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Do oxidative stress conditions impairing photosynthesis in the light manifest as photoinhibition?

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We compared the effect of photoinhibition by excess photosynthetically active radiation (PAR), UV-B irradiation combined with PAR, low temperature stress and paraquat treatment on photosystem (PS) II. Although the experimental conditions ensured that the four studied stress conditions resulted in approximately the same extent of PS II inactivation, they clearly followed different molecular mechanisms. Our results show that singlet oxygen production in inactivated PS II reaction centres is a unique characteristic of photoinhibition by excess PAR. Neither the accumulation of inactive PS II reaction centres (as in UV-B or chilling stress), nor photo-oxidative damage of PS II (as in paraquat stress) is able to produce the special oxidizing conditions characteristic of acceptor-side-induced photoinhibition.

Keywords: photosynthesis; oxidative stress; singlet oxygen; DanePy fluorescence

1. INTRODUCTION

Increased production of reactive oxygen species (ROS) has been associated with a number of stress conditions in plants (for reviews, see Hendry 1994; Inze & Van Montagu 1995; Smirnoff 1995; Hideg 1997). ROS have been recognized in at least one of the following roles: elicitors or propagators of oxidative damage or signal molecules for repair processes. Their production site and function may be different under different stress conditions, and also depend on the antioxidant capacity of the plant. The toxicity of ROS is caused by their reactivity, which also makes them difficult to detect and identify, especially *in vivo*.

Unusually, light also appears to be a stress factor and the primary target of light stress is the site of light energy use: the photosynthetic apparatus (Powles 1984). Although in the field all stress conditions occur in light, the interpretation is probably not simple. Photoinhibition by excess photosynthetically active radiation (PAR) damages the reaction centre of the photosystem (PS) II complex of the photosynthetic apparatus, which is located in the thylakoid membrane of higher plant chloroplasts. Photoinhibition has been extensively studied *in vitro*, in isolated membrane preparations and a definitive sequence of events has emerged. In this model, excess PAR leads to impairment of PS II electron transport, which is followed by selective degradation of the D1 PS II reaction centre protein and by more general membrane protein and lipid damage (for reviews, see Barber & Andersson 1992; Prasil *et al.* 1992; Aro *et al.* 1993).

Direct observation of ROS by spin trapping electron paramagnetic resonance (EPR) spectroscopy demonstrated that photoinhibition by excess PAR is an oxidative stress (Hideg *et al.* 1994*a*; Hirayama *et al.* 1996; Yruela *et al.* 1996). Among the particular pathways of damage an important one is recognized as acceptor-side-induced photoinhibition, which occurs when photosynthetically active, oxygen-evolving preparations are illuminated with excess PAR in the presence of oxygen. In this process, double reduction of the first PS II quinone acceptor Q_A results in increased reaction centre chlorophyll triplet formation (Vass *et al.* 1992; Vass & Styring 1992) and, consequently, in singlet oxygen production. There are strong indications that the above ROS are involved in the specific cleavage of the D1 protein: singlet oxygen-generating substances cause D1 protein fragmentation to the same specific fragments as does acceptor-side-induced photoinhibition by excess PAR (Mishra *et al.* 1994; Okada *et al.* 1996). Since the principal cause of photoinhibition in this model is the imbalance between energy intake and processing, it is plausible that any stress condition that suppresses photosynthetic electron transport or exhausts excess energy relaxation mechanisms would manifest as photoinhibition. The aim of the present work was to analyse this question on the basis of ROS detection *in vivo*.

Singlet oxygen production in acceptor-side-induced photoinhibition has been confirmed *in vitro*, in isolated photosynthetically active membrane preparations. Singlet oxygen has been detected with various techniques

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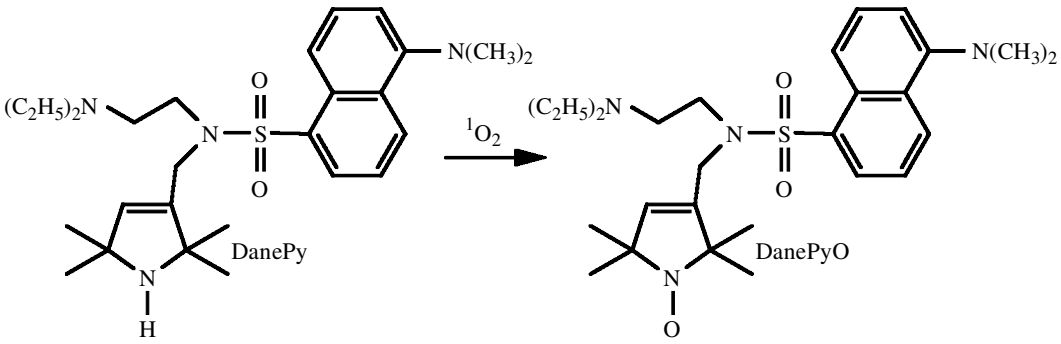

Figure 1. Chemical structure of DanePy and its nitroxide radical form DanePyO.

including spin trapping EPR spectroscopy (Hideg *et al.* 1994*b*), infrared chemiluminescence (MacPherson *et al.* 1993) and chemical trapping (Telfer *et al.* 1994). Extension of our EPR results to *in vivo* (leaf studies) was hampered by technical difficulties caused by the high water content of the leaves and the short lifetime of the probe within the leaf (Hideg *et al.* 2000). A new technique was needed. DanePy is a double sensor consisting of a fluorophore, dansyl, and a sterically hindered amine attached to a pyrroline ring (figure 1) (Kálai *et al.* 1998). This compound is fluorescent and diamagnetic. Reaction with singlet oxygen converts the amine into a nitroxide radical. The resulting compound (DanePyO) is paramagnetic (data not shown) and has lower fluorescence than DanePy (figure 2*a*). Using this sensor, we were able to demonstrate singlet oxygen production in leaves exposed to photoinhibition by excess PAR (Hideg *et al.* 1998*a*).

Our earlier *in vitro* studies showed that among various light stress conditions singlet oxygen production seemed to be a unique characteristic of PS II under acceptor-side-induced photoinhibition. Neither donor-side-induced photoinhibition (Hideg *et al.* 1994*a*) nor ultraviolet B (UVB) irradiation (Hideg & Vass 1996) appeared as singlet oxygen-mediated stress; both were associated with other types of ROS, mainly hydroxyl radicals. In the present study, we carried out a comparative study of PS II function and D1 protein degradation, *in vivo*, in photoinhibited leaves and in leaves exposed to other types of oxidative stress (low temperature, herbicide or UVB irradiation).

2. MATERIAL AND METHODS

Tobacco (*Nicotiana tabacum* L.) plants were grown in a greenhouse, under 80–100 $\mu mol\,m^{-2}s^{-1}$ PAR, 20–25 °C. Leaf disks were cut from the tip region of six-week-old leaves avoiding the midrib section. These samples were kept on wet tissue paper on a thin layer of water with their adaxial sides up and exposed to one of the following stress conditions: (i) photoinhibition by 1500 $\mu mol\,m^{-2}s^{-1}$ PAR at room temperature, (ii) irradiation with 25 $\mu mol\,m^{-2}s^{-1}$ UVB (280–320 nm) and 100 $\mu mol\,m^{-2}s^{-1}$ PAR at room temperature, (iii) chilling at 5 °C under 400 $\mu mol\,m^{-2}s^{-1}$ PAR, or (iv) 7.5 mM paraquat at room temperature under 100 $\mu mol\,m^{-2}s^{-1}$ PAR.

PAR was provided through an optical fibre guide from a KL-1500 (DMP, Switzerland) lamp. This illumination caused no local warming of the samples, even when 1500 $\mu mol\,m^{-2}s^{-1}$ PAR were applied. In paraquat stress, leaf disks were pre-incubated with paraquat in the dark for 1 h before illumination and paraquat was contained in the floating medium.

For PS II activity and D1 protein measurements, thylakoid membranes were prepared from the leaf disks immediately after the cessation of the stress. Photosynthetic oxygen evolution was measured with oxygen polarography (Hansatech, UK) using dimethyl-benzoquinone as electron acceptor. Net D1 protein content was determined using Western blotting with an antibody raised against a synthetic peptide corresponding to the C-terminus of pea D1 protein, a kind gift from Dr P. Nixon (Imperial College of Science, Technology and Medicine, London, UK) after separation of thylakoid membrane proteins by SDS polyacrylamide gel electrophoresis.

Singlet oxygen detection was based on the reaction of singlet oxygen with DanePy (3-(*N*-diethylaminoethyl)-*N*-dansyl)-aminomethyl-2,5-dihydro-2,2,5,5-tetramethyl-1*H*-pyrrole), yielding a nitroxide radical (DanePyO, 3-(*N*-diethyl-aminoethyl)-*N*-dansyl) aminomethyl-2,5-dihydro-2,2,5,5-tetramethyl-1*H*-pyrrol-1-yloxyl), which is EPR active and has lower fluorescence than DanePy (Kálai *et al.* 1998). Leaf disks were infiltrated with 50 mM DanePy and fluorescence emission spectra were recorded with a Quanta Master QM-1 (Photon Technology Int., Inc., South Brunswick, NJ, USA) using 345 nm excitation as described earlier (Hideg *et al.* 1998*a*). Singlet oxygen trapping is characterized as relative fluorescence quenching, $-\Delta F/F$, at the 532 nm emission maximum of DanePy (figure 2*b*).

3. RESULTS

Figure 2 illustrates the measurement of singlet oxygen production in leaves using DanePy. As shown in figure 2*a*, there is a marked difference between the fluorescence emission of DanePy and its nitroxide radical form, DanePyO. In the experiment shown in figure 2*a*, leaves were infiltrated by one of these two forms of the double sensor. On the basis of this difference in fluorescence intensity, singlet oxygen production can be characterized as fluorescence quenching of DanePy (Kálai *et al.* 1998; Hideg *et al.* 1998*a*). Figure 2*b* shows that this quenching occurred when a DanePy-infiltrated leaf was exposed to photoinhibition.

In order to characterize the stress-induced damage to PS II we measured the loss of electron transport activity and the relative amount of D1 protein. Experimental parameters were set in order to ensure that the loss of PS II activity followed approximately the same time-course during the four different types of stress conditions applied (data not shown). Inactive reaction centres were defined as ones containing D1 protein but unable to

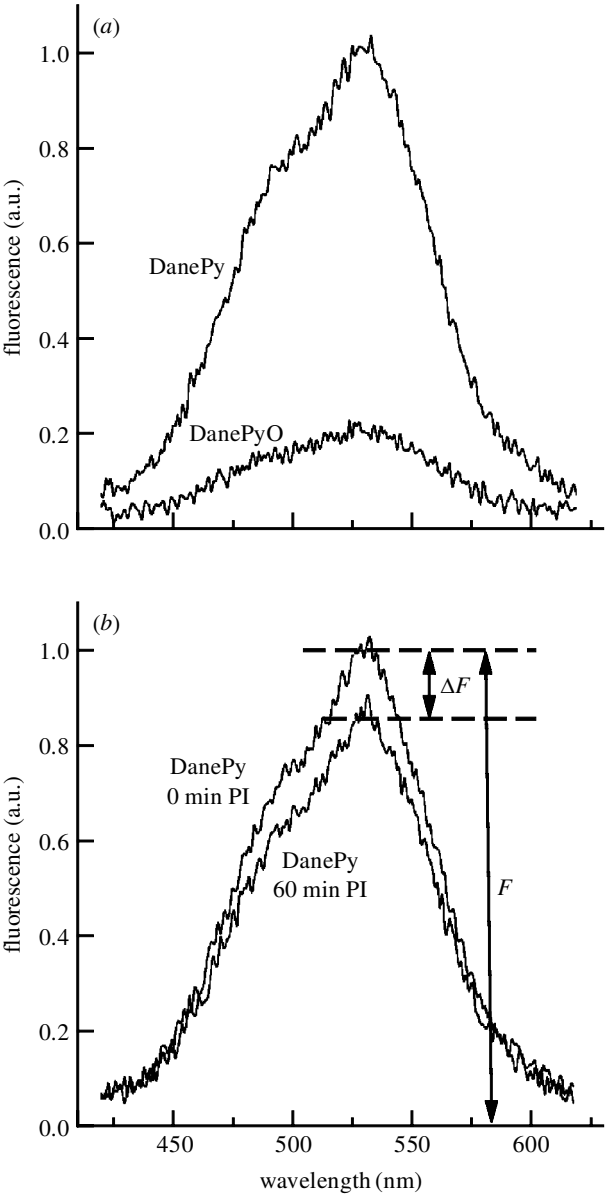

Figure 2. (*a*) Fluorescence emission spectra of DanePy and its nitroxide radical form DanePyO infiltrated into tobacco leaf disks. (*b*) Fluorescence emission spectrum of DanePy in tobacco leaves before and after 60 min photoinhibition (PI) by 1500 μmol $m^{-2}s^{-1}$ PAR at room temperature. All spectra were measured using $\lambda_{exc} = 345$ nm and are shown normalized to the fluorescence emission maximum of DanePy in the untreated leaf. Parameters used in the definition of relative fluorescence quenching, $\Delta F/F$ are also shown in (*b*).

evolve oxygen upon illumination. When leaves were exposed to photoinhibition at room temperature, there was no marked decrease in the amount of D1 protein, thus the accumulation of inactive centres corresponded to the loss of PS II activity.

Figure 3 shows the comparison of the amount of inactive PS II centres and singlet oxygen production in the case of four different oxidative stress conditions: photoinhibition, UVB irradiation, low temperature and treatment with paraquat. Figure 3*a* shows that singlet oxygen production during photoinhibition *in vivo* is proportional to the amount of inactive PS II centres (Hideg *et al.* 1998*a*). This result supports the model postulating functionally inactive centres as sources of singlet oxygen. Similarly to photoinhibition by PAR, the other type of light stress, irradiation with UVB, also resulted in the accumulation of inactive PS II centres, although to a smaller extent. Contrary to photoinhibition, there was no marked singlet oxygen production until the late phase of UVB treatment (figure 3*b*). Chilling at 5 °C resulted in both the accumulation of inactive PS II and singlet oxygen production, although to a smaller extent than photoinhibition (figure 3*c*), while neither inactive PS II accumulation, nor singlet oxygen production was characteristic of treatment by paraquat (figure 3*d*).

4. DISCUSSION

Photoinhibition by excess PAR may deactivate photosynthetic electron transport and cause oxidative damage in plants (Prasil *et al.* 1992; Aro *et al.* 1993; Krause 1994). The amount of PAR that is capable of initiating photoinhibition depends on the efficiency of the energy dissipation mechanisms and the capacity of the antioxidant defence system (for reviews, see Demmig-Adams & Adams 1992; Asada 1994). The primary site of damage is in PS II, where excess PAR generates stable reduced abnormal quinone states that lead to the production of singlet oxygen via chlorophyll triplet states (Vass & Styring 1992, 1993). In agreement with the established model of acceptor-side-induced photoinhibition, we have recently shown that singlet oxygen is produced under high light intensities *in vivo*, in inactive PS II reaction centres (Hideg *et al.* 1998*a*). Because stress conditions in the field usually combine, it is feasible to ask whether the impairment of PS II by another stress could decrease the threshold intensity above which PAR is in excess for photosynthesis and thus generate singlet oxygen-mediated photoinhibition. In the experiments demonstrated above, three different types of oxidative stress were combined with PAR and their ability to promote singlet oxygen production was examined. In order to perform comparative experiments, conditions were set in a way that ensured that PS II electron transport activity was damaged to the same extent during the same periods of time by the various stress conditions.

UVB irradiation affects plants at many levels causing a complex oxidative stress (for review, see Vass 1997). In the thylakoid membrane, the primary target of UVB is at the Mn cluster of the oxygen-evolving system (Renger *et al.* 1989; Vass *et al.* 1999), although the quinone acceptors of PS II are also affected (Greenberg *et al.* 1989; Vass *et al.* 1996). As we showed earlier, in isolated thylakoid membranes this PS II impairment by UVB does not result in marked singlet oxygen production (Hideg & Vass 1996). Our present data confirm this observation in leaves. Moreover, we found that a combination of UVB irradiation and PAR did not promote singlet oxygen production. Singlet oxygen appeared only in severely damaged leaves, after long irradiation, probably accompanying membrane lipid peroxidation. There is no marked singlet oxygen production at the earlier phase of damage, although inactive reaction centres accumulated (figure 3*b*). The absence of singlet oxygen production supports the recent model based on studies of cyanobacteria in which the initial site of PS II inactivation by UVB light *in vivo* is not at

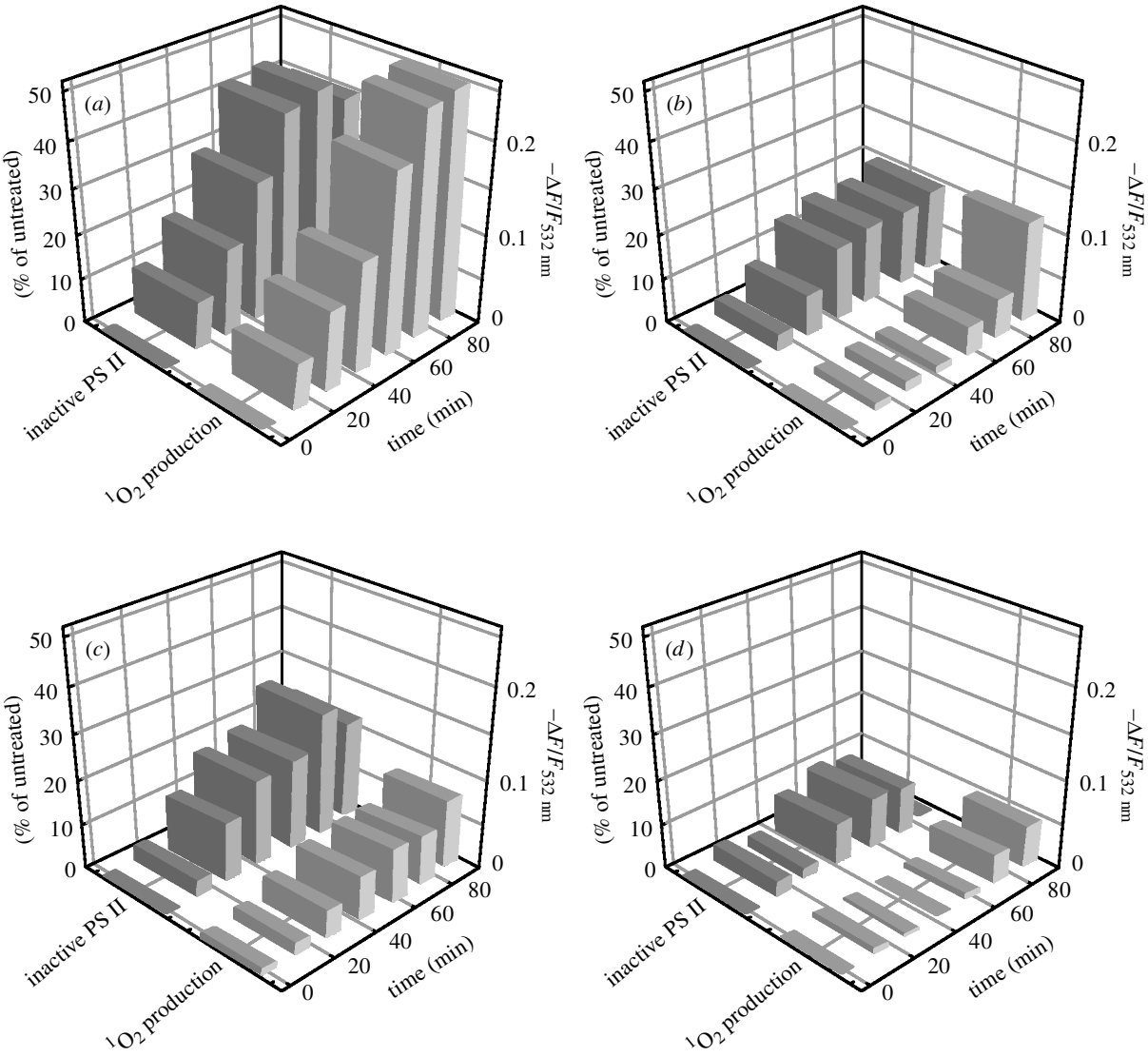

Figure 3. Comparison of singlet oxygen production and the amount of inactive PS II reaction centres in leaves under various stress conditions. The latter are defined as centres with intact protein structure but functionally impaired. Singlet oxygen production was measured as quenching of DanePy fluorescence (see § 2 for details). Leaves were exposed to (*a*) photoinhibition by 1500 μmol $m^{-2}s^{-1}$ PAR at room temperature, (*b*) irradiation with 25 μmol $m^{-2}s^{-1}$ UVB (280–320 nm) and 100 μmol $m^{-2}s^{-1}$ PAR at room temperature, (*c*) chilling at 5 °C under 400 μmol $m^{-2}s^{-1}$ PAR, or (*d*) 7.5 mM paraquat at room temperature under 100 μmol $m^{-2}s^{-1}$ PAR.

the quinone acceptors (Vass *et al.* 1999). Damage by UVB may be facilitated by PAR in centres with inactivated oxygen-evolving complexes according to the mechanism of donor-side-induced photoinhibition, but this hypothesis needs further testing.

Chilling results in an oxygen- and light-dependent inactivation of photosynthesis. It has been reported that the extent of PS II inhibition by low temperature depends on the photon flux density during chilling and no inhibition was observed in the dark (Bowles *et al.* 1983). In this way, chilling may be regarded as low-temperature-enhanced photoinhibition (Hetherington *et al.* 1989). Our results show that although inactive PS II reaction centres accumulated during illumination at low temperature and singlet oxygen was produced, both occurred to a smaller extent than during PAR at room temperature (figure 3*c*). This suggests that although chilling can be partially regarded as causing enhancement of photoinhibition by low temperature, this is not the only mechanism of PS II damage by low temperature. Observation of hydroxyl (Hideg & Björn 1996) and superoxide (Hodgson & Raison 1991) radical production in chilling exposed leaves, as well as the retarding effect of various added antioxidants (Wise & Naylor 1995), supports the role of alternative ROS-mediated damage.

Contrary to the above three stress conditions, oxidative damage by paraquat originates outside PS II. Paraquat is univalently reduced by PS I to its cation radical, which rapidly donates electrons to oxygen, producing superoxide radicals (Babbs *et al.* 1989). Such superoxide production at PS I exceeds the antioxidant ability of the superoxide dismutase–ascorbate–peroxidase system (for a review, see Asada 1994) and excess superoxide and other ROS propagate oxidative damage to other membrane components. In this way, PS II is also affected and D1 is degraded (Hideg *et al.* 1998*b*), but there is practically no inactive

PS II accumulation (figure 3*d*). Accordingly, singlet oxygen production was also low (figure 3*d*). Although PAR is necessary for the action of paraquat, damage caused by this internal radical source (the paraquat radical) results in almost simultaneous electron transport inactivation and D1 degradation. In this way, functional PS II impairment is probably the result of general (D1 and other) protein and membrane degradation, and acceptor-side-induced photoinhibition does not occur.

It is important to note that inactive PS II reaction centres are not the only potential sources of singlet oxygen. Stress-induced membrane disintegration may lead to free chlorophyll formation, which readily reacts with oxygen to form singlet oxygen (Halliwell 1982). Prolonged exposure of isolated chloroplasts to photo-oxidative stress caused lipid peroxidation, which was accompanied by singlet oxygen production (Takahama & Nishimura 1975). The singlet oxygen production that we observed after prolonged exposure to UVB (figure 3*b*) or paraquat stress (figure 3*d*), and found unrelated to the accumulation of inactive PS II centres, may originate from such reactions.

5. CONCLUSIONS

Besides being the driving force of photoinhibition, PAR is essential for damage by low temperature and by paraquat and it may modify the effect of UVB light. Although experimental conditions for the four studied stresses were set to result in the same time-course of PS II inactivation, damage clearly followed different pathways. Inactive PS II reaction centres accumulated during photoinhibition, UVB irradiation and chilling, while there was little accumulation in paraquat stress. Singlet oxygen production correlated well with the growth in the amount of inactive PS II reaction centres during photoinhibition and also to some extent in chilling. There was no such correlation in leaves exposed to UVB irradiation and PAR. Our results show that singlet oxygen production in inactivated PS II reaction centres is a unique characteristic of photoinhibition by excess PAR. We found that, although PAR may make chilling stress more severe by promoting photoinhibition, this was not the exclusive pathway of oxidative damage at low temperature. Moreover, neither the accumulation of inactive PS II reaction centres by UVB, nor photo-oxidative damage of PS II by paraquat was able to produce the special oxidizing conditions characteristic of acceptor-side-induced photoinhibition.

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