Changes of Redox Activity during the Development of Rape

Maria Filek[a,b,*], Magdalena Mirek[a], and Monika Długołecka[a]

[a] Institute of Plant Physiology, Polish Academy of Sciences, 30-239 Kraków, Niezapomisajek 21, Poland. Fax: +48 12 425 33 20. E-mail: mariafilek@excite.com
[b] Institute of Botany, Pedagogical Academy, 31-054 Kraków, Podbrzezie 3, Poland

* Author for correspondence and reprint requests

Z. Naturforsch. **61 c**, 548–552 (2006); received January 2/February 12, 2006

Redox activity was measured in vegetative and generative apical parts (5 mm of the stem) and youngest leaves of winter (cv. "Górczański") and spring (cv. "Młochowski") rape. Both genotypes were cultured under the same growth conditions (17/15 °C day/night, 16 h photoperiod), but winter rape was additionally vernalized (5/2 °C day/night, 56 days) in order to induce the generative development. The cyclic voltammetric method was used to measure the redox potential of samples in the presence of Fe^{3+} ions. Changes in the redox activity were compared with changes in riboflavin content and activities of antioxidative enzymes: superoxide dismutase (SOD) and catalase (CAT). The higher level of Fe^{3+} ions and riboflavin detected in generative apices and leaves of winter and spring varieties indicated that electrons (and their donors) were present at a lower level in these organs in comparison with the vegetative ones. On the contrary, SOD and CAT activity were lower in generative than in vegetative organs. This confirms changes in the redox balance and involvement of oxygen radicals in the generative development of rape plants. The similarity of the measured parameters between winter and spring varieties indicates that the observed changes are independent of the way of generative induction (vernalization). Riboflavin can serve as one of the electron carriers between other oxidation-reduction substances.

Key words: Redox Activity, Generative Development, Rape

Introduction

Oxidation and reduction (redox) are chemical processes by which a change in the oxidation numbers of a chemical element occurs. In cell signaling the term redox potential is introduced to describe a regulatory process in which the signal is delivered through redox reactions.

Redox signaling is used by a wide range of organisms to induce protective responses against oxidative damage and to reset the original state of "redox homeostasis" after temporary exposure to reactive oxygen substrate (ROS) (Dröge, 2002). However, a substantial body of evidence exists now which shows that living organisms have not only adapted to an unfriendly coexistence with free radicals, but have, in fact, developed mechanisms for the advantageous use of free radicals (Smith *et al*., 1996; Dröge, 2002).

In plants, chloroplast genome expression is regulated by redox reactions (Allen, 1994; Baena-González *et al.*, 2001). Also, transcriptional factors from viral, bacterial, and mammalian sources are known to be under redox control (Allen, 1993). Moreover, the influence of membrane potential on voltage-gated Ca^{2+} channels is well known in many plant cells (Bush, 1995), particularly with regard to cell growth and development. Especially, the generative development seems to be controlled by redox activity because of a possibility of manipulation of this process by external electric field application. The inhibitory effect of the electric current on flower induction was observed in the long-day plant spinach (Montovon and Greppin, 1986), short-day plant *Chenopodium rubrum* (Adamec *et al*., 1989) and also for grafted rape plants (Filek *et al*., 2003), but only when the cathode was connected to the leaves (or shoots) and the anode to the roots. The opposite electric field orientation increased the percentage of flowering plants in partly vernalized winter wheat (Filek *et al*., 2002). Thus, the effect of electric current on the stimulation/inhibition of the generative development seems to be connected with transport (or synthesis) molecules carrying a charge and the influence on changes in redox homeostasis.

Free radicals and reactive non-radical species derived from radicals exist in biological cells and tissues at low but measurable concentrations (Sies, 1993). Mechanisms of redox homeostasis depend on the balance between ROS production and vari-

ous types of scavengers. Halliwell and Gutteridge (1989) have defined antioxidants as substances that are able, at relatively low concentrations, to compete with other oxidizable substrates and, thus, to significantly delay or inhibit the oxidation of these substrates. Certain antioxidative enzymes, including especially superoxide dismutase (SOD) and catalase (CAT), are potent ROS scavengers (Dröge, 2002). Moreover, flavin coenzymes participate in redox reactions in numerous metabolic pathways (McCormick, 1999). Flavins are critical for the metabolism through their ability to catalyze two-electron dehydrogenations of numerous substrates and to participate in one-electron transfers to various metal centres through their free radical states (Massey, 2000). Among flavins, riboflavin is crucial for the production of biological energy and can work as an antioxidant in conjunction with antioxidative enzymes (Massey, 2000).

The aim of the presented study was to determine the redox activity of vegetative and generative apices and leaves of winter rape. Winter rape's generative development is connected with periodic cooling (vernalization) and thus the inclusion of oxidative stress in this process is expected (Mahan and Mauget, 2005). Generative organs (leaves, bud formation) appear usually at a higher temperature, at about 3 weeks after rape vernalization (under controlled growth conditions). Under these conditions it seems possible to observe a new redox state established in generative plant organs. The redox activity of vegetative and generative rape plants was compared with the presence of the riboflavin oxidized form and with changes in the activity of antioxidative enzymes: SOD and CAT. Non-vernalized spring rape was introduced as a control to separate effects connected with the generative development and cooling.

Materials and Methods

Plants

Winter rape seeds cv. "Górczański", after sterilization, were placed in pots containing a mixture of peat, soil and sand in the ratio 1:1:1, and transferred into a conditioned chamber at the temperature of 17/15 °C day/night (16 h day). The plants were grown under these conditions for about 4 weeks, until they reached the stage of 5-leaf rosettes – the vegetative developmental stage which is optimal for vernalization of winter rape. Some of the plants were vernalized for 8 weeks at the temperature of 5/2 °C day/night (16 h day). After vernalization, plants were transferred back to the temperature of 17/15 °C day/night (16 h day) and grown until the stem elongation occurred and generative leaves appeared.

Spring rape seeds cv. "Młochowski" were cultured at the temperature of 17/15 °C day/night (16 h day) in order to obtain vegetative and generative plants. The youngest leaves and apices (5 mm of the apical part of the stem, without leaves) were cut off from vegetative and generative plants of winter and spring rape and frozen at −70 °C.

Redox activity measurements

In order to detect redox activity samples were homogenized in a buffer containing 1 mM Tris-HCl [tris(hydroxymethyl)aminomethane hydrochloride], 0.5 mM $CaCl_2$, 50 mM KCl. The redox reaction was initiated by the introduction of 1 mM $K_3[Fe(CN)_6]$ (Federico and Giartosio, 1983; Berovic *et al.*, 2000). 20 μl samples were injected into a HPLC system (HPLC/ED, 5040 Cell, Beckman Instruments INC, Fullerton, CA, USA). The oxygen was purged from each solution with N_2 before recording the scan. The cyclic voltammety studies (System Gold, 125 NM Solvent Module) were performed to measure the redox potential of molecules in the solution. The potential between a working electrode (Au) and a reference electrode (Pd) varied linearly from the experimentally defined initial value −800 mV to +1600 mV and then back again when a cyclic voltammogram [current (I) versus potential (V)] was recorded. The Au electrode was cleaned using the polishing kit before every experiment. The quality of the Au electrode was controlled by cyclic voltammetry in 0.1 M H_2SO_4 (Oslonovitch *et al.*, 2003). Potassium ferrocyanide was used as a standard for voltage/concentration calibration (Chevion *et al.*, 2000). Changes in the redox activity of samples were calculated from calibration curves as changes in the Fe^{3+} concentration.

Determination of riboflavin content

Flavins were extracted by the procedure of Geoghegan *et al.* (2000). The buffer included 50 mM potassium phosphate, pH 7, and 0.3 mM EDTA. The flavin extract was purified by column chromatography on a Sephadex G-50 column (90 × 2.5 cm diameter). Oxidized forms were detected spectrophotometrically at λ = 480 nm (Curley *et al.*, 1991).

Activity of antioxidative enzymes

For superoxide dismutase (SOD) and catalase (CAT) activity measurements samples were homogenized at 4 °C with 50 mM KP (K_2HPO_4 + KH_2PO_4) buffer and 0.1 mM EDTA (pH 7). After centrifugation, at 10 000 × *g* for 15 min, the supernatant was dialyzed for 24 h.

The activity of CAT was measured spectrophotometrically (λ = 240 nm) by a modified method of Aebi (1984). The reaction mixture was initiated by 0.03 M solution of H_2O_2 in phosphate buffer (0.05 M KP + EDTA, pH 7.0). The kinetics of enzyme reactions was examined after 60 s and 120 s using KINLAB (Perkin-Elmer Corp., Norwalk, CT, USA) software.

The activity of SOD was measured at λ = 595 nm by the modified method of McCord and Fridovich (1969). One unit was defined as the amount of enzyme necessary for 50% inhibition of cytochrome c in a coupled system with xanthine and xanthine oxidase. The reaction kinetics for the enzyme was examined 60 s and 120 s after the initiation of the reaction using KINLAB (Perkin-Elmer Corp.) software.

Proteins were determined according to Bradford (1976), using bovine serum albumin as a standard.

Results and Discussion

Measurements of Fe^{3+}-induced redox activity were performed in order to estimate if the generative development of rape is connected with changes of oxidized and reduced molecules. It was found that the addition of 1 mM $K_3[Fe(CN)_6]$ to the samples induced higher reduction (lower level of Fe^{3+} ions) in apices and leaves of vegetative plants in comparison with the generative ones (Fig. 1A). This effect was detected in both winter and spring varieties. The higher reduction of ferric ions indicates a higher level of electrons in vegetative organs. This was confirmed by measurements of the riboflavin content (Fig. 1B). The lower level of riboflavin detected in vegetative apices and leaves points to the reaction sequence involving the reduction of riboflavin to dihydroriboflavin. Two electrons and two hydrogen atoms are transferred to the isoalloxazine ring system during this process.

The most important adsorbent of electrons in plant cells is oxygen. Plants have evolved a number of mechanisms to protect themselves against

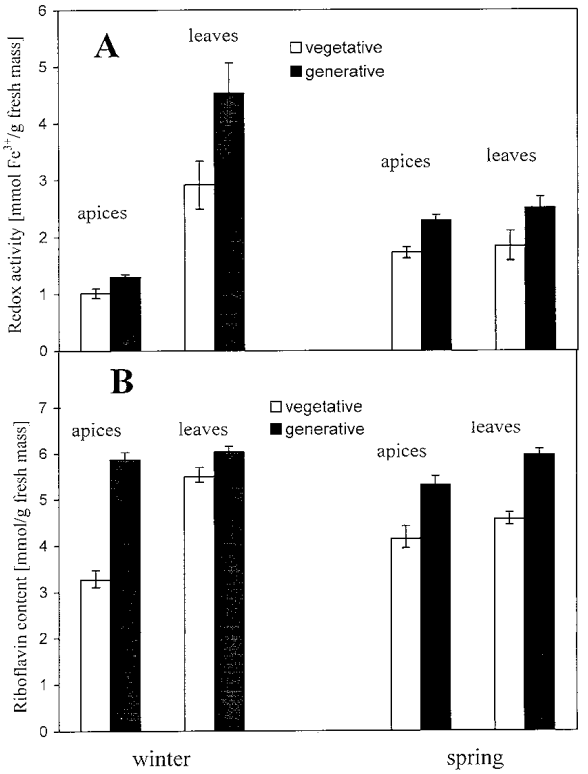

Fig. 1. Redox acivity calculated as Fe^{3+} content (A) and riboflavin content (B) for vegetative and generative apices and leaves of winter and spring rape. For details see Materials and Methods. Values represent averages from 5 replicates ± SE.

oxygen radicals ($O_2^{\bullet -}$ and H_2O_2) (Noctor and Foyer, 1998; Dröge, 2002). Superoxide dismutase (SOD) is one of the most important enzymes, which catalyzes the dismutation of $O_2^{\bullet -}$ to H_2O_2. In organisms, H_2O_2 can be destroyed by catalase (CAT). It is assumed that an increase in antioxidant enzymes activity informs about an increase in oxygen radicals concentration. In the presented studies measurements of the superoxide enzymes SOD and CAT showed a higher level of their activity in vegetative apices and leaves of both winter and spring organs in comparison with the generative ones (Figs. 2A and B). This confirms a higher level of electrons in vegetative organs of rape plants in comparison with the generative ones.

The presence of the higher level of electrons in vegetative organs of both winter and spring rape suggests that this developmental stage is connected with more negative polarization of organs

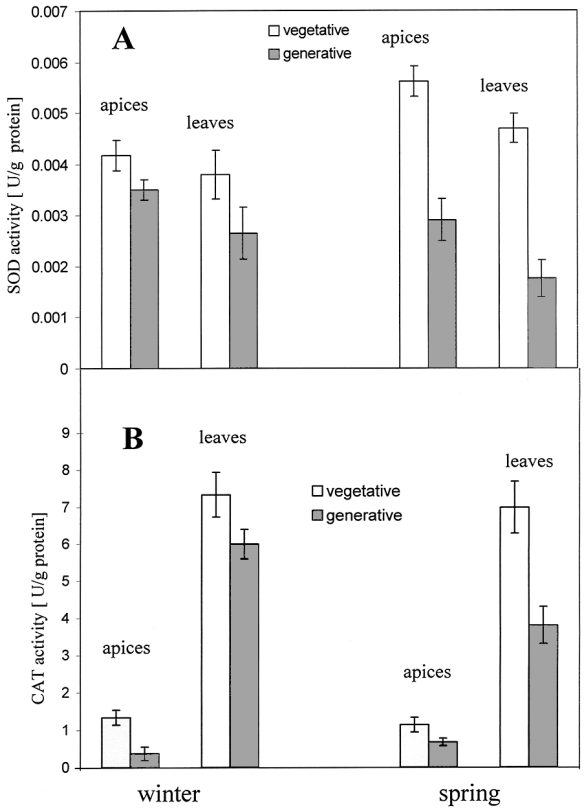

Fig. 2. Superoxide dismutase (SOD) activity (A) and catalase (CAT) activity (B) for vegetative and generative apices and leaves of winter and spring rape. For details see Materials and Methods. Values represent averages from 5 replicates ± SE.

(accumulation of negatively charged molecules) than it appears in the generative stage. On the basis of electric field induction/blocking of rape flowering it was expected that generative organs are more negatively charged than the vegetative ones: a significant stimulation of the generative development was obtained after the introduction of a positive electrode to apices (Filek *et al.*, 2002, 2003). However, it can be assumed that this increase in negative polarization of vegetative organs is necessary only in the first stage of the generative induction. Such trigger of ROS production in cells during the generative induction can mediate a positive feedback effect in signal transduction since intracellular signaling is often enhanced by ROS or by a pro-oxidative shift of the intracellular redox state (Sundaresan *et al.*, 1995). The molecular details of the oxidative enhancement are not entirely clear. However, the work on different signaling pathways revealed certain consistent patterns that have important physiological implications.

ROS have traditionally been considered to be toxic to lipid peroxidation or oxidative DNA degradation products (Marrs, 1996). However, in recent years, it has become apparent that plant cells generate low levels of ROS endogenously in response to a number of biotic and abiotic stresses (Lamb and Dixon, 1997; Wohlgemut *et al.*, 2002). It was confirmed that increased accumulation of H_2O_2 and changes in the redox status alert the plant cell to an environmental change (Noctor and Foyer, 1998; Foyer and Noctor, 2003). Oxidative stress can therefore be defined as a disruption of the cellular redox balance. Practically, all cells and tissues convert continuously a small proportion of oxygen into superoxide. During normal metabolism, active oxygen species are generated as a side product in electron transport processes, such as photosynthesis and respiration. At low reaction rates ROS are capable of degrading of cytokinins (Lo *et al.*, 1996; Frebortova *et al.,* 2004), important hormones for cell differentiation and also participate in redox regulation of genes (Deora and Lander, 2000). Thus, they could also participate in the induction of the generative development. However, during the generative development a high level of ROS could have a destructive influence on cell metabolism. The decrease in SOD and CAT observed in fully developed generative apices and leaves suggests a decrease in ROS present in this developmental stage. Riboflavin can play a role of a mediator in the establishment of a new redox balance in generative organs. Riboflavin as a precursor to flavin nucleotides (FMN, FAD) is involved in energy metabolism through redox reactions (Massey, 2000). The higher level of riboflavin observed in generative organs (Fig. 1A) can be connected with a decrease in its reduction to dihydroriboflavin because of lower electron (and/or other charges) concentration in generative organs. These changes in redox parameters had a similar direction for both winter and spring rape organs. Thus it seems that a decrease in reduction is characteristic for the generative development of rape plants independently of the way of the generative induction.

Adamec L., Macháčková I., Krekule J., and Nováková M. (1989), Electric current inhibits flowering in short-day plant *Chenopodium rubrum* L. J. Plant Physiol. **134**, 43–46.

Aebi H. (1984), Catalase *in vitro*. Methods Enzymol. **105**, 121–125.

Allen J. F. (1993), Redox control of transcription sensors, response regulators, activators and repressors. FEBS Lett. **332**, 203–207.

Allen J. F. (1994), Redox control of gene expression and the function of chloroplast genomes: a hypothesis. Photosynt Res. **36**, 95–102.

Baena-González E., Baginsky S., Mulo P., Summer H., Aro E. M., and Link G. (2001), Chloroplast transcription at different light intensities. Glutathione-mediated phosphorylation of the major RNA polymerase involved in redox-regulated organellar gene expression. Plant Physiol. **127**, 1044–1052.

Berovic M., Rošelj M., and Wondra M. (2000), Possibilities of redox potential regulation in submerged citric acid bioprocessing on beet molasses substrate. Food Technol. Biotechnol. **38**, 193–201.

Bradford M. (1976), A rapid and sensitive method for the quantitation and sensitivity of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem. **72**, 248–254.

Bush D. S. (1995), Calcium regulation in plant cells and its role in signalling. Annu. Rev. Plant Physiol. Plant Mol. Biol. **46**, 95–122.

Chevion S., Roberts M. A., and Chevion M. (2000), The use of cyclic voltammetry for the evaluation of antioxidant capacity. Free Radic. Biol. Med. **28**, 860–870.

Curley G. P., Carr M. C., Mayhew S. G., and Voordouw G. (1991), Redox and flavin-binding properties of recombinant flavodoxin from *Desulfovibrio vulgaris* (Hildenborough). Eur J. Biochem. **202**, 1091–1100.

Deora A. A. and Lander H. M. (2000), Regulation of signal transduction and gene expression by reactive nitrogen species. In: Antioxidant and Redox Regulation of Genes (Sen C. K., Sies H., and Baeuerle P. A., eds.). Academic Press, Orlando, FL, pp. 147–178.

Dröge W. (2002), Free radicals in the physiological control of cell function. Physiol. Rev. **82**, 47–95.

Federico R. and Giartosio C. E. (1983), A transmembrane electron transport system in maize roots. Plant Physiol. **73**, 182–184.

Filek M., Biesaga-Kościelniak J., Marcińska I., Krekule J., and Macháčková I. (2002), Direct electric current partly replaces the chilling effect in vernalisation of winter wheat. J. Plant Physiol. **159**, 795–797.

Filek M., Biesaga-Kościelniak J., Marcińska I., Krekule J., Macháčková I., and Dubert F. (2003), The effect of electric current on flowering of grafted scions of non-vernalized winter rape. Biol. Plant. **46**, 625–628.

Foyer C. H. and Noctor G. (2003), Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. Physiol. Plant. **119**, 355–364.

Frebortova J., Fraaije M. W., Galuszka P., Sebela M., Pec P., Hrbac J., Novak O., Bilyeu K. D., English J. T., and Frebot I. (2004), Catalytic reaction of cytokinin dehydrogenase: preference for quinones as electron acceptors. Biochem. J. **380**, 121–130.

Geoghegan S. M., Mayhew S. G., Yalloway G. N., and Butler G. (2000), Cloning, sequencing and expression of the gene for flavodoxin from *Megasphaera elsdenii* and the effects of removing the protein negative charge that is closest to N(1) of the bound FMN. Eur. J. Biochem. **267**, 4434–4444.

Halliwell B. and Gutteridge J. M. C. (1989), Free Radicals in Biology and Medicine, 2nd ed. Clarendon, Oxford, UK.

Lamb C. and Dixon R. A. (1997), The oxidative burst in plant disease resistance. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48**, 251–275.

Lo Y. Y. C., Wong J. M. S., and Cruz T. F. (1996), Reactive oxygen species mediate cytokine activation of c-Jun NH_2-terminal kinases. J. Biol. Chem. **271**, 15703–15707.

Mahan J. R. and Mauget S. A. (2005), Antioxidant metabolism in cotton seedlings exposed to temperature stress in the field. Crop Sci. **45**, 2337–2345.

Marrs K. (1996), The functions and regulation of glutathione S-transferases in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **47**, 127–158.

Massey V. (2000), The chemical and biological versatility of riboflavin. Biochem. Soc. Trans. **28**, 283–296.

McCord J. M. and Fridovich I. (1969), Superoxide dismutase, an enzymic function for erytrocuperein (hemocuperein). J. Biol. Chem. **244**, 6049–6055.

McCormick D. B. (1999), Riboflavin. In: Nutrition in Health and Disease (Shils M., Olson J. A., Shike M., and Ross A. C., eds.). Williams & Wilkins, Baltimore, pp. 391–399.

Montovon M. and Greppin H. (1986), Dévelopment apcal de l'épinard et l'application d'un potential électrique de contrainte. Saussurea (Genéve) **17**, 85–91.

Noctor G. and Foyer C. H. (1998), Ascorbate and glutathione: keeping active oxygen under control. Annu. Rev. Plant Physiol. Plant Mol. Biol. **49**, 249–279.

Oslonovitch J., Li Y.-J., Donner C., and Krischer K. (2003), The $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ charge transfer reaction on Au(111) revisited in the presence and absence of a two-dimensional, condensed organic film. J. Electroanal. Chem. **541**, 163–174.

Sies H. (1993), Strategies of antioxidant defense. Eur. J. Biochem. **215**, 213–219.

Smith C. E., Ruttledge T., Zeng Z., O'Malley R. C., and Lynn D. G. (1996), A mechanism for inducing plant development: The genesis of a specific inhibitor. Proc. Natl. Acad. Sci. USA **93**, 6986–6991.

Sundaresan M., Yu Z.-X., Ferrans V. J., Irani K., and Finkel T. (1995), Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. Science **270**, 296–299.

Wohlgemut H., Mittlestrass K., Kschieschan S., Bender J., Weigel H.-J., Overmyer K., Kangasjärvi J., Sandermann H. J., and Langebartels C. (2002), Activation of an oxidative burst is a general feature of sensitive plants exposed to the air pollutant ozone. Plant Cell. Environ. **25**, 717–726.