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EPR spin labeling measurements of thylakoid membrane fluidity during barley leaf senescence

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ABSTRACT

Physical properties of thylakoid membranes isolated from barley were investigated by the electron paramagnetic resonance (EPR) spin labeling technique. EPR spectra of stearic acid spin labels 5-SASL and 16-SASL were measured as a function of temperature in secondary barley leaves during natural and dark-induced senescence. Oxygen transport parameter was determined from the power saturation curves of the spin labels obtained in the presence and absence of molecular oxygen at 25 °C. Parameters of EPR spectra of both spin labels showed an increase in the thylakoid membrane fluidity during senescence, in the headgroup area of the membrane, as well as in its interior. The oxygen transport parameter also increased with age of barley, indicating easier diffusion of oxygen within the membrane and its higher fluidity. The data are consistent with age-related changes of the spin label parameters obtained directly by EPR spectroscopy. Similar outcome was also observed when senescence was induced in mature secondary barley leaves by dark incubation. Such leaves showed higher membrane fluidity in comparison with leaves of the same age, grown under light conditions. Changes in the membrane fluidity of barley secondary leaves were compared with changes in the levels of carotenoids (car) and proteins, which are known to modify membrane fluidity. Determination of total car and proteins showed linear decrease in their level with senescence. The results indicate that thylakoid membrane fluidity of barley leaves increases with senescence; the changes are accompanied with a decrease in the content of car and proteins, which could be a contributing factor.

Introduction

Leaf senescence constitutes the final stage of leaf development resulting in deterioration of leaf function and death. It is a highly organized process during which proteins are degraded and nutrients recycled and mobilized to seeds, storage organs or new vegetative growth (Himelblau and Amasino, 2001). Changes that occur during leaf senescence have been intensively studied and they include chlorophyll (chl) catabolism (Ougham et al., 2008), loss of photosynthetic competence (McRae et al., 1985), increase in production of reactive oxygen species (Zimmermann and Zentgraf, 2005) as well as changes in expression of senescence associated genes (Lim et al., 2007). However, changes in physical properties of thylakoid membranes of chloroplasts during senescence received relatively little attention. There are several studies concerning changes in lipid fluidity of microsomal membranes and plasma membranes from senescing leaves (Leshem et al., 1984; Roberts et al., 1987), fruits (Legge et al., 1986), cotyledons (McKersie et al., 1978) and flowers (Borochov et al., 1976; Legge et al., 1982; Thompson et al., 1982), while only one deals with senescence related changes in thylakoid fluidity (McRae et al., 1985).

Thylakoids are highly specialized systems of membranes inside chloroplasts. They are the site of light dependent reactions of photosynthesis. Lipid composition of thylakoid membranes is characterized by high amounts of polyunsaturated galactolipids (Lichtenthaler, 1999), and absence of sterols (Weeb and Green, 1991). Consequently, as compared to many other biological

Abbreviations: β-car, β-carotene; car, carotenoids; chl, chlorophyll; EPR, electron paramagnetic resonance; Lut, lutein; Nx, neoxanthin; Vx, violaxhantin; Zea, zeaxanthin.

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membranes, the thylakoid membranes are relatively fluid. The importance of membrane fluidity is clearly evident in respect to plant responses to changes of environmental conditions. Exposure of plants to elevated temperatures leads to decrease in the fluidity of thylakoid membrane and changes in double bond index (Raison et al., 1982). The damage of thylakoid membranes may lead to a significant decrease in photosynthesis and decreased biomass production in grain yield for crop plants. Membrane fluidity of thylakoids can be modified by many factors. Increase in the saturated to unsaturated fatty acid ratio and depletion of unsaturated fatty acids account for the decrease in membrane fluidity (Fobel et al., 1987; Yruela et al., 2001). Similar effect is observed when sterols are incorporated into chloroplast thylakoid membranes (Ford and Barber, 1983). Incorporation of carotenoids (car) and operation of the xanthophyll cycle modify membrane fluidity (Lazrak et al., 1987; Gruszecki and Strzalka, 1991); so does the accumulation of lipid peroxidation products (Mayak et al., 1983; Chen and Yu, 1994). Finally, the lipid to protein ratio regulates the fluidity of membranes and a decrease in this ratio is an indication of a more rigid environment (Strzalka and Subczynski, 1981; Strzalka and Machowicz, 1984; Quartacci et al., 2000).

The present study examines which parameters are associated with changes in physical properties of thylakoids isolated from barley senescing leaves. Simultaneous measurements of thylakoid membrane fluidity, oxygen transport parameter, chl and carotenoid quantification, and protein level have been made for mature and naturally senescing secondary barley leaves, and during dark induced senescence of these leaves.

Materials and methods

Plant material and treatments

"Golden Promise" barley (*Hordeum vulgare*) was cultivated inside the greenhouse in a day and night rhythm (day: 16 h with 150 μE light at 24 °C; night: 8 h darkness at 18 °C). In cereals such as barley, senescence seems to be regulated at the level of the individual leaf. Nutrients are mobilized from the older leaves to the younger leaves and eventually to the flag leaves. For this reason we choose leaf that emerges second after sowing (secondary leaf) as the material for experiments. Thylakoid membranes were isolated from secondary leaves harvested 18, 20, 23, 27, 31, 35 and 39 days after sowing. Each sample was an average of 10 secondary leaves and was done in a duplicate. Dark-induces senescence was induced on mature secondary leaves (24 days after sowing) by 5 days dark incubation of whole plants. As a control, mature secondary leaves of equal age were harvested from plants grown under light conditions.

Isolation and spin labeling of thylakoid membranes

Barley leaves (1.0 gram) were homogenized with 100 ml of buffer A (pH 7.6, 50 mM Hepes, 0.4 M sucrose, 10 mM NaCl) on ice. Homogenate was filtered through 4 layers of cheese cloth into tubes for centrifugation and centrifuged in K23 centrifuge for 90 s at 500 × *g*. Pellet was discarded and supernatant was transferred into clean tubes and centrifuged for 7 min at 1000 × *g*. Resulting pellet was suspended in 20 ml of buffer B (pH 7.6, 0.2 M sucrose, 20 mM Hepes, 0.1 M KCl, 5 mM $MgCl_2$) and centrifuged for 7 min at 1000 × *g*. Final pellet was suspended in 5 ml of buffer C (osmotic shock buffer pH 7.6, 20 mM Hepes, 0.1 M KCl, 5 mM $MgCl_2$) and left for 5 min on magnetic stirrer on ice. Then 5 ml of buffer D (pH 7.6, 0.4 M sucrose, 20 mM Hepes, 0.1 M KCl, 5 mM $MgCl_2$) was added and the suspension was centrifuged for 10 min at 2000 × *g*. Pellet of thylakoid membranes was suspended in buffer B at the concentration of chl of 1 mg/ml. All procedures were performed under dim green light and temperature of 4 °C.

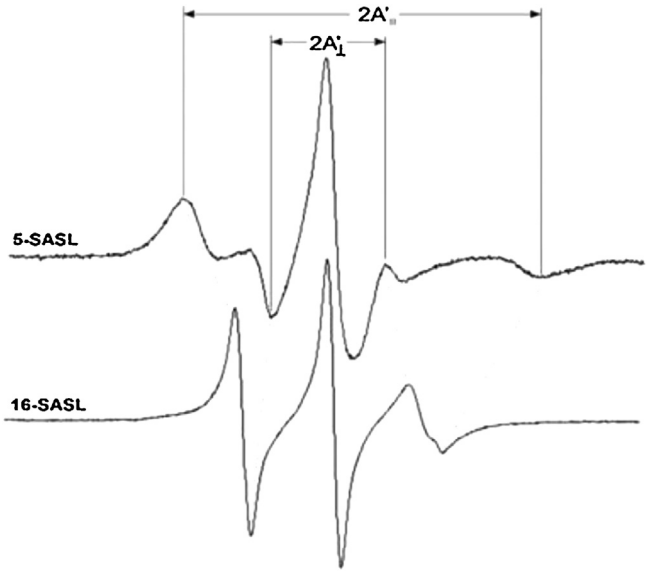

Fig. 1. EPR spectra of doxylstearic acid spin labels in thylakoid membrane recorded at 25 °C for samples equilibrated with nitrogen. The measured parameter is indicated. Outermost splitting parameter ($2A_{II}'$) gives information about the general mobility of the spin label in the membrane. It thus reflects the degree of membrane fluidity.

Spin labeling was performed according to Ligeza et al. (1998). Briefly, 15 μl of 2 mM chloroform solution of n-doxylstearic acid was dried on the bottom of a 0.5 ml Eppendorf tube. 300 μl of thylakoid membrane suspension was added and vortexed for 15 min at room temperature. After that, the sample was centrifuged for 5 min in Eppendorf AG centrifuge at 14,500 × *g*. The pellet was suspended to chl concentration of 1 mg/ml and left on ice until measurement.

EPR measurements and oxygen transport parameter calculation

Physical properties of thylakoid membranes isolated from barley leaves were investigated by electron paramagnetic resonance (EPR)-spin labeling. Two stearic acid spin labels 5-SASL and 16-SASL that monitor molecular dynamics at different depths of the membrane were used. 5-SASL has a nitroxide group (N—O•) localized close to the headgroup region of the membrane, while the N—O• group of 16-SASL localizes in the membrane center. The suspension of thylakoids in a gas permeable capillary made of TPX was positioned inside the resonator. The samples were equilibrated either with air or with nitrogen gas, which was also used for temperature control. EPR spectra were recorded at physiological range of temperatures in order to see if the changes in the membrane fluidity are identical for all temperatures and the outermost splitting parameter $2A_{II}'$ was measured for 5-SASL (Fig. 1) while rotational correlation times τ_{2B} and τ_{2C} were calculated for 16-SASL as described by Berliner (Berliner and Reuben, 1989). Both parameters reflect the local fluidity of the membrane: their smaller values indicate the greater motional freedom of the spin-labeled fatty acids in the thylakoid membrane and its higher fluidity. Oxygen transport parameter (W) was obtained from power saturation curves of 5 and 16-SASL recorded in the presence and absence of molecular oxygen at 25 °C, using an analogous procedure as described in (Wisniewska and Subczynski, 1998) for $Fe(CN)_6^{3+}$ accessibility parameter. An experimental power saturation curve, which is the plot of the EPR signal amplitude (Y') versus the square root of incident microwave power ($\sqrt{P}$) was fitted to the