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New sensors for monitoring reactive oxygen in plants under light stress

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Introduction

Biotic stress conditions may cause oxidative damage in plants. When the antioxidant system and repair mechanisms are unable to prevent or counterbalance damage, photosynthetic productivity declines. Reactive oxygen species (ROS) are key elements in several environmental stress conditions and are considered in a variety of roles: as primary elicitors of damage, as propagators, as by-products, and recently also as messenger molecules for the activation of defense or repair.

Spin trapping EPR spectroscopy can be useful in detecting ROS, but its application is limited *in vivo*, in leaves with high water content. Double (spin and fluorescent) ROS sensors consist of a fluorophore and a spin trap. During their functioning, conversion of the latter into an EPR active nitroxide results in partial fluorescence quenching (Kálai et al 1998, Hideg et al 2000a).

Besides being the driving force of photosynthesis and an important signal, light may also become a biotic stress factor in plants. Both aspects of stress by sunlight, photoinhibition (PI) by excess photosynthetically active radiation (PAR) and UV-B irradiation result in the inactivation of photosystem (PS) II electron transport and damage to a PS II core protein (D1). On the other hand, there are also differences in the effect of the two light stresses, not only at morphological but also at molecular level, for instance the primary site of electron transport inactivation in PS II, the cleavage site of D1, whether they cleave PS II core proteins other than D1, and the oxygen dependence of their effect *in vitro* (for reviews on PI and UV-B see Aro et al 1993, Barber 1994 and Vass 1997, Tevini and Teramura 1998, respectively).

The aim of our study was to investigate whether these differences are reflected in stress induced ROS production *in vivo*. Using a dansyl-based double sensor, DanePy, we found evidence for singlet oxygen production in tobacco leaf disks exposed to PI (Hideg et al 1998) but not in UV-B exposed ones (Hideg et al 2000b). On the other hand, *in vitro* spin trapping experiments evidenced UV-induced ROS production (Hideg and Vass 1996). Here we compare the oxidative nature of PI by PAR and UV-B stress *in vivo*, using the singlet oxygen sensor DanePy and new double sensors specific to ROS other than singlet oxygen.

Materials and methods

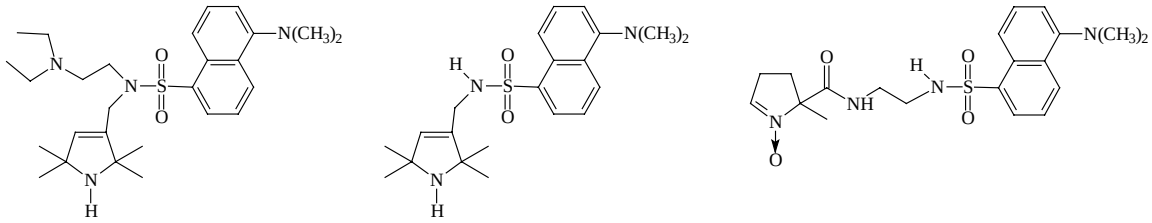
Arabidopsis thaliana plants were grown in the greenhouse, under 80-100 μmol m^{-2} s^{-1} PAR, 20-25°C for 5 weeks from germination. Detached leaves were kept on wet tissue paper with their adaxial sides up and exposed to either PI by 1800 μmol m^{-2} s^{-1} PAR from a KL-1500

(DMP, Switzerland) lamp or to 27 μmol m^{-2} s^{-1} UV-B from a VL-215M lamp (maximal emission at 312 nm, Vilbert-Lourmat, France), through a cellulose acetate filter (Courtaulds Chemicals, U.K.). For ROS detection, leaves were infiltrated with one of the ROS sensors (in 0.5 – 5 mM water solution, containing < 5% ethanol) within 15-20 s. The fluorescence emission spectrum of the ROS sensor was recorded at room temperature, with a Quanta Master QM-1 (Photon Technology Int. Inc., USA) spectrofluorimeter using 330 nm excitation. ROS detection was based on the decrease of sensor fluorescence, as described earlier (Hideg et al. 1998).

In order to avoid keeping the ROS sensor in the leaf for longer times, UV-B experiments were carried out in two series. In one, leaves were infiltrated with the ROS sensor, then the sample was exposed to UV-B for 30 min, during which the fluorescence of the ROS sensor was checked periodically. In an other series, plants were irradiated with UV-B without infiltration for 30 min, resulting in approx. 40% inactivation of PS II (measured as photosynthetic efficiency on the basis of variable chlorophyll fluorescence, data not shown), then infiltrated, and a reference fluorescence spectrum of the ROS sensor was measured. This was followed by an other 30 min UV-B treatment. ROS production between 30-60 min of UV-B irradiation was characterized comparing changes in ROS sensor fluorescence to the reference spectrum measured at 30 min. The two sets of ROS fluorescence data were combined normalizing the first reference point of the second set to the last data in the first set.

Results and discussion

Figure 1 shows the three dansyl-based double sensors used in this study. Their selectivity is characterized in Table 1 on the basis of decrease in their fluorescence in response to various, chemically generated ROS.



DanePy HO-1889NH L-2204

Fig. 1. The ROS sensors applied in this study.

DanePy, 5-Dimethylamino-naphthalene-1-sulfonic acid (2-diethylamino-ethyl)-(2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)-amide; HO-1889NH, 5-Dimethylamino-naphthalene-1-sulfonic acid (2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)-amide; L-2204, 2-Methyl-1-oxy-3,4-dihydro-2*H*-pyrrole-2-carboxylic-acid [2-(5-dimethylamino-naphthalene-1-sulfonylamino)-ethyl]-amide

HO-1889NH is similar to DanePy, but lacks a side chain (Fig. 1). This structural change results in lower sensitivity to $^1O_2$, but causing an additional reactivity to $O_2^{-\cdot}$ which is not characteristic to DanePy (Table 1). The third compound, L-2204, in which dansyl was connected to a more general spin trap, was sensitive to all four types of ROS tested.

Table 1. Selectivity of the applied ROS sensors.

	no addition	$^1O_2$	H_2O_2	$^{\cdot}OH$	$O_2^{-\cdot}$
DanePy	:= 100 %	35	97	98	93
HO-1889NH	:= 100 %	60	98	96	65
L-2204	:= 100 %	52	36	39	57

ROS induced changes in the fluorescence emission of the various ROS sensors are given as % of maximumal fluorescence intensity (measured without ROS). Consequently, smaller values (%) correspond to higher sensitivity. Singlet oxygen ($^1O_2$) was generated from illuminating 30 µmol chlorophyll in detergent, hydroxyl radicals ($^{\cdot}OH$) were produced from 0.2 µmol H_2O_2 – which was also applied independently – and 0.2 µmol Fe(II). Superoxide anion radicals ($O_2^{-\cdot}$) were generated from illuminating 60 µmol riboflavin.

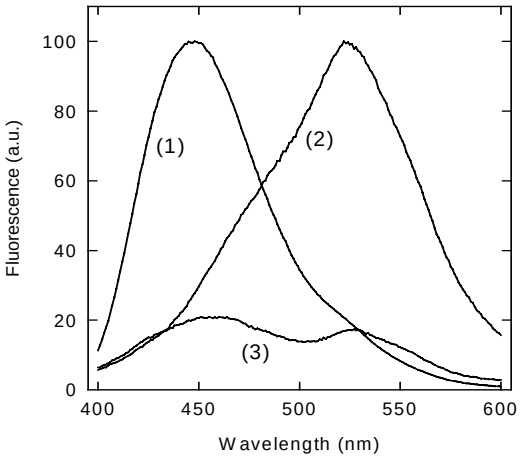


Fig. 2. Fluorescence emission spectra of *Arabidopsis* leaves infiltrated with (1) L-2204 or (2) HO-1889NH, or with water (3).

Figure 2 shows fluorescence emission spectra of the ROS sensors infiltrated into *Arabidopsis*. DanePy and HO-1889NH have very similar spectra, therefore only the latter is shown. The third spectrum in Fig. 2 shows the blue-green autofluorescence of the leaf (Goulas et al 1990), measured after infiltration with water only. ROS production was measured as relative decrease in the sensor fluorescence, corrected for blue-green fluorescence. The latter was also influenced by stress treatments and was measured separately in leaves without ROS sensors, in parallel experiments.

Figure 3 shows that the response of *Arabidopsis* leaves to PI and UV-B is different in terms of ROS production. In line with our earlier data measured in tobacco leaf disks (Hideg et al 2000b), we also found in the *Arabidopsis* experiment that $^1O_2$ was trapped by DanePy in PI exposed but not in UV-B irradiated leaves (Fig. 3A and B).

Experiments with HO-1889NH showed more similarity between the ROS response of the leaf to PI or UV-B. Both conditions resulted in intense ROS production, from the early phase of stress. By the time the photosynthetic yield has decreased to 50% (after 20-25 min PI or 35-40 min UV-B irradiation, data not shown), HO-1889NH fluorescence was lower than 50% of the initial value. Because – unlike DanePy –, HO-1889NH is reactive to $O_2^{-\cdot}$, the difference in their fluorescence quenching indicates superoxide production in both light stress conditions. $O_2^{-\cdot}$ production appears more intense in UV-B irradiated samples than during PI.

Experiments with L-2204, an ROS sensor with broader selectivity than the two others, also showed differences in the effect of PI and UV-B. Surprisingly, although L-2204 was almost as reactive to $^1O_2$ and $O_2^{-\cdot}$ *in vitro* as HO-1889NH (Table 1), its fluorescence did not change to the same extent in the *in vivo* PI experiment (Fig. 3A). However, one should note that the selectivity of the sensors to chemically generated ROS does not necessarily correspond to a scale of their reactivity *in vivo*. The lack of larger L-2204 fluorescence quenching may result from a lower reactivity of the sensor *in vivo* or from its localization. The latter is more likely, as L-2204 trapped more ROS in UV-B treated samples (Fig. 3B). Some of these could be the $^{\cdot}OH$ identified in UV-B stressed thyakoids earlier (Hideg and Vass 1996).

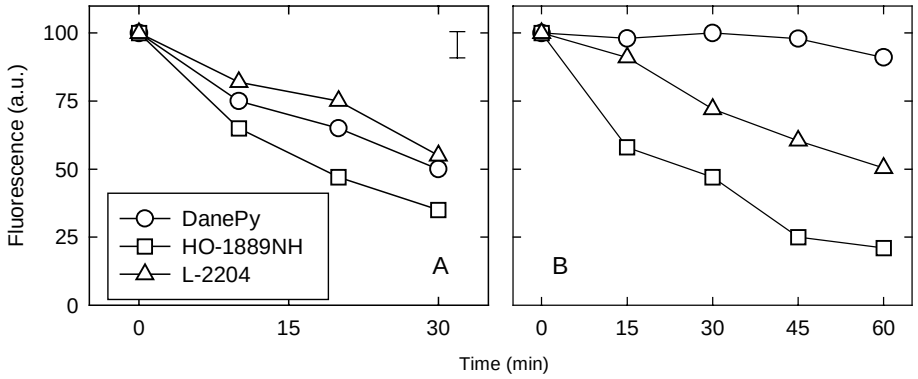


Fig. 3. ROS production induced by (A) PI and (B) UV-B irradiation in *Arabidopsis* leaves. Leaves were infiltrated with one of the following ROS sensors: DanePy (circles), HO-1889NH (squares) or L-2204 (triangles). ROS was measured as a decrease in sensor fluorescence, as total fluorescence and were corrected for the blue-green autofluorescence of the leaf itself.

In summary, our preliminary data show that the two main aspects of light stress from sunlight, PI and UV-B irradiation result in the production of different ROS *in vivo*. While $^1O_2$ production appears as a unique characteristic of PI, free radicals are also produced, although following different patterns. Their production may be the result of incomplete functioning of the water-water cycle (reviewed by Asada 1999), but other sources are also feasible and need to be investigated further.

Acknowledgments

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