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Structural and biochemical response of chloroplasts in tolerant and sensitive barley genotypes to drought stress

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ABSTRACT

The aim of this research was to characterize the changes of structural organization of chloroplasts of sensitive (Maresi) and tolerant (Cam/B1) barley genotypes upon soil drought (10 days), which was applied in two stages of plant growth, i.e. seedlings and flag leaves. The electron paramagnetic resonance (EPR) technique was used for the determination of changes in the concentration and nature of long-lived radicals and metal ions (Mn, Fe), measured directly in the structures of fresh leaves, occurring after stress treatment. Stronger variations of EPR parameters were found after drought stress application in the flag-leaf phase and for sensitive genotype. Chloroplasts of Cam/B1 were characterized by a larger surface area and less degradation of their structure during drought stress in comparison to Maresi. The data obtained from Raman spectra showed that better stress tolerance of the genotype was accompanied by greater accumulation of carotenoids in chloroplasts and was correlated with an increase in carotenoid radicals. The increase of the value of the electrokinetic potential (relative to control), which was slightly larger for the chloroplasts of Maresi than of Cam/B1, indicated the chemical reconstruction of the membrane leading to a reduction of their polarity during drought action.

1. Introduction

In spite of numerous articles describing the physiological and biochemical factors involved in the mechanism of plants' response to drought stress, this very important environmental phenomenon, resulting in a potential diminishing of the yield, is still not fully recognized. Many cellular biochemical processes are engaged in the regulation of photosynthesis and maintaining the redox homeostasis (Pinheiro and Chaves, 2011). It was suggested that under water deficit (Chaves et al., 2009; Farooq et al., 2009) and other stresses (Filek et al., 2010b; Romero-Puertas et al., 2004), alterations in photosynthesis may occur as the first symptoms of stress action. These effects are the results of reducing the light absorption by photosynthetically active centers caused by damage of photosynthetic systems PSI and PSII by reactive oxygen species (ROS) (Geissler et al., 2009; Pinheiro and Chaves, 2011; Zhang et al., 2011). This process led to a decrease in the efficiency of chloroplasts and changes in their structures (Filek et al., 2010b).

During environmental stresses, enhanced formation of ROS is considered the main factor responsible for the oxidation of the most significant biological compounds, leading to disruption of mechanisms of physiological processes and the reorganization of cell structure (Acharya and Assmann, 2009). It has been shown that excessive ROS generation is facilitated by the presence of transition metal ions, mainly Fe and Cu (Labanowska et al., 2016a,b; Stohs and Bagchi, 1995). The mechanism of their action was based on the Fenton-like reaction of superoxide and hydroxyl radicals' formation. Our earlier works (Labanowska et al., 2013a,b, 2014, 2016a) showed that, in plant materials with higher concentrations of iron and copper ions, the amount of stable radicals such as tyrosyl, semiquinone and carbon center radicals was higher and increased under oxidative stress (Filek et al., 2010b, 2015; Labanowska et al., 2013a), stronger in sensitive genotypes of plants (Labanowska et al., 2012b). The proposed mechanism of stable radicals' creation assumed the stabilization of unpaired electrons originating from ROS, in organic matrix (Labanowska et al., 2012a, 2013a, 2014, 2016a). Bou-Abdallah (2010) found that oxidation of

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Fe(II) by O_2 to Fe(III) – O – Fe(III) in plant tissues led to the formation of protein radicals on the tyrosine molecule. In turn, copper ions play an important role in establishing hydroquinone – benzoquinone redox balance (Stohs and Bagchi, 1995) and can influence the formation of semiquinone radicals (Kaim, 2003). On the other hand, transition metal ions (mainly Mn, Cu and Fe) bound in protein complexes of antioxidative enzymes, present in chloroplasts and engaged in photosynthesis mechanism, are also involved in defense mechanisms against ROS excess, which is generated under oxidative stress. Considerable attention has also been directed to carotenoids (Li et al., 2008), active in protection against photooxidative damage in photosynthesis (Lu and Li, 2008). The final amount of ROS in cells is determined by the equilibrium between their production and scavenging reactions.

Our previous EPR studies (Filek et al., 2015) performed on barley plants exposed to drought stress allowed characterization of the differences between sensitive and tolerant genotypes in the creation of long-living radicals. However, EPR measurements were carried out on lyophilized samples, which disturbed water relations in plants, and moreover, during milling of lyophilized leaves, mechanically induced free radicals could be formed (Dyrek et al., 2013; Kuzuya et al., 1999). Therefore, the present work was performed on fresh leaves of plants in two developmental stages (seedlings and flag leaves) of control and drought stressed barley. This technique proved to be suitable for differentiating between leaves originating from tolerant and sensitive genotypes. Moreover, EPR spectroscopy allows the investigation of plant material without preliminary biochemical preparation of chloroplasts. Although EPR is limited to systems containing unpaired electrons, it can be applied to examine biological tissues because they contain various paramagnetic centers such as transition metal ions (Mn, Fe and Cu) occurring, for example, in enzymes and other biological structures. Furthermore, during life processes, numerous radicals are formed. Analysis of EPR spectra provides valuable information about the kind and chemical character of paramagnetic centers, their surroundings and interactions between them. In addition to qualitative information, quantitative data on the paramagnetic centers concentrations can also be obtained from the spectra. The EPR technique shows high sensitivity and its non-destructive character permits *in vivo* study. Low concentrations and short lifetimes of some radicals, for example ROS, limit the application of this method. However, this can be overcome by multiple scanning of the sample and by using the spin-trapping method, respectively (Miller and Brudvig, 1991; Spasojević et al., 2011; Zhang et al., 2003). The small number of paramagnetic centers and the presence of water, with high dielectric constant, in biological samples, obstruct measurements. In this case, the application of lower frequencies makes it possible to record samples of larger volume, but at the same time, the resolution of the spectra decreases. Refrigeration or lyophilization of biological sample is then used.

Raman spectroscopy, similarly as the EPR technique, was used to study the subtle changes in concentration of carotenoids, localized in chloroplast membranes. In addition, chloroplast structures and their modifications under drought conditions were determined on the basis of microscopic observations and electrokinetic potential measurements. Changes of electrokinetic potential of chloroplasts, as the indicator of the reorganization of membrane lipids after stress action were registered in our earlier work (Filek et al., 2010a).

2. Materials and methods

2.1. Plant material

Two barley genotypes: drought-resistant Cam/B1 (Cam/B1//CI008887/CI05761, 2-line) from Syria (ICARDA International Centre for Agricultural Research in the Dry Areas, Aleppo) and drought-sensitive Maresi (German genotype, 2-line, from Gene Bank in Prague, Czech Republic) were cultured under conditions described in detail previously (Filek et al., 2015). Both genotypes are presently in the collection of the Institute of Plant Genetics (Poznan, Poland) and difference in their tolerance to the drought stress was confirmed by measurements of chlorophyll fluorescence. Kalaji et al. (2016) claims that this method gives detailed information on the status and function of Photosystem II reaction centers and can be a valuable means of analyses of photosynthetic responses to environmental stresses. It was found that for investigated two barley cultivars after 10 days of drought all fluorescence parameters were lower for sensitive genotype than for tolerant one (Filek et al., 2015). As shown by Oukarroum et al. (2007), DFI parameter, a measure of stress induced changes in the photosystem PSII activity, which can be found from the chlorophyll fluorescence data, was also higher for the tolerant genotype of barley. The studies performed on leaves originating from drought-tolerant (Cam/B1) and drought-sensitive (Maresi) barley genotypes indicated an increase of amount of associated with chloroplasts actin filaments and essential changes in their structure induced by drought stress mainly in stress tolerant cultivars (Sniegowska-Swierk et al., 2015). According to authors, the various responses of both genotypes to drought stress might result from their specific genetic differences.

After sterilization, seeds were sown in the pots with a capacity of 9 dm^3 and filled with a mixture of soil (SUBSTRAT Natural; SCOTTS, Poland) and sand (7:2, w/w) at a 16-h photoperiod, an irradiance of 650 μmol (photon) m^{-2} s^{-1} (provided by high pressure sodium lamps, 400 W; Philips SON-T AGRO, Brussels, Belgium), at 50% air humidity, in an air-conditioned greenhouse. Initially, 15 seedlings were placed in pots, and after germination (4 days), the number of plants was reduced to 10. The temperature of growth was maintained at 20/17°C (day/night) until the phase of stem elongation. The humidity of the substrate in the pots was determined by monitoring the weight (based on the water retention curve) and was stabilized to 8% water content of the dry weight of the soil (i.e. 3.2 pF). The drought stress (3.65%, the value corresponding to 4.0 pF) was applied in two phases of the plant growth i.e. in seedling stage (after the appearance of the 4th leaf) and in flag-leaf stage (after the appearance of flag leaf) and was continued for 10 days. Control plants were cultivated with 10% water content. The experiment was repeated three times and plants from 5 pots (for all treatments) were used to analysis. Analyses were performed on the 2nd leaf (the seedlings stage) and on the flag leaf (the flag-leaf stage) for both control and drought stressed plants.

EPR spectroscopy measurements were performed on appropriate leaves immediately after their collection. EPR spectra were also recorded for thylakoid membranes, β-carotene samples (Sigma Aldrich) and ferritin (Sigma Aldrich).

2.2. Microscopic observation

The material (leaves) was fixed in Carnoy solution (100% ethanol:glacial acetic acid; 3:1 v:V). Samples, after washing and dehydrating in ethyl alcohol of increasing concentration, were saturated with LR GOLD resin (Fluka LR Gold embedding kit for microscopy). Resin hardening material was cut with ultramicroton LEICA (Ultracut UCT) into sections having a thickness of 1500 nm. The sections were stained on hot with methylene blue for 10 min. They were flushed several times with distilled water, and then sealed in DPX Mountant for histology. The observation of preparations and documentation of results was performed with a BX50 microscope (Olympus) with NIS Elements AR 3.00 NIKON software, which was used to quantify the number of chloroplasts per cell and their surface area.