis thaliana. Physiol Mol Biol Plants 6:117-127.

Mur LAJ, Mandon J, Cristescu SM, Harren FJM, Prats E (2011) Methods of nitric oxide detection in plants: A commentary. Plant Sci 181(5):509-519.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15(3):473-497.

Pedroso MC, Magalhaes JR, Durzan D (2000) A nitric oxide burst precedes apoptosis in angiosperm and gymnosperm callus cells and foliar tissues. J Exp Bot 51:1027-1036.

Pető A, Lehotai N, Lozano-Juste J, León J, Tari I, Erdei L, Kolbert Zs (2011) Involvement of nitric oxide and auxin in signal transduction of copper-induced morphological responses in *Arabidopsis* seedlings. Ann Bot 108:449-457.

Sun ZN, Wang HL, Liu FQ, Chen Y, Tam PKH, Yang D (2009) BODIPY based fluorescent probe for peroxynitrite detection and imaging in living cells. Org Lett 11:1887-1890.