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Dansyl- and rhodamine-based fluorescent sensors for detecting singlet oxygen and superoxide production in plants *in vivo*

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ABSTRACT Reactive oxygen species (ROS) play a key role in a variety of biotic and abiotic stress conditions, therefore their direct measurement *in vivo* is of special importance. In this study, we describe two dansyl-based ROS sensors, the singlet oxygen specific DanePy; HO-1889NH, which is reactive to both singlet oxygen and superoxide radicals and a rhodamine-based singlet oxygen sensor, HO-2941. In order to characterise the potential use of these chemosensors in plant experiments *in vivo*, we characterise their ROS trapping ability, fluorescence emission spectra and localisation in spinach leaves.

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KEY WORDS

Double (fluorescent and spin) sensors
singlet oxygen
superoxide radicals
laser scanning microscopy

A wide range of environmental conditions such as UV irradiation, photoinhibition by excess photosynthetically active radiation, pathogen infection, drought, pollutants, are potentially harmful to plants, leading to disruption of the balance between the production and removal of highly damaging reactive oxygen species (ROS). In this way, developing methods for direct *in vivo* identification and quantification of these ROS is of special importance in plant stress studies.

Materials and Methods

Mature spinach leaves were purchased at the local market and used on the same day. For ROS detection, leaves were infiltrated through a pinhole with 1 mM solutions of 3-[*N*-(b-diethylaminoethyl)-*N*-dansyl]aminomethyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole (DanePy; Kálai et al. 1998), 3-(*N*-dansyl)aminomethyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole (HO-1889NH; Kálai et al. 2001), or with 3-N-(2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-carboxoamidopropyl) sulforhodamine (HO-2941).

The fluorescence emission spectrum of ROS sensors was recorded at room temperature, as described earlier (Hideg et al. 1998). Excitation wavelengths were 330 nm and 533 nm for dansyl- (DanePy, HO-1889NH) and for rhodamine-based (HO-2941) sensors, respectively.

For ROS selectivity experiments, fluorescence was measured in a quartz cuvette during continuous stirring in 0.2 mM solution of one of the ROS sensors in Na-phosphate buffer (50 mM, pH 7.2). Relative changes in sensor fluorescence were induced by trapping various, chemically generated ROS. Singlet oxygen ($^1O_2$) was generated from illuminating 30 µmol detergent extracted chlorophyll, hydroxyl radicals ($^{\bullet}OH$) were produced from Fenton reaction, from 0.2 mM H_2O_2 – which was also applied independently – and 0.2 mM Fe (II). Superoxide anion radicals ($O_2^{-\bullet}$) were generated from illuminating 0.06 mM riboflavin.

For microscopy, 5 x 5 mm leaf cuttings were put between two UV-transparent microscope cover glasses and measured using a confocal laser scanning system (laser scanning microscopy; LSM 510, Karl Zeiss, Germany) in combination with an inverted microscope (Axiovert 100 M, Karl Zeiss, Germany). Adaxial sides of the leaf segments faced the 351 nm Ar laser or 543 nm HeNe laser excitation and fluorescence emission was observed through filters: 505-550 nm for green (dansyl-based sensors), 560-615 for yellow (rhodamine-based sensor) and above 650 nm for red (chlorophyll) fluorescence. Images were scanned at 0.8 s per frame, averaging 4 images.

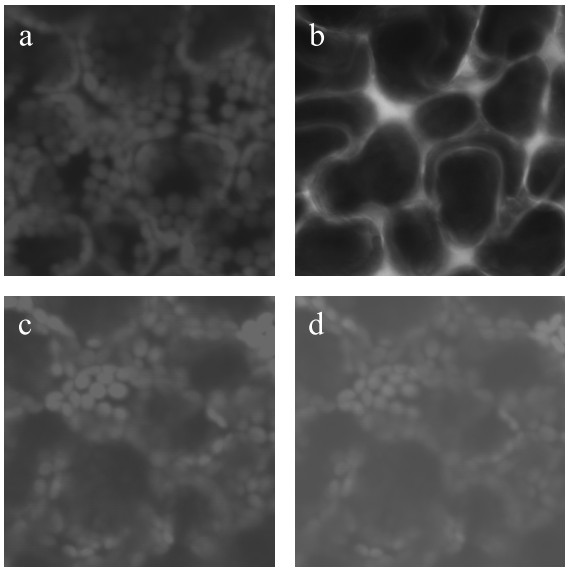

Figure 1. LSM images (a,c) red ($\lambda > 650$ nm), (b) yellow ($560 < \lambda < 615$ nm), and (d) green ($505 < \lambda < 550$ nm) fluorescence detected in (a,b) DanePy and (c,d) HO-2941 infiltrated spinach leaves at 15 mm depth from the adaxial leaf surface. Excitation was (a-c) 351 nm and (d) 543 nm. Image size 200 x 200 µm.

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Table 1. Relative changes in the fluorescence emission of the ROS sensors upon reacting with various ROS from chemical sources.

	no addition	$^1O_2$	H_2O_2	$^{\Sigma}OH$	$O_2^{-\Sigma}$
DanePy	:= 100 %	35%	97%	98%	93%
HO-1889NH	:= 100 %	60%	98%	96%	65%
HO-2941	:= 100 %	81%	95%	102%	æ

Results and Discussion

Dansyl-based ROS sensors are fluorescent in the 430-550 nm region, superimposing on the UV-inducible blue-green autofluorescence of green leaves (Chapelle et al 1984) (data not shown), presenting the use of rhodamine-based 550-650 nm fluorescent sensors more advantageous.

Table 1 illustrates the specificity of the applied ROS traps to various ROS. As reported earlier, DanePy was reactive to $^1O_2$ and fairly unreactive to other ROS (Hideg et al. 2000). In the present experiments it showed the largest fluorescence quenching in the presence of singlet oxygen among all tested compounds. A small structural modification in HO-1889NH as compared to DanePy made the sensor less sensitive to externally added $^1O_2$, but reactive to $O_2^{-\bullet}$ as well. The rhodamine-based HO-2941 showed moderate sensitivity to $^1O_2$ and practically no response to H_2O_2 or $^{\bullet}OH$ (Table 1). Due to the modifying effect of riboflavin on HO-2941, it was not possible to test the reactivity of this sensor to $O_2^{-\bullet}$ by this method.

When considering *in vivo* applications, it is crucial to investigate the localisation of the ROS traps in leaves. This was studied using laser scanning microscopy for gathering fluorescence data from inside the leaf in a non-invasive way. In Figure 1, the localisation of fluorescence from the ROS sensors DanePy and HO-2941 are compared with that of chlorophyll fluorescence. Images show that DanePy penetrates into the chloroplasts, while the rhodamine-based HO-2941 does not, limiting the *in vivo* applicability of the latter in photosynthesis-related stress studies, but offering the possibility of parallel studies using the two sensors. Data obtained with HO-1889NH were very similar to that with DanePy and are therefore not shown (Hideg et al. 2002).

In summary, ROS trapping abilities and known localisation of the chemosensors shown in this study makes them applicable in a variety of plant stress studies *in vivo.*

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