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Reactive oxygen species from type-I photosensitized reactions contribute to the light-induced wilting of dark-grown pea (*Pisum sativum*) epicotyls

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Type-II, singlet oxygen-mediated photosensitized damage has already been shown to occur in epicotyls of dark-germinated pea (*Pisum sativum* L.) seedlings upon illumination, resulting in fast turgor loss and wilting. In this study we show evidence that the palette of reactive oxygen species (ROS) is more complex. Hydrogen peroxide, superoxide and hydroxyl radicals are also formed, suggesting the occurrence of type-I reactions as well. Moreover, hydrogen peroxide injection into the epicotyls in the dark was able to provoke wilting directly. Formation of hydroxyl radicals could also be triggered by the addition of hydrogen peroxide in the dark, preferentially in the mid-sections where wilting occurs, showing that potential mediators of a Fenton reaction are present in the epicotyls, but unevenly distributed. Localization of light-inducible ROS formation fully (hydrogen peroxide) or partially (superoxide radicals) overlaps with the distribution of monomer protochlorophyllide complexes, showing that these pigment forms are capable of provoking both type-I and type-II reactions.

Introduction

Native arrangements of pigment–protein complexes are critical during early plant development. Tissues containing protochlorophyllide (Pchlide) or chlorophyllide (Chlide) molecules as monomers are light sensitive, capable of promoting light-induced production of reactive oxygen species (ROS) (Erdei et al. 2005). Most of the dark-developed angiosperm tissues are particularly light sensitive. Photo-oxidative processes are prevented if the pigments aggregate into macrodomains, which are oligomers of ternary complexes of the enzyme subunits NADPH:protoclorophyllide oxidoreductase (EC 1.3.1.33, POR) (Armstrong et al. 2000) characteristic mainly for prolamellar bodies (PLBs) of leaf etioplasts. In contrast, etioplasts in epicotyls or other stem-related organs contain few and small PLBs (Böddi et al. 1994, McEwen et al. 1994, Skribanek et al. 2008) and the Pchlide pigments are predominantly in a monomer state (Böddi et al. 1994, 1998, McEwen et al. 1994, Skribanek et al. 2000, 2008). Low temperature (77 K) fluorescence emission spectra of etiolated pea epicotyls measured with 440 nm excitation show a maximum at around 631 nm and a shoulder of low amplitude at 655 nm. The maximum is an overlap of a 629 and a 636 nm band, both corresponding to Pchlide monomers connected

Abbreviations – Chlide, chlorophyllide; DAB, 3,3′-diaminobenzidine; EPR, electron paramagnetic resonance; NBT, nitroblue tetrazolium; Pchlide, protochlorophyllide; PLB, prolamellar body; POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; PPFD, photosynthetic photon flux density; ROS, reactive oxygen species; TEM, transmission electron microscope.

to different non-chromophore molecules (Böddi et al. 1998); while the shoulder indicates the presence of the oligomer Pchlide (i.e. at the same time as the POR oligomers). There is an increasing gradient of Pchlide along the epicotyls with the highest amounts present in the uppermost region (Böddi et al. 1994). The ratio of the monomer to oligomer complexes decreases in the upward direction (Böddi et al. 1994).

In an earlier study, we showed that photosensitized singlet oxygen ($^1O_2$) production, lipid peroxidation and subsequent turgor loss occurred in the middle regions of the epicotyls, resulting in an irreversible wilting when dark-grown pea seedlings were illuminated (Erdei et al. 2005). In this study we examined whether this reaction is limited to type-II, $^1O_2$-mediated photochemistry or whether illumination also provoked other ROS production, such as reduced oxygen forms characteristic of type-I reactions.

Materials and methods

Plant material

Seven-day-old dark-germinated pea (*Pisum sativum* L. cv. 'Zsuzsi') seedlings (10–15 cm long) were used in the experiments. The seeds were pre-germinated on wet filter paper in Petri dishes for 2–3 days, and then the plants were grown in hydroponic culture in tap water at room temperature in the dark. Sample collection was carried out in dim green light.

ROS detection with color-responsive indicators

3,3′-diaminobenzidine (DAB) or nitroblue tetrazolium (NBT) were used as hydrogen peroxide or superoxide indicators, respectively. A peroxidase-catalyzed reaction between DAB and H_2O_2 leads to the formation of an insoluble brown precipitate (Malmgren and Olsson 1977, Thordal-Christensen et al. 1997). Pale yellow NBT is reduced by superoxide to blue–gray formazan that results in tissue staining (Beyer and Fridovich 1987). Both oxidized DAB and NBT can be directly visualized in etiolated tissue. Seedlings were exposed to white light [600 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD)] for 20 min, subsequently infiltrated in a glass tube with 5 m*M* DAB and incubated for 18 h in darkness. For NBT visualization, seedlings were infiltrated with 0.6 m*M* NBT, incubated in the dark for 30 min and then illuminated with white light (600 μmol m^{-2} s^{-1} PPFD). The blue–gray color of formazan showed a positive reaction after 10 min. These staining procedures were applied with vacuum infiltration at room temperature.

Treatment with exogenous H_2O_2

We tested whether wilting could be induced in the dark by exogenous H_2O_2. In order to obtain a homogeneous H_2O_2 distribution, etiolated epicotyls were injected spirally, at intervals of 1 cm along the epicotyls, with a Na–K phosphate buffer (pH 7.0) containing 0.7 m*M* H_2O_2. Control plants were injected similarly with phosphate buffer only. After injection, all samples were kept in the dark for 30 min.

Detection of Pchlide/Chlide with fluorescence microscopy

Samples were taken for microscopic observation to clarify the horizontal distribution of pigments. The middle regions of the epicotyls were cut into 0.5–1.0 mm thick sections with a Plant Microtome MT-2 (NK System, Osaka, Japan). The sections were placed on slides into a drop of 0.05 *M* phosphate buffer, pH 7.0 containing 50 m*M* sodium ascorbate to prevent photo-oxidation of the chlorophyllous pigments. The sections were observed by fluorescence microscope (OLYMPUS BX50, Olympus, Tokyo, Japan). To observe the red (600–750 nm) fluorescence of chlorophyllous pigments, a WBV filter was used to produce blue excitation light. Fluorescence images were recorded using Micropublishers 5.0 adjusted by the software Qcapture (Microsoft, Redmond, WA, USA).

Low temperature fluorescence spectroscopy

For 77 K fluorescence measurements the middle region of the epicotyls were divided into five longitudinal sections: the central cylinder region and four equal cortex regions each containing one leaf trace (for details see Fig. 1). Fluorescence emission spectra of these samples were measured with a Fluoromax-3 (Jobin Yvon Horiba, Longjumeau, France) spectrofluorometer with 0.1 s integration time and 0.5 nm data collection frequency. The excitation wavelength was 440 nm, the excitation and emission slits were 2 and 5 nm, respectively. All spectra were corrected to the wavelength-dependent variations of the detection. Baseline correction and 5-point linear smoothing were performed using SPSERV-V.14 software (copyright: Cs. Bagyinka, Institute of Biophysics, Biological Research Centre, Szeged, Hungary). The data represent the average of three independent measurements.

H_2O_2 detection with electron microscopy

Subcellular localization of H_2O_2 was detected with a transmission electron microscope (TEM), utilizing Bestwick's cytochemical method, which is based on

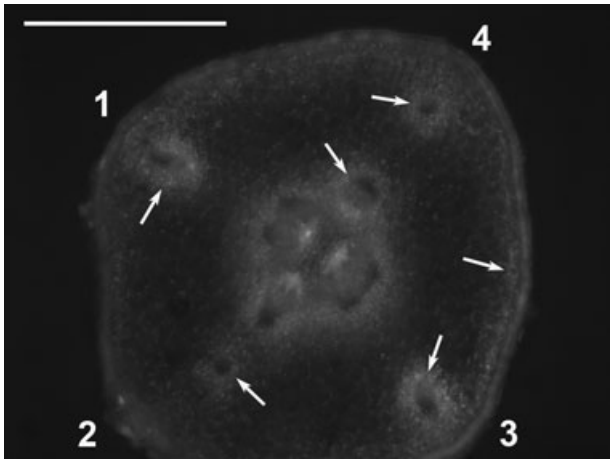

Fig. 1. Grey-scale fluorescence image of a cross section from the middle region of a 7-day-old dark-grown pea epicotyl. Excitation: xenon lamp and WBV filter. This blue light resulted in red fluorescence (600–750 nm) of chlorophyllous pigments. Arrows indicate the regions with most intense fluorescence. Numbers refer to the different longitudinal sections studied with fluorescence spectroscopy (see Table 1). 1 and 3: represent leaf traces with fiber bundles, 2: leaf trace of the closest scale shaped leaf, 4: leaf trace opposite to the scale shaped leaf. Bar: 1 mm.

the generation of cerium perhydroxide precipitates from cerium chloride and H_2O_2 (Bestwick et al. 1997). Small parts of control and treated epicotyls were incubated in freshly prepared 5 m*M* $CeCl_3$ in 50 m*M* MOPS buffer, pH 7.2 for 30 min before fixation. Samples were collected 30 min after exposure to 300 µmol m^{-2} s^{-1} PPFD. $CeCl_3$-treated samples without illumination were used as a control. Tissue pieces were fixed in two steps (2% glutaraldehyde for 3 h and 1% osmium tetroxide for 2 h, both fixatives were solved in 0.1 *M* K–Na–phosphate buffer, at pH 7.2) and embedded in Durcupan resin after dehydration in an ethanol series. Sections (60–80 nm) were cut by a Reichert-Ultracut E microtome (Vienna, Austria), mounted on uncoated copper grids and stained with 5% uranyl acetate dissolved in methanol for 4 min and lead citrate for 6 min (Reynolds 1963). The sections were investigated using a Hitachi 7100 TEM (Tokyo, Japan) at 75 kV accelerating voltage.

EPR spectroscopy

Segments (3 cm) were cut from various regions (apical-upper, middle and basal-lower regions) of the epicotyls under dim green light and incubated in a spin trapping assay using ethanol/α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) (1%/40 m*M*, in 50 m*M* K–phosphate buffer, pH 7.0) according to Renew et al. (2005). In this experiment, $^{\bullet}OH$ reacts with ethanol to a carbon-centered hydroxyethyl radical, which is trapped by POBN resulting in a stable long-lived spin adduct (Ramos et al. 1992). After 2 h incubation, samples were exposed to 300 µmol m^{-2} s^{-1} PPFD for 30 min, then immediately placed into glass sample tubes and positioned optimally in the cavity of the electron paramagnetic resonance (EPR) spectrometer. Control samples were not illuminated but kept in darkness for 30 min after incubation. EPR spectra were recorded using a Bruker ESP300 computer-controlled X-band spectrometer Bruker (Rheinstetten, Germany) equipped with an ER4103TM microwave cavity. Spectra from the samples containing the POBN spin trap were recorded at room temperature (9.74 GHz microwave frequency, 100 kHz modulation frequency, modulation amplitude 2 G, 63 mW microwave power) as the sum of 10 scans (each taking about 42 s) in 1024 points. For quantification, spectra were baseline corrected and filtered using a 15-point polynomial filter. EPR absorption peaks were integrated and this value was used for quantitative assessment of $^{\bullet}OH$ trapped in the samples.

Results

To study the localization and distribution of the Pchlide in the epicotyl tissues, fluorescence microscopy was used. These studies showed three main tissue regions with red fluorescence as follows: the subepidermal cortex layer, the parenchyma cells around the four leaf traces in the cortex and the parenchyma cells around the vessels of the central cylinder (Fig. 1, arrows). It is interesting to note that two opposing leaf traces had strong fiber crowns (Fig. 1, numbers 1 and 3) connected to their external sides, while the other two opposing leaf traces had no fiber bundles.

These tissue regions were divided in longitudinal sections and their 77 K fluorescence emission spectra were recorded separately. In all spectra, the maximum was found at 631–632 nm and a small shoulder was found around 655 nm. However, amplitude ratios of fluorescence intensities at 631 and 655 nm, representing ratios of Pchlide monomers to Pchlide in oligomer complexes varied (Table 1).

To localize the ROS formation, special indicator molecules were infiltrated into pea epicotyls. Superoxide radicals ($O_2^{-\bullet}$) were detected by NBT staining (Fig. 2A, B), this ROS form was only observed in epicotyls exposed to relatively high PPFD (600 µmol m^{-2} s^{-1}). The reaction of NBT with $O_2^{-\bullet}$ resulted in formazan production within 10 min. The cross section shows that $O_2^{-\bullet}$ was detected in the subepidermal cell layers. A complete lack of staining in the non-irradiated sample (Fig. 2C, D) proves

Table 1. The ratio of fluorescence emission at 631 and 655 nm in the different regions of the middle segments of 7-day-old etiolated pea epicotyls. For measurements the epicotyls were separated into five longitudinal sections: the central cylinder region and four equal cortex regions each containing one leaf trace (for details and numbering see Fig. 1). The data represent the average of three independent measurements.

	Leaf trace with fiber bundle (1)	Leaf trace of the closest scale shaped leaf (2)	Leaf trace with fiber bundle (3)	Leaf trace opposite to that of scale shaped leaf (4)	Central cylinder
631/655 ratio	4.8	5.6	6.5	5.6	4.0

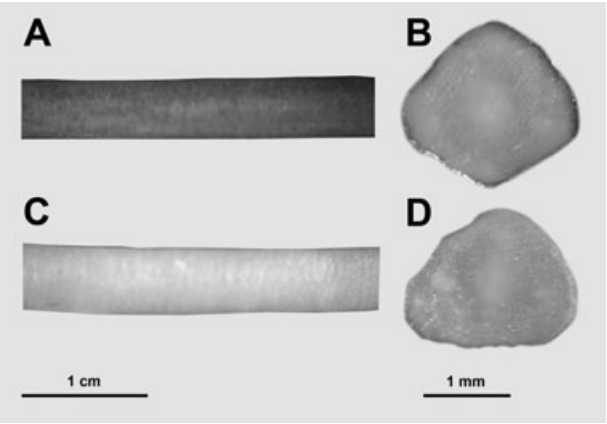

Fig. 2. Surface view of a 3 cm segment from the middle parts of 7-day-old etiolated pea epicotyls (A and C) and cross sections from the central parts of the same epicotyls (B and D). A and B: The sample was incubated with 0.6 m*M* NBT for 30 min in the dark, subsequently exposed to white light (600 $\mu mol\, m^{-2}\, s^{-1}$) for 10 min. C and D: The sample was infiltrated with NBT and incubated in the dark for 40 min (control).

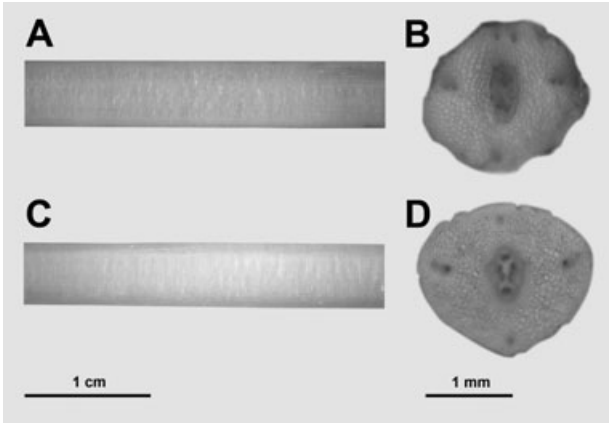

Fig. 3. Surface view of a 3 cm segment from the middle parts of 7-day-old etiolated pea epicotyls (A and C) and cross sections from the central parts of the same epicotyls (B and D). A and B: The sample was exposed to white light (600 $\mu mol\, m^{-2}\, s^{-1}$) for 20 min and subsequently infiltrated with DAB and incubated for 18 h in the dark. C and D: The sample was not illuminated, but infiltrated with DAB and incubated in the dark for 18 h (control).

that $O_2^{-\bullet}$ production was not as a result of mechanical injury caused by infiltration or cutting of the samples.

DAB infiltration was used to examine H_2O_2 production in response to the same irradiation. In Fig. 3A, B, DAB staining indicates H_2O_2 production in illuminated epicotyls. The staining was intense in the subepidermal cell layers of the cortex, in cells surrounding the leaf traces and in the central tissue cylinder. These identified regions are not necessarily the primary production sites, as this ROS molecule can diffuse through cell membranes. However, they overlap with the regions in which the highest Pchlide fluorescence was observed (Fig. 1). ROS production could not be caused by mechanical injury through sample treatment, because no color change was found in non-irradiated samples (Fig. 3C, D).

H_2O_2 production was confirmed at the ultrastructural level by $CeCl_3$-based cytochemical staining. Cerium perhydroxide precipitation was observed in illuminated samples, mainly along radial cell walls of epidermal cells (Fig. 4A) while no precipitates were found in control epicotyls, which were not illuminated (Fig. 4B). Weak Ce staining was also observed in the walls of cortical cells of illuminated samples.

Detection of H_2O_2 suggested the possibility that this ROS may cause wilting directly. Injecting H_2O_2 into the epicotyls caused wilting without illumination, as illustrated in Fig. 5. No wilting was observed in epicotyls, which were injected with phosphate buffer only. Illumination during the preparation of samples and taking photos (30–50 $\mu mol\, m^{-2}\, s^{-1}$ PPFD for 1–2 min) did not provoke any wilting. In the H_2O_2-injected samples, wilting started approximately 15–20 min after injection and was complete within 30 min.

The presence of $O_2^{-\bullet}$ and H_2O_2 in illuminated epicotyls suggested the possibility of further ROS-forming oxidation reactions, such as $^{\bullet}OH$ production. This was tested with spin trapping EPR spectroscopy. Figure 6 shows that the sextet lines of the POBN-hydroxyethyl spin adduct, a characteristic of $^{\bullet}OH$ in the sample, was most pronounced in the mid-sections of illuminated epicotyls. As illustrated by EPR spectra in Fig. 6A, this signal was also observed in samples kept in the dark, although with smaller amplitudes. This lower intensity signal was observed in all samples, independent of light conditions (Fig. 6B) and is possibly an artifact from spin trap impurities. The production of $^{\bullet}OH$ was

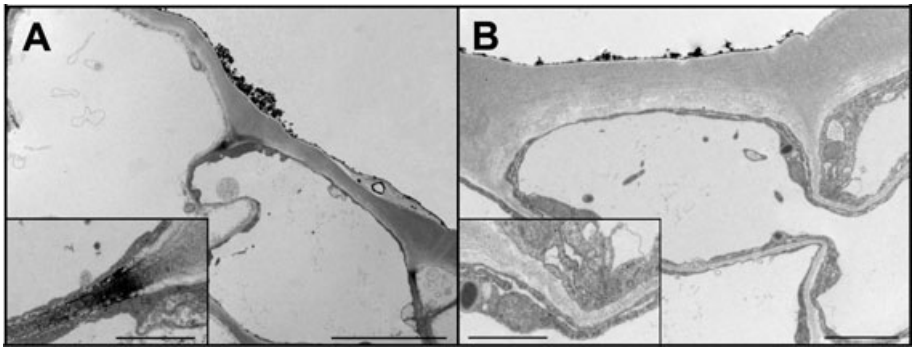

Fig. 4. Electron micrographs from the middle sections of 7-day-old etiolated pea epicotyls treated with 5 m*M* cerium chloride for 30 min before fixation. A: Epicotyl illuminated for 30 min with white light (300 μmol m^{-2} s^{-1}). Bar: 10 μm. B: Non-illuminated control sample. Bar: 2 μm. Insets: Enlarged details of a radial cell walls. Bar: 1 μm.

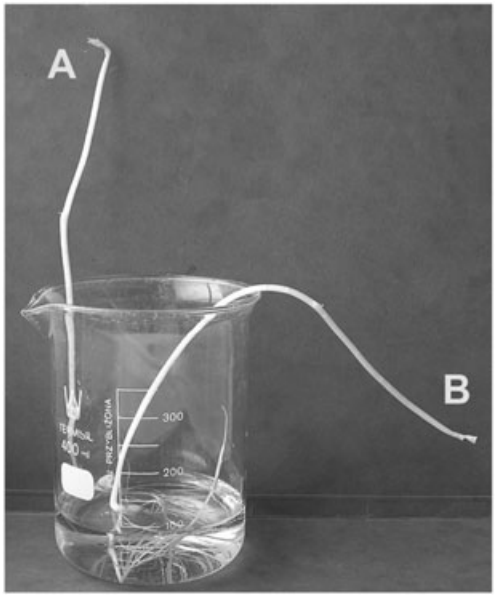

Fig. 5. Seven-day-old dark-grown pea seedlings. A control sample (A) was injected with phosphate buffer (50 m*M* pH 7.2). The experimental sample (B) was injected with 0.7 m*M* H_2O_2 in the same buffer. Injections were carried out in dim green light; the samples were kept in the dark for 30 min before taking the photographs.

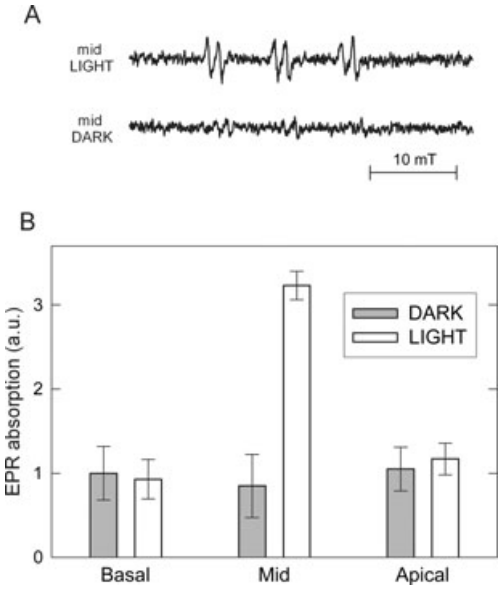

Fig. 6. EPR absorption of the POBN-hydroxyethyl adduct diagnostic for $^{\bullet}OH$. (A) EPR spectra of two segments cut from the middle sections of 7-day-old etiolated pea epicotyls, incubated with ethanol/POBN for 2 h, then either illuminated with 300 μmol m^{-2} s^{-1} PPFD (trace-labeled LIGHT) or kept in darkness (DARK) for 30 min. (B) Integrated EPR absorptions of the POBN-hydroxyethyl adduct measured in epicotyl segments from basal, middle or apical regions after the above treatments. Data were normalized to the average value of non-illuminated basal samples (n = 5, error bars are standard deviations).

observed when samples were injected with H_2O_2 in the dark (data not shown).

Discussion

We have shown in earlier studies the participation of a $^1O_2$-mediated photo-oxidative mechanism in the induction of light-provoked wilting in dark-germinated pea epicotyls (Erdei et al. 2005). However, photosensitized ROS generating reactions in biological tissues are usually complex and involve more than one photochemical pathway. In the present study, various selective ROS-specific assays were used to prove the presence of three different ROS: $O_2^{-\bullet}$, H_2O_2 and $^{\bullet}OH$, in illuminated pea epicotyls. Light-dependent occurrence of all three ROS was detected directly (Figs. 2, 3 and 6) under the same experimental conditions, which caused wilting.

The observation that the $H_2O_2 \rightarrow {}^{\bullet}OH$ conversion did not require irradiation (data not shown) suggests that the primary photoproduct is either H_2O_2 or $O_2^{-\bullet}$. Observations showing that H_2O_2 injection alone (without illumination) is capable of causing wilting after 10–30 min (Fig. 5), and also triggers ${}^{\bullet}OH$ promptly, suggest that (1) cofactors of a Fenton reaction are present at higher concentrations in the middle regions of dark-grown epicotyls and (2) ${}^{\bullet}OH$ radicals from this reaction initiate subsequent membrane damage. It has been shown that ${}^{\bullet}OH$ radicals could cause cell wall loosening and thus mediate extension growth in maize coleoptiles and sunflower hypocotyls (Chen and Schopfer 1999, Fry 1998, Liszkay et al. 2003, Schopfer et al. 2002). The source of ${}^{\bullet}OH$ in these samples was also suggested to be the inorganic metal catalyzed Fenton reaction, but – unlike in the photosensitized mechanism we propose – initiated by a controlled enzymatic H_2O_2 source. This inorganic metal could be iron, stored as ferritin in plastids of epi- and hypocotyl tissues (McEwen et al. 1994), but probably is also present in small amounts in 'free' ionic form. In our experiments, higher yields of ${}^{\bullet}OH$ from an uncontrolled photo-reaction may damage the plasma membrane which then contributes to wilting. Detecting Ce perhydroxide precipitates mainly along the radial cell walls (Fig. 4A) of illuminated epicotyls supports this hypothesis. Damage of plasma membranes of epidermis cells at the radial cell walls may lead to water loss and changed mechanical properties of the epicotyls.

It is important to note that the applied ROS detecting methods, with the exception of spin trapping, are qualitative, not quantitative. However, actual amounts of the spin trap in the sample are unknown, as well as its trapping efficiency or actual life-times of the spin adduct. The most intense ROS production was observed in the middle section of the epicotyls. The possible reason for this phenomenon is the coincidence of high proportion and inhomogeneous distribution of monomer Pchlide (Table 1, Böddi et al. 1994, 1996) acting as photosensibilizer, poorly developed and unequally organized mechanical tissues (Fig. 1), as well as the presence of Fenton catalysts in this region. Lower sections are lignified and contain much less pigment than the upper sections (Böddi et al. 1994). The regions above the studied middle sections contain more developed etioplasts and higher amounts of Pchlide oligomers, which do not sensibilize ROS production (Armstrong et al. 2000, Böddi et al. 1994).

Our results support the occurrence of a $H_2O_2 \rightarrow {}^{\bullet}OH$ transformation pathway. Furthermore, detecting $O_2^{-\bullet}$ and the light-dependent nature of both ROS production and wilting suggest a photosensitizer $\rightarrow O_2^{-\bullet} \rightarrow H_2O_2 \rightarrow {}^{\bullet}OH$ series of reactions. The sensitizer of the initial reaction can be Pchlide, which was shown to undergo radical formation before its conversion to Chlide by the light-driven enzyme POR in vitro, in a model system (Belyaeva et al. 2001). A transient charge separation product has also been shown to participate in the initial photochemistry of Pchlide $\rightarrow$ Chlide transition; however, this is not a radical product if NADPH is present to accept the extra charge in the POR-catalyzed reaction occurring in etioplasts (Heyes et al. 2003). NADPH shortage occurs in epicotyls of pea (Böddi et al.

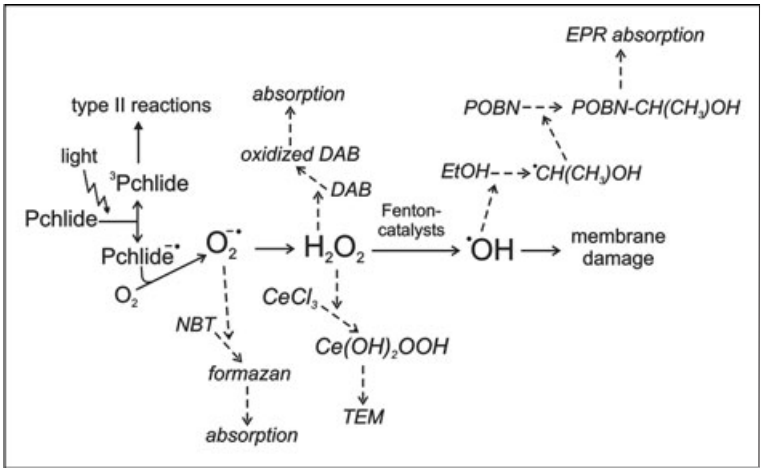

Fig. 7. Scheme of the proposed type-I photosensitized reactions (solid arrows) leading to the wilting of epicotyls of dark-germinated pea (*P. sativum* L.) seedlings upon illumination. Methods used for detecting various ROS are shown with dashed arrows. Chemicals used in ROS assays and their products are shown in italics. Possible type-II photoreactions, leading to singlet oxygen-mediated oxidative damage have already been discussed (Erdei et al. 2005).

2005) and most pigments are not bound to POR, nor to NADPH. The fact that monomeric Pchlide and/or Chlide can be possible photosensitizers of these reactions is further confirmed by the coincidence of maximal Pchlide or Chlide fluorescence in the same regions of epicotyl tissues (Fig. 1) where highest H_2O_2 production has been localized (Fig. 3). In the epicotyls, where pigments in the absence of PLBs are mainly in monomer state (Böddi et al. 1998) chances of Pchlide radical formation seem to be higher, and thus the occurrence of electron transfer to oxygen in a type-I photodynamic reaction is more likely. Photochemical production of superoxide by saponified chlorophyll and its breakdown products in solution was shown by Jahnke and Frenkel (1975).

In summary, our data show that apart from the earlier demonstrated energy transfer to oxygen from excited monomer state pigments resulting in $^1O_2$ production (Erdei et al. 2005), electron transfer and thus photosensitized oxygen reduction also occurs. Membrane damage by additional ROS products, H_2O_2 or more likely by $^{\bullet}OH$ also contributes to disordered water status of the epicotyls and their wilting in the light. A scheme of proposed series of reactions and applied methods is shown in Fig. 7.

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