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Changes in production of reactive oxygen species in illuminated thylakoids isolated during development and senescence of barley

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ABSTRACT

This paper presents a detailed analysis of thylakoids isolated from secondary barley leaves harvested 18, 22, 25, 29, 32, 35 and 39 days after sowing (DAS). Goal of the analysis was to investigate the production of different reactive oxygen species (ROS) during development and senescence of barley. Generation of superoxide anion ($O^{\bullet}_2-$) and hydrogen peroxide (H_2O_2) increases during development of barley reaching the highest value right after the onset of senescence (between 25 and 29 DAS), thereafter the levels of both ROS start to decrease until 35 DAS when production of H_2O_2 increases again. In comparison with $O^{\bullet}_2-$ and H_2O_2, generation of singlet oxygen ($^1O_2$) showed continuous production of low amounts thought the duration of experiment. Oxidative damage to the thylakoid membrane was assessed by measuring lipid peroxidation. Results showed gradual increase in lipid peroxidation with progress of plant development with highest increase occurring at the late stages of senescence. A possible factor contributing to the elevation in the production of ROS could be an increase in membrane fluidity observed in our previous study. Fluidization of the membrane, allows for better penetration of oxygen inside the membrane, which can lead to an increase in the production of ROS. Indeed, the production of ROS started to increase together with observed fluidization of the membrane from 22 to 29 DAS. Thereafter, production of ROS started to decline till 35th DAS. On the last day of the measurement, chl is at 25% of its initial value, lipid peroxidation reaches the highest value and H_2O_2 increases again.

1. Introduction

Leaf senescence is an important physiological process of higher plants, and is often regarded as the final stage of leaf development (Wu et al., 2012). It is a highly organised process during which proteins are degraded and nutrients recycled and mobilized to seeds, storage organs or new vegetative growth (Himelblau and Amasino, 2001). Leaf senescence is particularly important for cereal plants. Cereal plants that have a late onset and slower rate of leaf senescence have been proven to increase yield (Borrell et al., 2001). On the other hand, premature senescence induced by stress results in reduced yield and quality in crops. Furthermore, plant senescence can have negative effects on post-harvest storage (Page et al., 2001). For this reason, a better understanding of senescence processes can have beneficial effects on the productivity and quality of grain and the storage life of harvested tissues.

Although in recent years there has been significant progress in the area of leaf senescence, the important regulatory mechanisms of this complex process are not well understood. One of the major events in leaf senescence is the production of reactive oxygen species (ROS). These include relatively low reactive partially reduced oxygen species such as hydrogen peroxide (H_2O_2), superoxide $O^{\bullet}_2-$ an excited singlet oxygen molecule and the most reactive—hydroxyl radicals ($OH^{\bullet}$) (Lee et al., 2012; Prochazkova et al., 2001). Most ROS in plant cells are formed directly or indirectly via the dismutation of superoxide, which arises as a result of single electron transfer to molecular oxygen in electron transfer chains principally during the Mehler reactions in the chloroplast (Asada et al., 1974; Mehler, 1951). Other important sources of ROS generation include; NADPH oxidases (NOX) commonly known as respiratory burst oxidase homologs (Rbohs) (Sagi and Fluhr, 2006)

Abbreviations: car, carotenoids; chl, chlorophyll; DAS, days after sowing; EPR, electron paramagnetic resonance; LPPs, lipid peroxidation products; MF, membrane fluidity; PET, photosynthetic electron transport; ROS, reactive oxygen species.

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and peroxisomes which have the capacity to rapidly produce and scavenge H_2O_2 and $O^{\bullet}{}_2^-$ (Sandalio et al., 2013). The formation of ROS is generally considered to be a damaging process causing oxidative damage to DNA, proteins and membrane lipids. However, they also play an important role in cellular signalling pathways in plants (Apel and Hirt, 2004; Li et al., 2009; Ramel et al., 2012 Scarpeci et al., 2008). This is especially true for H_2O_2. Transcriptomic analyses of *Arabidopsis* plants have revealed hundreds of H_2O_2-responsive genes (Yun et al., 2010), while numerous studies demonstrated important role of H_2O_2 during the regulation of various abiotic and biotic stresses (Quan et al., 2008). Furthermore, H_2O_2 plays an important role during the senescence process where it was shown that it could be used as a signal to promote senescence in different plant species, and to be part of a complex regulatory network (Bieker et al., 2012). Importance of H_2O_2 for many physiological processes is not surprising, especially if we consider its properties as the most stable and least reactive ROS which can easily cross the membrane (Quan et al., 2008; Yang and Poovaiah, 2002) and has a half-life of 1 ms (Gechev et al., 2006). . In comparison to H_2O_2, $O^{\bullet}{}_2^-$ has a shorter half-life of approximately 2–4 μs (Gechev et al., 2006). Its potential to act as signalling molecule is limited by its inability to pass through the membranes (Takahashi and Asada, 1983) and quick conversion to H_2O_2 in the presence of SOD (Asada, 2006). Numerous studies reported an increase in the production of $O^{\bullet}{}_2^-$ in the course of natural and artificially induced senescence (McRae and Thompson, 1983; Pastori and Del Rio, 1997) and possible roles during senescence (Scarpeci et al., 2008) and abiotic stresses (Rodriguez-Serrano et al., 2006). Finally, $^1O_2$ is the most reactive with short lifetime of 3.1–3.9 μs in pure water (Krasnovsky, 1998). Nevertheless, it was demonstrated that $^1O_2$ is capable of diffusing a distance of over 270 nm in rat nerve cells (Skovsen et al., 2005) and that $^1O_2$ produced in the photosystem II is capable of leaving the thylakoid membrane and reaching the cytoplasm or even the nucleus (Fischer et al., 2007). As with other ROS, $^1O_2$ has a dual effect. As an oxidizing agent it can react with various biological molecules causing damage and leading to cell death (op den Camp et al., 2003) and it can also play a signalling role activating the expression of different genes (Kim et al., 2008; Laloi et al., 2007). It was reported that $^1O_2$ is the main cause for senescence-associated oxidative stress in the chloroplasts of sage (Munné-Bosch et al., 2001) and that the mass generation of singlet oxygen was measured in the early stages of hormone treated barley (Springer et al., 2015).

This study is a continuation of our previous work, in which we investigated the physical properties of barley thylakoid membranes during the course of senescence, and how changes in the levels of different carotenoids and proteins impact these changes (Jajić et al., 2014). In this article, we investigate the production of three main ROS; $O^{\bullet}{}_2^-$, H_2O_2, and $^1O_2$ during the development and senescence of barley leaves by using highly advanced methods for the specific detection of ROS. These include electron paramagnetic resonance (EPR) spin trapping and use of time resolved singlet oxygen spectroscopy. Furthermore, we will try to link the increase in the production of ROS with the increase in membrane fluidity observed in our previous study and investigate damage caused to the membrane by assessing the magnitude of lipid peroxidation, which is a widely used stress indicator of plant membranes.

2. Materials and methods

2.1. Plant material and treatments

"Golden Promise" barley (*Hordeum vulgare*) was cultivated inside the greenhouse under long-day (16 h light/8 h dark) conditions at 22°/18 °C at a light intensity of 150 μE. In cereals such as barley, senescence seems to be regulated at the level of the individual leaf. Nutrients are mobilized from older leaves to younger leaves and eventually to flag leaves. For this reason we choose the leaf that emerges second after sowing (secondary leaf) as the material for the experiments. Thylakoid membranes were isolated from secondary leaves harvested 18, 22, 25, 29, 32, 35 and 39 days after sowing. Each sample was an average of 10 secondary leaves and was done in duplicate.

2.2. Isolation of thylakoid membranes

Thylakoids were isolated from barley leaves (1.0 g) as described by (Khorobrykh et al., 2002) with some modifications. In short, 1.0 g of leaf material was ground in 100 mL of buffer A (pH 7.6, 0.4 M sucrose, 25 mM Hepes–KOH, 20 mM NaCl, 5 mM MgCl2) on ice. The homogenate was filtered through 4 layers of cheese cloth and centrifuged at 500 × *g* for 90 s. the pellet was discarded and the supernatant was transferred into a clean tube and centrifuged at 1000 × *g* for 7 min. The resulting pellet was suspended in 20 mL of buffer B (pH 7.6, 20 mM NaCl, 5 mM $MgCl_2$, and 25 mM Hepes–KOH) for 1 min to be osmotically shocked. An equal volume of buffer C (pH 7.6, 0.8 M sucrose, 20 mM NaCl, 5 mM $MgCl_2$, 25 mM Hepes–KOH) was added, and the suspension was centrifuged at 2000 × *g* for 5 min. The pellet was twice washed by suspending it in buffer A, followed by centrifugation at 2000 × *g* for 5 min. The washed pellet was suspended in 2 mL of buffer D (pH 7.6, 0.4 M sucrose, 20 mM NaCl, 5 mM $MgCl_2$, 10 mM Hepes–KOH) and stored on ice. All procedures were performed under dim green light and a temperature of 4 °C.

2.3. Pigment quantification

The pigments from leaves were determined spectrophotometrically according to the following procedure (Lichtenthaler and Buschmann, 2001). Approximately 100 mg of secondary barley leaves was ground, placed in an Eppendorf tube and mixed with 1 mL of extraction solvent (80% aqueous acetone). The suspension was left for 2 h at −20 °C and centrifuged at 14,500 × *g* for 2 min. the supernatant was collected, diluted to an absorbance value of 0.5–1.0 and measured at 649, 665 and 470 nm.

2.3.1. Measurement of photosynthetic electron transport (PET) capacity

Photosynthetic electron transport (PET) was measured directly with a use of 2,6-dichlorophenol-indolphenol (DCPIP) according to (Allen and Holmes, 1986). When DCPIP accepts electrons, it changes from a blue to a colourless state which can be monitored spectrophotometrically at the wavelength of 590 nm. Measurement was performed by illumination of thylakoids at 10 s intervals with high light and measurement of absorption at 590 nm. Reaction medium contained thylakoids diluted with buffer D to a chl concentration of 10 μg chl mL^{-1} and 100 mM DCPIP as electron donor or 100 mM DCPIP with 100 mM diphenyl carbazide (DPC) as electron acceptor. The same procedure was repeated with thylakoids suspended in mixture of dimethyl sulfoxide and buffer D (80% DMSO, 20% buffer D) to check the influence of DMSO on the activity of the electron transport chain.

2.4. Determination of superoxide anion

The superoxide anion ($O^{\bullet}{}_2-$) was measured by EPR-spin trapping using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as a spin trap. The detection of $O^{\bullet}{}_2-$ was performed according to (Van Doorslaer et al., 1999) with some modifications. Isolated thylakoids (1 mg chl/mL) were diluted with DMSO to a chl concentration of 200 μg chl mL^{-1}. The reaction mixture (80% DMSO, 20% buffer