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Developmental changes in energy dissipation in etiolated wheat seedlings during the greening process

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Abstract

We studied the developmental changes in photosynthetic and respiration rates and thermal dissipation processes connected with chloroplasts and mitochondria activity in etiolated wheat (*Triticum aestivum* L., var. Irgina) seedlings during the greening process. Etioplasts gradually developed into mature chloroplasts under continuous light [190 μmol(photon) m^{-2} s^{-1}] for 48 h in 5-day-dark-grown seedlings. The net photosynthetic rate of irradiated leaves became positive after 6 h of illumination and increased further. The first two hours of de-etiolation were characterized by low values of maximum (F_v/F_m) and actual photochemical efficiency of photosystem II (PSII) and by a coefficient of photochemical quenching in leaves. F_v/F_m reached 0.8 by the end of 24 h-light period. During greening, energy-dependent component of nonphotochemical quenching of chlorophyll fluorescence, violaxanthin cycle (VXC) operation, and lipoperoxidation activity changed in a similar way. Values of these parameters were the highest at the later phase of de-etiolation (4–12 h of illumination). The respiration rate increased significantly after 2 h of greening and it was the highest after 4–6 h of illumination. It was caused by an increase in alternative respiration (AP) capacity. The strong, positive linear correlation was revealed between AP capacity and heat production in greening tissues. These results indicated that VXC in chloroplasts and AP in mitochondria were intensified as energy-dissipating systems at the later stage of greening (after 4 h), when most of prolamellar bodies converted into thylakoids, and they showed the greatest activity until the photosynthetic machinery was almost completely developed.

Additional key words: alternative respiration; energy dissipation; greening; violaxanthin cycle; wheat.

Introduction

Light is the source of energy and the dominant signalling input for photosynthetic organisms. At present, it is clear that under nonlimiting light conditions plants gain far more energy than they can utilize for photosynthetic CO_2 fixation (Noguchi and Yoshida 2008, Wilhelm and Selmar 2011). Moreover, light stress is thought to be a normal condition for photosynthesis in plants (Wilhelm and Selmar 2011). Excessive light energy induces generation of reactive oxygen species (ROS). Most of ROS produced originate from chloroplasts, especially under light (Foyer and Noctor 2003). ROS are also generated in peroxisomes and mitochondria (Foyer and Noctor 2003, Navrot *et al.* 2007). ROS can act as signalling molecules, especially in response to various stresses (Gechev *et al.* 2006). On the other hand, surplus of ROS is harmful because of their detrimental effect on

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Abbreviations: AL – actinic light; AP – alternative pathway of respiration; Ax – antheraxanthin; Car – carotenoids; Ch – chloroplast; Chl – chlorophyll; Chlide – chlorophyllide; CP – cytochrome pathway of respiration; DM – dry mass; ETC – electron transport chain; F_t, F_o', and F_m – stationary, minimal, and maximal fluorescence in leaves adapted to the actinic light [190 μmol(photon) m^{-2} s^{-1}, PAR]; FM – fresh mass; F_v/F_m – maximum photochemical efficiency of PSII; lut – lutein; LPA – lipoperoxidation activity; M – mitochondrion; NPQ – nonphotochemical quenching; Nx – neoxanthin; PAR – photosynthetically active radiation; PLB – prolamellar body; P_N – net photosynthetic rate; Pchlide – protochlorophyllide; PSII – photosystem II; q – rate of heat production; q_E – energy-dependent component of nonphotochemical fluorescence quenching; q_N and q_P – coefficients of nonphotochemical and photochemical quenching, respectively; R_D – dark respiration measured as CO_2 emission rate; ROS – reactive oxygen species; SHAM – salicylhydroxamic acid; TBARS – thiobarbituric acid-reactive-substances; TCA – tricarboxylic acid cycle; V_{alt} and V_{cyt} – capacity of alternative and cytochrome pathway of respiration, respectively; V_t – total respiration measured as O_2 uptake rate; VDE – violaxanthin de-epoxidase; Vx – violaxanthin; VXC – violaxanthin cycle; Φ_{PSII} – actual photochemical efficiency of PSII; Zx – zeaxanthin; β-Car – β-carotene.
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all important macromolecules: proteins, pigments, lipids, and nucleotides. The excess of ROS may affect cell structures, due to their free diffusion through the cellular compartments (Sweetlove *et al.* 2002) including chloroplast envelope membranes (Mubarakshina *et al.* 2010). Plants developed protection and repair mechanisms to prevent photooxidative damage and to maintain physiological state of cell metabolism. Among them, energy-dissipating systems functioning in chloroplasts and mitochondria play a crucial role in the defense against excessive light energy.

In chloroplasts, thermal dissipation of light energy prevents triplet Chl formation. Under excessive light, up to 90% of the absorbed light can be dissipated *via* the nonphotochemical quenching (NPQ) pathway within the light-harvesting Chl-carotenoid antenna complexes of PSII – LHCII (Horton *et al.* 1994, Demmig-Adams and Adams 1996, Ruban *et al.* 2001, 2007). Several processes can contribute to NPQ, but its major fraction is dependent on the energization of the thylakoid ΔpH formed under the influence of illumination and it is called q_E (Quick and Stitt 1989, Krause and Weis 1991, Horton *et al.* 1994). q_E is also related to the xanthophyll cycle functioning (VXC) (Demmig-Adams 1990, Horton *et al.* 1994, Niyogi *et al.* 1998, Gilmore 1997, Latowski *et al.* 2004, Nowicka *et al.* 2009). VXC is the light-dependent interconversion of xanthophyll pigments: violaxanthin (Vx), antheraxanthin (Ax), and zeaxanthin (Zx). Two enzymes, violaxanthin de-epoxidase (VDE) and zeaxanthin epoxidase (ZE), are engaged in the VXC (Latowski *et al.* 2004). Localized in thylakoid lumen and activated by acidic pH, VDE catalyses the de-epoxidation of Vx to Zx. It was suggested that process of making LHCII-bound Vx available for enzymatic de-epoxidation is a process that is dependent not only on acidification but the light-intensity as well (Gruszecki *et al.* 2009). The mechanism of q_E is still under debate. Zx is thought to bind with PsbS, a subunit of PSII, accepting excitation energy from Chl and yielding in massive energy dissipation (Szabó *et al.* 2005). It cannot be excluded that the xanthophyll dependence of q_E is due to other pigment-binding proteins, probably of the Lhcb type (Chl *a/b*-binding proteins) (Bonente *et al.* 2008).

The other protective, energy-dissipating mechanism is connected with re-oxidation of the reduction equivalents (NADPH) produced in chloroplasts and exported *via* different shuttles to other cell compartments, in particular to mitochondria. The nonphosphorylating pathways (alternative oxidase pathway (AP), the type II NAD(P)H dehydrogenases and uncoupled proteins) in the respiratory electron-transport chain (ETC) are considered to be efficient dissipation systems for the reductants (Noguchi and Yoshida 2008). Among the non-phosphorylating pathways, the electron flow *via* AP has a greater impact on energy conservation and dissipation since it bypasses proton-pumping Complexes III and IV in the respiratory ETC. AP capacity may be up to 40% and even more of the total mitochondrial respiration, especially during the period of stress (Covey-Crump *et al.* 2007, Rachmilevitch *et al.* 2007, Garmash and Golovko 2009) including excessive light conditions (Kumar *et al.* 2007, Yoshida *et al.* 2008). ROS, particularly H_2O_2, formed during stress, was shown to act in a signal transduction pathway (Gechev *et al.* 2006, Møller and Sweetlove 2010) with AOX modulating the strength of the signal (Vanlerberghe *et al.* 2009, Juszczuk *et al.* 2012). At present, there is evidence for the importance of alternative respiration in optimizing photosynthesis and protection against photoinhibition (Kumar *et al.* 2007, Noguchi and Yoshida 2008, Yoshida *et al.* 2008, Dinakar *et al.* 2010). AP is thought to dissipate excess reductants produced in chloroplasts and imported from these organelles to mitochondria *via* malate/oxaloacetate shuttle (Padmasree and Raghavendra 1999).

Greening process in plant is light-induced biogenesis of plastids and maturation of thylakoid membranes in chloroplasts. This process is characterized by Chl formation (protochlorophyllide-chlorophyllide transformation), which is catalyzed by light-dependent protochlorophyllide reductase (LPOR). In seedlings grown in the dark (in angiosperms), proplastids develop into etioplasts containing prolamellar bodies (PLBs), which rapidly develop into thylakoids under light (Solymosi and Schoefs 2010). During this structural reorganization, the energy dissipation processes can intensify to protect developing seedlings from oxidative stress induced by illumination.

In the present study, we investigated the developmental changes in photosynthetic and respiration rates and thermal dissipation processes associated with chloroplasts and mitochondria activity in etiolated wheat seedlings during greening under continuous light conditions. Our goal was to find out how the activity of the energy-dissipating systems (violaxanthin cycle and alternative respiration) depends on the level of photosynthetic machinery development.

Materials and methods

Plant material and culture condition: Wheat (*Triticum aestivum* L., cv. Irgina) seeds were germinated in tap water. Three-day-old seedlings were transferred into 3-dm^3 boxes with half strength Knop medium (Polesskaya and Alekhina 1995) and they were grown in the dark for 2 d in a growth chamber (*KBWF 720*, *Binder*, Tuttlingen, Germany) at 23°C and 70% relative humidity. Thereafter, the etiolated seedlings were exposed to continuous light [190 μmol(photon) m^{-2} s^{-1}, PAR] at 21°C and allowed to green for 48 h. The nutrient solution was changed every day. The light was provided by luminescent lamps (*TL-D 30W*, *Philips*, Amsterdam, The Netherlands).

All measurements were carried out on the first leaf blade, the top segment (1.5 cm) of which was removed and the next 2–3 cm long segment was taken for the experiment. The leaves exposed to light at 0, 1, 2, 4, 6, 12, 24, and 48 h were used.

Electron microscopy: Samples were fixed in 2% glutaraldehyde for 3 h and postfixed in 1% OsO_4 for 2 h. 70 mM Na-K phosphate buffer (pH 7.4) was used to prepare fixatives and rinsing. After tissue dehydration in series of alcohol and acetone dilutions, the samples were embedded in Epon-812 resin. Ultrathin sections were cut with *BS 490Å* ultramicrotome (*Tesla*, Brno, CR). Sections were stained with uranyl acetate and lead citrate, and examined with an electron microscope (*BS 500*, *Tesla,* Brno, CR).

Pigment extraction and quantification: Leaf samples [about 0.2–0.3 g(FM)] were collected and frozen in liquid N_2. Leaf Chl and carotenoid (Car) contents were measured by *UV-1700* spectrophotometer (*Shimadzu*, Tokyo, Japan) in acetone extracts at 662 (Chl *a*), 644 (Chl *b*), and 470 nm (total Car) according to Lichtenthaler (1987). Separations and quantifications of individual Cars were done by reversed-phase high-performance liquid chromatography (HPLC) system according to Gilmore and Yamamoto (1991). The HPLC setup comprised the following equipment: *Smartline HPLC Pump 1000, Smartline UV Detector 2500, Smartline Manager 5000* (*Knauer*, Berlin-Zehlendorf, Germany), and the *Diasphere-110-C_{18}NT* column 4.0 × 250 mm, 5 μm particle size (*Biochemmack*, Moscow, Russia). The pigments were eluted for 34 min with gradient solvent systems A (acetonitrile:methanol:water, 75:12:4, v/v/v) and B (methanol:ethyl acetate, 68:32, v/v) at a flow rate of 0.033 cm^3 s^{-1}. Identification of Car was carried out by comparing the HPLC retention times with corresponding standards.

For VXC activity, plants were kept in darkness for 1 h, then exposed to white light (1,200 μmol m^{-2} s^{-1}) for 30 min and again placed in darkness for 1 h. Sets of leaves for the HPLC analysis were taken before and after the light exposure and after subsequent darkness.

Gas exchange and Chl fluorescence: CO_2-exchange was measured with an open system with *Li-7000* (*Li-COR Inc.*, Lincoln, NE, USA) infrared gas analyzer operating on a differential scheme. The middle part of leaf blade was placed into thermostated leaf chamber filled with humidified atmospheric air using a compressor. The chamber was equipped with water jackets; temperature in the chambers (21–22°C) was monitored with the thermostate *LT 300* (*LOIP*, St.-Petersburg, Russia). To determine the net photosynthetic rate (P_N), the leaf was exposed to 190 μmol(photon) m^{-2} s^{-1}. Illumination was provided by mercury lamp (*DRLF 1000W*, *Elecrosvet*, Chelyabinsk, Russia). Measurements of P_N in response to PAR were made at five PAR levels [1,000; 440, 190, 90, and 45 μmol(photon) m^{-2} s^{-1}]. The dark respiration rate (R_D) in leaves was determined as emission of CO_2 in darkness.

Chl fluorescence was measured using a portable PAM (pulse-amplitude modulation) fluorometer *PAM-2100* (*Walz*, Effeltrich, Germany) according to the Handbook of Operation 2003 (*Walz*, Effeltrich, Germany). At the beginning of measurements, stationary (F_t), minimal (F_o'), and maximal (F_m') Chl fluorescence in leaves adapted to the actinic light [190 μmol(photon) m^{-2} s^{-1}] were determined. Then, after dark incubation for 30 min, the leaves were given a saturating pulse (SP) at 4,000 μmol(photon) m^{-2} s^{-1} (1 s) and the maximum quantum yield of PSII (F_v/F_m) was measured. F_o was measured using a weak red light modulated at 600 Hz. The maximal fluorescence (F_m) was induced by a short pulse (0.8 s) of saturating light at 4,000 μmol(photon) m^{-2} s^{-1} (SP). The maximal quantum yield of PSII (F_v/F_m) was calculated as $(F_m – F_o)/F_m$. The actual photochemical efficiency of PSII (Φ_{PSII}) was determined as $(F_m' – F_t)/F_m'$. Photochemical quenching (q_P) and nonphotochemical quenching (q_N) of Chl fluorescence were calculated as $(F_m' – F_t)/(F_m' – F_o')$ and $(F_m – F_m')/(F_m – F_o')$, respectively.

For q_N induction kinetic measurements, plants were dark-adapted for 30 min, and F_o and F_m were determined. Thereafter, leaves were illuminated for 12 min by the actinic light (AL). To study the q_N induction, saturating white light pulses [4,000 μmol(photon) m^{-2} s^{-1} with duration of 1 s] were applied and F and F_m' were measured every 20 s during actinic illumination. The relaxation of NPQ data were obtained by saturating light pulses applied every 100 s during 20 min of dark incubation. Time courses for induction and relaxation of q_N were calculated as $(F_m – F_m')/(F_m – F_o)$.

Leaf respiration: O_2 uptake rate in a leaf was measured using a Clark-type oxygen, thermoelectrically controlled electrode (*Oxytherm System*, *Hansatech Inst*., Pentney, Norfolk, England) at 21°C. Measurements were carried out by suspending small leaf slices [0.015 g(FM)] in the reaction vessels of the electrode unit containing 1.5 mL HEPES buffer (50 mM, pH 7.2) in the presence of KCN (2 mM, inhibitor of cytochrome respiration, CP) to measure alternative pathway respiration (AP) capacity (V_{alt}), salicylhydroxamic acid (SHAM, inhibitor of AP; dissolved in ethanol, 3 mM) to measure CP capacity (V_{cyt}), and to measure the total respiration in the absence of any inhibitors (V_t). Optimal concentrations of specific inhibitors were determined by titration of plant tissue pieces with increasing concentrations of the inhibitors to saturate O_2 uptake (Møller *et al*. 1988). The residual respiration rate measured as O_2 uptake rate, insensitive to the both inhibitors, didn't exceed 10% of the total respiration rate.

Rate of heat production: The rate of metabolic heat production (q) of detached leaves was measured in the dark by differential scanning microcalorespirometry method (Hansen *et al.* 1994) at 20°C using *Biotest 2* (*Institute of Basic Biological Problems of Russian Academy of Sciences,* Pushchino, Moscow, Russia) working in isothermal regime (Boiko *et al.* 2009). These determinations were carried out by placing 80 to 100 mg of leaf sample into 1-cm^3 stainless steel calorimeter ampoules; setting the temperature and waiting about 20 min for steady-state rates of heat production. The rates of q were calculated as $[q_1 - (q_{H_1} + q_{H_2})/2) \times 0.22]$ and expressed as [mV g^{-1}(DM)], where q_1 was heat production of the sample, q_{H_1} and q_{H_2} were zero value of the rate of heat production before and after measurement, 0.22 μV was a calibration coefficient of the *Biotest 2*.

Lipid peroxidation: Assay of thiobarbituric acid-reactive-substances (TBARS) in plant tissues for estimation of lipid peroxidation was performed using the method of Heath and Packer (1968). Absorbance of sample fraction was read at 532 nm subsequent to subtraction of nonspecific absorption at 600 nm. The TBARS concentration [nmol g^{-1}(FM)] was calculated using the extinction coefficient 155 mM^{-1} cm^{-1}.

Statistical analysis: The presented results were the mean values with standard errors (SE) (n = 3–15) from three or four independent experiments. After checking for normal distribution of variables, data were analyzed using one way and mulivariant *ANOVA* followed by *Duncan*'s test (P<0.05). Tests were implemented using *Statistica 6.1* software (*StatSoft. Inc.*, Tulsa, OK, USA).

Results

Ultrastructure of mesophyll cells: The etioplasts of 5-day-dark-grown leaves contained regular PLBs and starch grains (Fig. 1*A*). After 1 h of illumination, the plastids contained a few thylakoid membranes, which protruded from PLBs (Fig. 1*B*). After 6 h of greening, PLBs were absent; and plastid membranes were completely reorganized into thylakoids (Fig. 1*C*). Chloroplasts still contained starch grains. Mitochondria became bigger and attained a slightly oval shape (Table 1). After 6 h of the greening process, proximity of mitochondria and chloroplasts was often observed (Fig. 1*C–E*). By the end of the 2nd day of illumination, chloroplasts had well developed grana consisting of 6.1 ± 1.7 thylakoids. By this time, starch grains were absent in the cells.

Photosynthetic pigments: Etiolated leaves contained Car [0.23 mg g^{-1}(DM)] and almost undetectable amounts of Chl (Fig. 2). During the greening process, the amount of Chl, mainly Chl *a*, increased significantly. After 48 h of greening, the concentrations of Chl and Car increased 30 and 7 times, respectively. The relative amount of Car in the total pigment pool decreased during the greening process.

HPLC analysis revealed the presence of β-carotene (β-Car), Vx, Ax, lutein (Lut), neoxanthin (Nx), and Zx in the pool of Car (Table 2). Content of β-Car, Lut, Nx, and Vx increased significantly during the greening. Concentration of Ax and Zx reached their maximum 4 h after the start of illumination. By the end of the 2nd day of continued illumination, the concentration of Zx – the key pigment of VXC cycle – decreased to the level detected in etiolated leaves. At the same time, the content of Ax as the intermediate VXC cycle pigment was 2.5 times lower than in the etiolated leaves.

To examine how VXC operates during the greening process, we exposed dark-acclimated plants to high light [1,200 μmol(photon) m^{-2} s^{-1}] and then again to darkness. HPLC separation of extracts from leaf samples taken

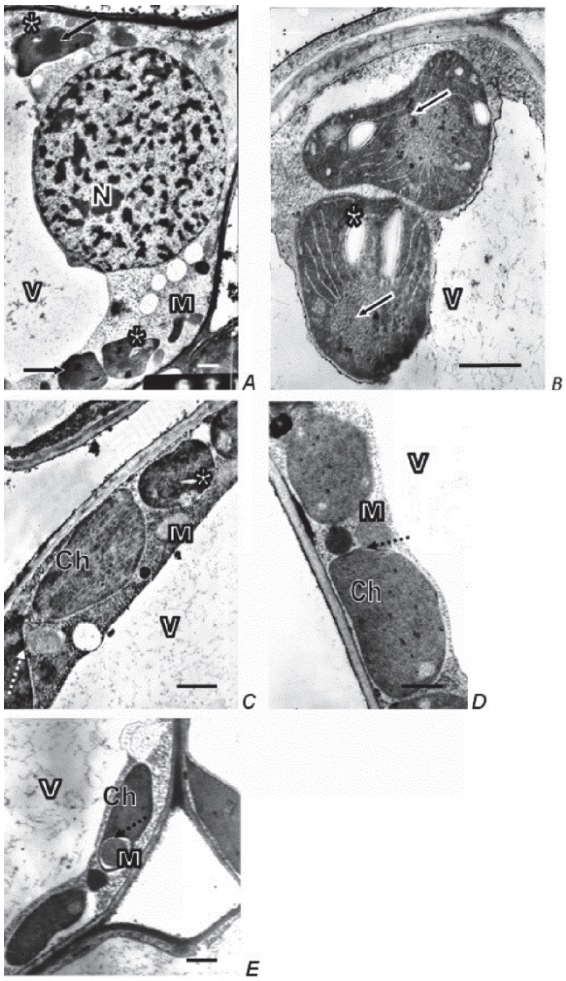

Fig. 1. Electron micrographs of mesophyll cells transverse section of etiolated leaves (*A*), and after illumination for 1 h (*B*), 6 h (*C*), 24 h (*D,E*). Ch – chloroplast, M– mitochondrion, N – nucleus, V – vacuole; (*) – starch grain. *The arrows* indicate prolamellar body. *The discontinuous arrows* indicate zones of close contact between Ch and M. Bars – 1 μm.

Table 1. The mean number and size of chloroplasts (Ch) and mitochondria per mesophyll cell cross section of wheat greening leaves. a – width, b – length, S - area. Time indicates hours after the start of illumination. Data are presented as mean values ± SE (n = 15) taken during three independent experiments. Significant differences between mean values (*ANOVA*, *Duncan*'s test, P<0.05) are indicated by *different letters*.

Time [h]	Ch number	Ch with starch [% of the total Ch number]	Mitochondria number	Size of Ch [μm] a	b	S [μm^2]	Size of mitochondria [μm] a	b	S [μm^2]
0	7.9 ± 0.4^{a}	11.4 ± 2.6^{a}	6.8 ± 0.6^{ab}	1.8 ± 0.1^{a}	3.3 ± 0.3^{a}	4.7 ± 0.4^{a}	0.5 ± 0.02^{a}	0.7 ± 0.04^{a}	0.3 ± 0.02^{a}
6	9.6 ± 0.5^{b}	3.1 ± 0.4^{b}	7.0 ± 0.7^{ab}	2.0 ± 0.2^{ab}	3.3 ± 0.3^{a}	5. 1± 0.6^{a}	0.5 ± 0.05^{a}	1.0 ± 0.10^{b}	0.4 ± 0.05^{bc}
24	8.8 ± 0.4^{ab}	0	5.8 ± 0.5^{a}	2.0 ± 0.1^{ab}	3.8 ± 0.2^{a}	6.0 ± 0.3^{a}	0.6 ± 0.05^{b}	0.8 ± 0.05^{ac}	0.4 ± 0.03^{b}
48	9.0 ± 0.7^{ab}	0	8.7 ± 1.1^{b}	2.3 ± 0.1^{b}	5.6 ± 0.3^{b}	10.2 ± 0.8^{b}	0.7 ± 0.03^{b}	0.9 ± 0.03^{ac}	0.5 ± 0.02^{c}

Table 3. Parameters of chlorophyll fluorescence (F_v/F_m and Φ_{PSII} – maximum and actual photochemical efficiency of PSII, respectively; q_N and q_P – coefficients of nonphotochemical and photochemical quenching, respectively), CO_2 gas exchange (P_N – net photosynthetic rate, R_D – dark respiration measured as CO_2 emission rate), rate of heat production (q), and level of lipid peroxidation presented as TBARS content in wheat leaves during greening. Data are presented as mean values ± SE (n for P_N, R_d, q, TBARS content = 3–6; n for F_v/F_m, Φ_{PSII}, q_N, q_P = 5–12) taken during three independent experiments. Significant differences between mean values (*ANOVA*, *Duncan*'s test, P<0.05) are indicated by *different letters*.

Time [h]	F_v/F_m	Φ_{PSII}	q_P	q_N	P_N [nmol(CO_2) g^{-1}(DM) s^{-1}]	R_D [nmol(CO_2) g^{-1}(DM) s^{-1}]	TBARS [nmol g^{-1} (FM)]	q [mW g^{-1}(DM)]
0	-	-	-	-	-	23.0 ± 1.5^{abc}	15.1 ± 0.7^{a}	11.4 ± 1.5^{ab}
1	0.45 ± 0.03^{a}	0.22 ± 0.02^{a}	0.78 ± 0.01^{a}	0.32 ± 0.01^{a}	–30.0 ± 3.3^{a}	20.4 ± 1.6^{abd}	15.0 ± 1.0^{a}	8.2 ± 1.4^{a}
2	0.48 ± 0.02^{b}	0.33 ± 0.02^{b}	0.86 ± 0.01^{b}	0.38 ± 0.03^{b}	–13.2 ± 3.2^{b}	22.2 ± 1.8^{ab}	20.2 ± 0.9^{b}	8.5 ± 1.9^{a}
4	0.64 ± 0.02^{c}	0.54 ± 0.01^{c}	0.90 ± 0.01^{c}	0.50 ± 0.04^{c}	–9.1 ± 2.7^{b}	25.1 ± 2.1^{ac}	19.5 ± 1.0^{b}	14.8 ± 1.5^{b}
6	0.69 ± 0.01^{c}	0.58 ± 0.14^{d}	0.94 ± 0.01^{de}	0.56 ± 0.01^{d}	–1.8 ± 2.3^{c}	28.0 ± 1.9^{c}	28.9 ± 2.2^{c}	25.8 ± 2.4^{c}
12	0.74 ± 0.01^{d}	0.64 ± 0.16^{e}	0.93 ± 0.02^{d}	0.59 ± 0.01^{d}	25.5 ± 1.8^{de}	14.8 ± 1.5^{d}	32.4 ± 2.2^{c}	10.0 ± 1.2^{a}
24	0.76 ± 0.01^{d}	0.67 ± 0.12^{f}	0.96 ± 0.01^{e}	0.40 ± 0.03^{b}	20.6 ± 2.3^{d}	17.2 ± 1.7^{bd}	22.3 ± 2.0^{b}	11.3 ± 1.3^{ab}
48	0.80 ± 0.01^{e}	0.74 ± 0.01^{g}	0.94 ± 0.03^{de}	0.45 ± 0.04^{e}	30.7 ± 0.8^{e}	20.4 ± 1.0^{abd}	23.0 ± 1.6^{b}	10.4 ± 0.8^{a}

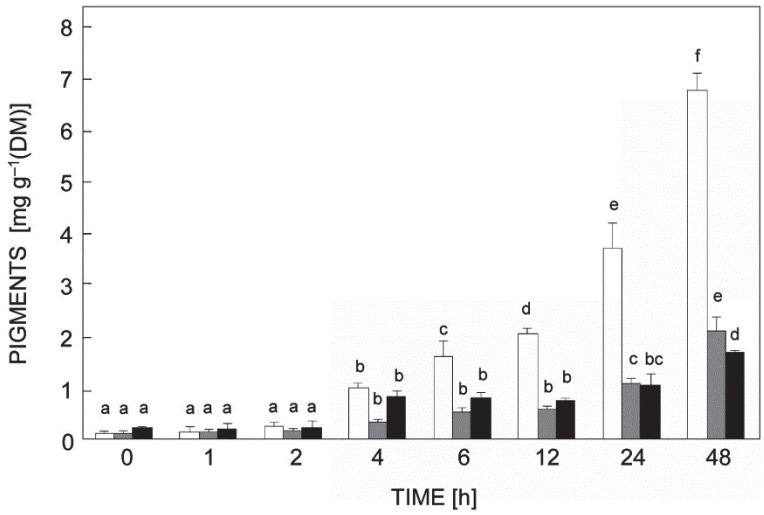

Fig. 2. Contents of chlorophyll *a* (*white*), chlorophyll *b* (*grey*), and carotenoids (*black*) in wheat leaves during greening. Data are presented as mean values ± SE (n = 4–5) taken during three independent experiments. Significant differences between mean values (*ANOVA*, *Duncan*'s test, P<0.05) are indicated by *different letters*.

Table 2. Contents of carotenoids ([mg g^{-1}(DM)]) in wheat leaves during greening. Carotenoids: Nx – neoxanthin, Vx – violaxanthin, Ax – antheraxanthin, Zx – zeaxanthin, β-Car – β-carotene. Time indicates hours after starting illumination. Data are presented as mean values ± SE (n = 4 – 5) taken during three independent experiments. Significant differences between mean values (*ANOVA, Duncan*'s test, P<0.05) are indicated by *different letters*.

Time [h]	Nx	Vx	Ax	Lutein	Zx	β-Car
0	0.005 ± 0.001^{a}	0.035 ± 0.004^{a}	0.031 ± 0.004^{ab}	0.096 ± 0.011^{a}	0.006 ± 0.001^{ab}	0.009 ± 0.001^{a}
1	0.007 ± 0.001^{a}	0.040 ± 0.003^{a}	0.037 ± 0.004^{b}	0.107 ± 0.014^{a}	0.009 ± 0.001^{b}	0.009 ± 0.001^{a}
2	0.008 ± 0.001^{a}	0.053 ± 0.009^{a}	0.046 ± 0.009^{c}	0.145 ± 0.028^{b}	0.015 ± 0.002^{c}	0.010 ± 0.002^{a}
4	0.021 ± 0.001^{b}	0.105 ± 0.003^{b}	0.060 ± 0.002^{d}	0.287 ± 0.006^{c}	0.036 ± 0.001^{d}	0.042 ± 0.001^{b}
6	0.031 ± 0.004^{b}	0.155 ± 0.015^{c}	0.034 ± 0.001^{ab}	0.326 ± 0.026^{c}	0.016 ± 0.001^{c}	0.070 ± 0.007^{c}
12	0.047 ± 0.004^{c}	0.204 ± 0.012^{d}	0.029 ± 0.002^{a}	0.397 ± 0.012^{d}	0.014 ± 0.001^{c}	0.167 ± 0.011^{d}
24	0.114 ± 0.003^{d}	0.282 ± 0.013^{e}	0.016 ± 0.002^{e}	0.673 ± 0.024^{e}	0.007 ± 0.001^{ab}	0.257 ± 0.007^{e}
48	0.164 ± 0.007^{e}	0.297 ± 0.012^{e}	0.013 ± 0.001^{e}	0.773 ± 0.041^{f}	0.004 ± 0.001^{a}	0.364 ± 0.025^{f}

at certain periods of time allowed us to observe changes in the contents of VXC pigments. During the greening process of the dark-acclimated leaves, Vx increased from 60 to 94% of the total pool of xantophyll cycle pigments (VAZ); relative contents of Ax and Zx decreased from 31 to 3% and from 9 to 3% of VAZ, respectively (Fig. 3). After 1 h of greening, Vx and Ax contents decreased during light exposure. There was no epoxidation observed after placing plants in darkness indicating disturbed VXC functioning. After 2 h of illumination, greater amount of Vx and Ax (25 and 18%, respectively) was de-epoxidized, but there was no normal epoxidation - only Ax was used as a substrate for ZE. Normal operation of VXC was observed after 4 h in the presence of light. However, the pools of converted xanthophylls were different. It was the highest after 6 and 12 h of greening, thereafter the pools of converted xanthophylls decreased.

Chl fluorescence: F_v/F_m and Φ_{PSII} increased during the greening process (Table 3). After two days of illumination, F_v/F_m was near 0.8. The values of q_P were equal to 0.78 after 1 h of greening. After 6 h of greening, q_P increased to about 0.94, and thereafter it did not change and remained high during the next hours. The values of q_N, reflecting the level of nonphotochemical quenching, were low and equal to 0.3–0.4 during the first two hours of greening. Then q_N increased and reached the highest values during the period from the 4th to the 12th h of greening, and thereafter q_N slightly decreased again (Table 3).

To gain more insight into the characteristics of q_E – the ΔpH-dependent, fast inducible nonphotochemical quenching, we studied the q_N induction and relaxation (Fig. 4). Time courses of q_N induction showed that from 1 to 2 h of greening the q_N parameter rose rather slowly and reached steady-state values in 200 s. During the next hours of greening, the q_N parameter increased faster. By 48 h, the leaves needed only 40 s to reach the steady state. The maximal q_N steady-state level was observed between the 6th and 12th h of greening. After switching off the actinic light, q_N decreased slowly during the first two hours of greening, but after 6 h of greening q_N decreased faster.

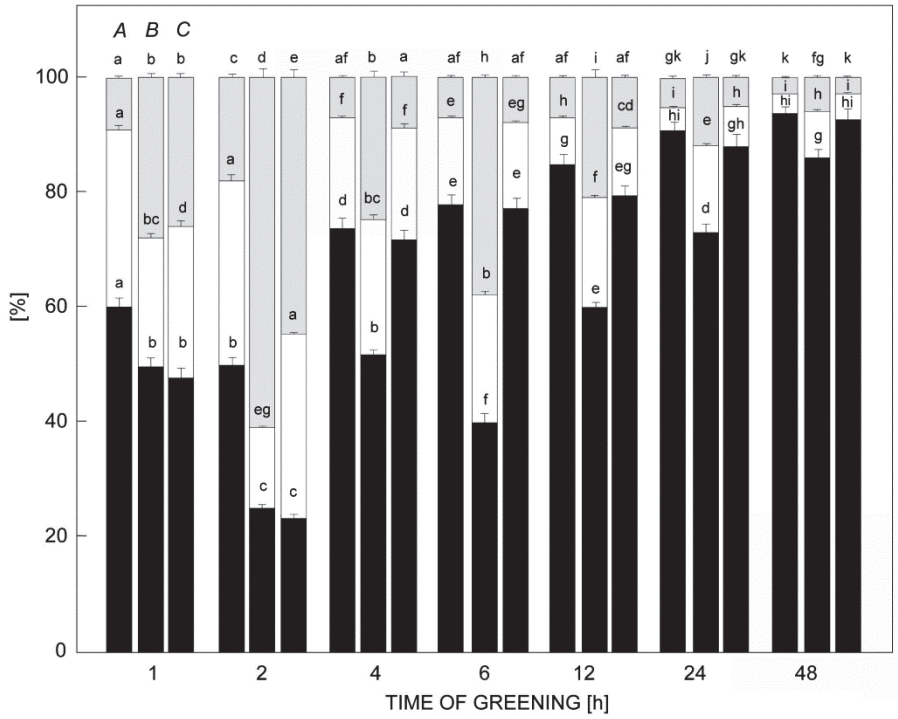

Fig. 3. Relative percentage of xanthophylls cycle pigments in wheat leaves during greening: *A* – control (after 1 h of darkness), *B* – after 30 min of light exposure [1,200 μmol(photon) m^{-2} s^{-1}] and subsequent 1 h of darkness (*C*). Pigments: violaxanthin (black), antheraxanthin (white), zeaxanthin (grey). Data are presented as mean values ± SE (n = 3) taken during three independent experiments. Time of greening and experimental conditions (*A,B,C*) were analyzed as independent factors. According to multivariant *ANOVA* analysis the effect of these factors was significant (P<0.05). Significant differences between mean values (*Duncan*'s test, P<0.05) are indicated by different letters.

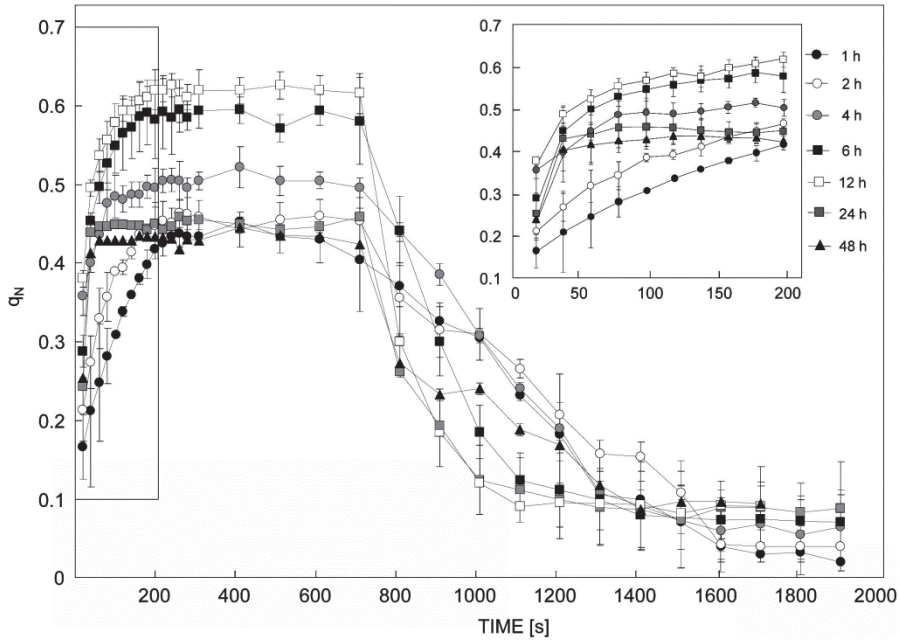

Fig. 4. Time courses for induction and relaxation of nonphotochemical quenching (q_N) in wheat leaves after different periods of greening. Before experiments, plants were dark-adapted for 30 min. After the first measurement, actinic light at intensity of 190 μmol(photon) m^{-2} s^{-1} (12 min) and then darkness (20 min) were applied. Time of greening is indicated by *different symbols*. Data are presented as mean values ± SE (n = 4–5) taken during three independent experiments. Time of greening [h] and time course for light induction of q_N [s] were analyzed as independent factors. According to multivariant *ANOVA* analysis the effect of these factors was significant (P<0.05). *The inset* represents time course for q_N induction after different periods of greening.

P_N remained negative in the presence of light during the first 6 h (Table 3). During this period, the CO_2-emission in the light decreased and plants reached the compensation point of CO_2-exchange. Thereafter P_N increased and was equal to 30 nmol(CO_2) g^{-1}(DM) s^{-1} after 48 h of illumination.

Respiration: R_D measured as the rate of CO_2 emission in etiolated seedlings was about 23 nmol(CO_2) g^{-1}(DM) s^{-1} (Table 3). After exposure to light, R_D slightly decreased. Thereafter, R_D again increased during the first 6 h of greening and it was the highest after 6 h of illumination. Thereafter, R_D decreased in average by 1.5 times.

The pattern of total dark respiration rate in the greening leaves of the seedlings measured as the rate of O_2 uptake (V_t) and CO_2 emission (R_D) was practically similar (Fig. 5, Table 3). V_t in etiolated leaves was equal to 3.6 nmol(O_2) g^{-1}(FM) s^{-1}. After 1 h of greening, V_t rate decreased. Then, V_t increased again and reached 5.5 nmol(O_2) g^{-1}(FM) s^{-1} to the end of 6th h of illumination (Fig. 5*A*). This rise was followed by a decline and V_t was about an average value of 4.2 nmol g^{-1}(FM) s^{-1} from 12 h to 48 h of illumination. The capacity of cytochrome pathway respiration (V_{cyt}) in etiolated leaves was about 2.4 nmol(O_2) g^{-1}(FM) s^{-1}. V_{cyt} decreased after 1 h of greening. V_{cyt} increased to 2.6 nmol(O_2) g^{-1}(FM) s^{-1} during the next 5 h of greening and it decreased thereafter to the initial level detected at the beginning of the greening process (Fig. 5*B*). Changes in alternative pathway respiration capacity (V_{alt}) were expressed more. In etiolated leaves, V_{alt} was relatively high [near 0.9 nmol(O_2) g^{-1}(FM) s^{-1}]. After exposure to light, this value decreased twice. During the next 4–6 h of greening, V_{alt} changed dramatically, increasing 6 times. Thereafter, V_{alt} decreased and it was an average value of 1.3 nmol(O_2) g^{-1}(FM) s^{-1} (Fig. 5*B*).

Fig. 5*C* shows the changes in respiratory pathway ratio during greening of the seedlings. The respiration of etiolated tissues proceeded mainly through CP (70%). After 4 h of greening, V_{cyt}/V_t, calculated on the basis of the average values of V_{cyt} and V_t, decreased and reached minimal level (50%) to the end of 6th h of illumination. Thereafter V_{cyt}/V_t value increased by 10% and it was maintained on this level to the end of the experiment. On the contrary, the relative part of AP capacity in the total respiration (V_{alt}/V_t) in etiolated tissues was approximately 25%. V_{alt}/V_t value increased from 20 to 50% during the first 6 h of greening and thereafter decreased to 30%.

Rate of heat production: Changes in q in leaf tissues during greening corresponded to the respiration dynamics (Table 3). In etiolated leaves, q was slightly higher than during the first 2 h of greening. Thereafter, q increased and it was the highest by the end of the 6-h period of the greening process. Then it decreased 2.5 times and it was maintained at this level up to the end of the experiment.

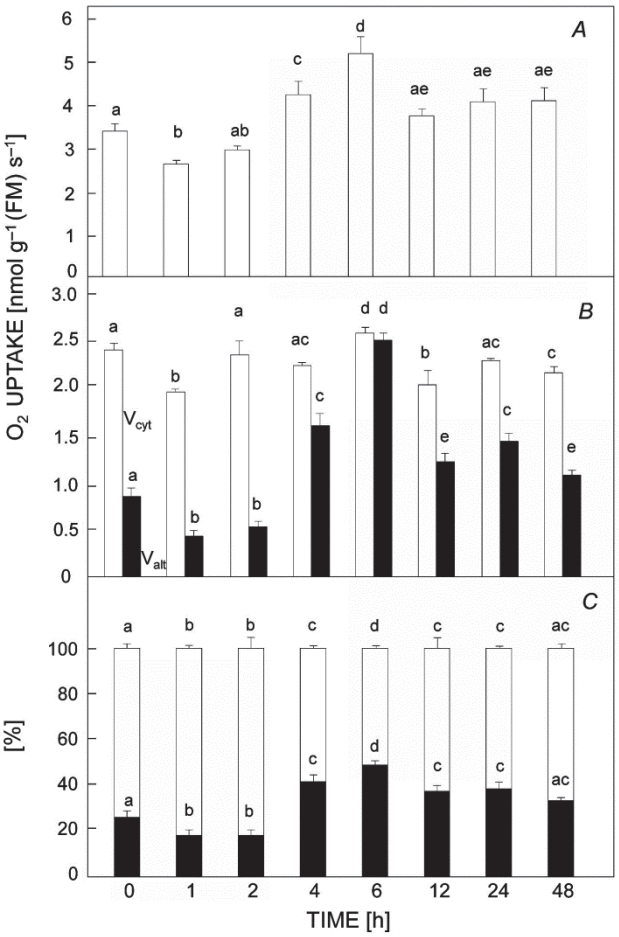

Fig. 5. Total respiration rate (*A*), capacities of cytochrome (*white*) and alternative respiration (*black*) (*B*), and the respiratory pathway ratio (*C*) of wheat leaves during the greening process. Time indicates hours after the start of illumination. Data are presented as mean values ± SE (n = 4–6) taken during four independent experiments. Significant differences between mean values (*Duncan*'s test, P<0.05) are indicated by *different letters*.

Lipid peroxidation: The absolute TBARS values in etiolated leaves were about 15 nmol g^{-1}(FM) (Table 3). Light intensified lipid peroxidation. By the end of 12-h period of greening, TBARS content in leaves was doubled. The TBARS content declined after the 1st day of illumination.

Discussion

The wheat seedlings were exposed to relatively low level of illumination [190 μmol(photon) m^{-2} s^{-1}]. This level was in the curvature region (the transition from the light-limited phase to the light-saturated plateau) of the light-

response curve of the net CO_2 exchange rate of the first mature wheat leaf (Fig. 6). The curvature region corresponds to the typical light intensities of plant habitat (Tooming 1984, Garmash and Golovko 1997).

During the initial few hours of greening, however, the etiolated leaves were probably forced to withstand an excess of light energy. It was found that P_N became positive only after 6 h of illumination (Table 3), when chloroplasts became more mature (Fig. 1). We estimated the functionality of the photosynthetic machinery and the cell membrane state by measuring Chl fluorescence and lipid peroxidation parameters (Table 3).

The first 2 h of de-etiolation were characterized by low values of F_v/F_m, Φ_{PSII}, and q_P in leaves. The low F_v/F_m was due to low values of F_o and F_m (data are not presented), which might be the result of the incomplete light-harvesting complexes. Thereafter, a gradual increase of F_v/F_m and Φ_{PSII} was detected. To the end of the 24-h light period, F_v/F_m reached about 0.8, which is the typical value for a healthy mature leaf. These results agree with the common dynamics of photosynthetic apparatus development under continuous light. The light-triggered reduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide), as a prerequisite for Chl synthesis, lasts for 4–6 h of illumination, when concentration of LPOR, as the most abundant protein associated to PLBs, didn't change dramatically (Solymosi and Schoefs 2010). According to our data, PLBs were already absent after 6 h of de-etiolation. However, the formation of fully functional photosynthetic machinery including building-up light-harvesting complexes and integration of PSI and PSII into the membrane requires longer illumination (more than 6 h) (Schoefs *et al.* 1998).

The changes of q_N, reflecting the nonphotochemical quenching of Chl fluorescence, and lipoperoxidation activity (LPA) were similar (Table 3) during the greening process. They were low after 1 h of illumination (Table 3), thereafter q_N and LPA increased and were the highest after 12 h of greening, and then they decreased again. The low values of q_N and LPA during the first few hours of de-etiolation confirmed that etioplasts have their own effective defense against photooxidation. There is evidence that LPOR plays a vital role in avoiding ROS formation (Schoefs and Franck 2003, Solymosi and Schoefs 2010).

The increase in q_N was likely due to activation of systems dissipating excessive light energy in developing chloroplasts. We studied the VXC activity along with the kinetics of q_N induction and relaxation. It is known that generation of q_E requires a lumen pH below 6 and proceeds during 10 min of illumination at saturating light intensities (Quick and Stitt 1989, Krause and Weis 1991, Horton *et al.* 1994, Kalituho *et al.* 2007).

According to our experiment, etiolated leaves accumulated Car, the major part of which were xanthophylls (Table 2). But during the first stage (1–2 h) of greening, VXC didn't function normally (Fig. 3). In addition, the slow induction and dark relaxation of NPQ during this period of de-etiolation confirmed that energy dissipation was not connected with the operation of VXC. It is known that etiolated leaves contain significant amount of xantophylls and VDE, but VXC doesn't protect Pchlide and Chlide molecules in etioplasts (Franck and Mathis 1980, Ignatov *et al*. 1983, Pfündel and Strasser 1988). Accumulation of xantophylls has been found to be essential for PLBs formation (Park *et al*. 2002, Cuttris *et al*. 2007).

The highest induction and relaxation as well as maximal values of q_N were observed between the 6th and the 12th h of illumination, when VXC functioned more effectively. During the next hours of the greening process, the induction of q_N was fast, but the maximal q_N values were lower. It correlated with the decreasing level of Zx and Ax in dark-acclimated leaves and VXC activity in general. These results are supported by the literature

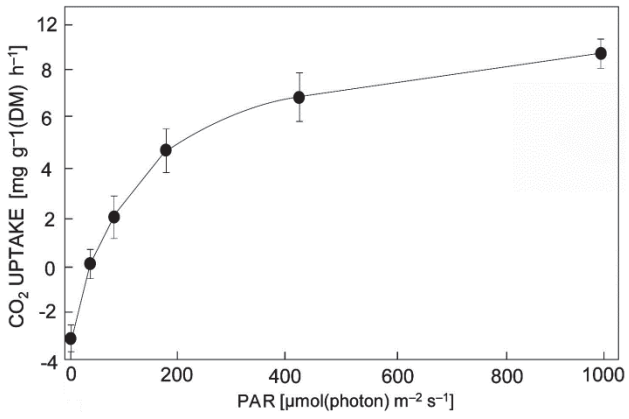

Fig. 6. Light-response curve of the net CO_2 exchange rate in the 1st mature leaf of the 7-d-old wheat seedlings. Data are presented as mean values ± SE (n = 4–6) taken during three independent experiments. PAR – photosynthetically active radiation.

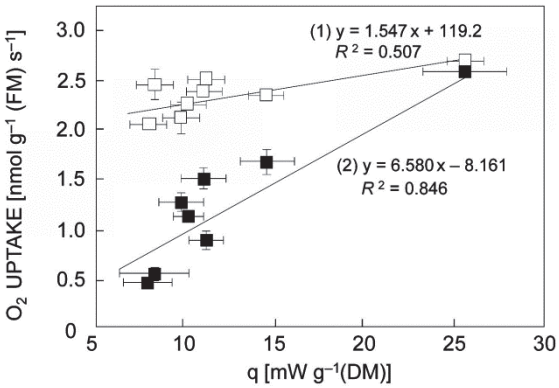

Fig. 7. Relationship between heat production rate (q) and capacities of cytochrome respiration (1) and alternative respiration (2) during wheat leaves greening. Data are presented as mean values ± SE (n = 3–6) taken during three or four independent experiments.

data. It was shown that the contact between Chl and Car became closer and, correspondingly, the efficiency of Chl triplet state quenching by Car increased during the later phase of cucumber cotyledons greening (after 3 h of continuous illumination) (Waloszek *et al.* 2002).

In illuminated leaves, the respiratory chain can have an important function in the interaction between chloroplasts and mitochondria and protection of the cell against photoinhibition (Kumar *et al.* 2007, Noguchi and Yoshida 2008, Yoshida *et al.* 2008, Dinakar *et al.* 2010). We found that the respiratory activity measured as the rate of O_2 uptake or CO_2 emission changed dramatically during the first 6 h of illumination (Table 3, Fig. 5). Changes in V_t were mainly associated with the changes in V_{alt}.

Respiratory pathways oxidize carbohydrates to generate ATP, which is used to drive the energy requiring anabolic processes, including maintenance and synthesis *de novo*. Calorespirometry can be used to rapidly measure the rate of heat, generated by catabolism (Hansen *et al.* 1994). It is known that in fast growing tissues only 20% of respiratory energy is stored in new biomass. The other part of energy is lost as heat, but 90% of that heat is a result of energy produced by catabolism (Semikhatova 1990). If electrons are transferred to the alternative pathway, no protons are pumped and the energy of the reductants is converted to heat. We revealed the positive linear correlation between V_{alt} and heat production in greening tissues (Fig. 7). This could indicate participation of the nonphosphorylating alternative pathway as energy-dissipating system in the de-etiolation process and the photosynthetic apparatus development.

Earlier, the increase in AP activity was observed, when measured by oxygen isotope fractionation during the greening process in soybean cotyledons (Ribas-Carbo *et al.* 2000). However, the basis for this shift in respiratory metabolism is not completely understood. We found that during the first 2 h of greening, the respiratory activity as well as AP capacity (V_{alt}) was low. V_t and V_{alt} increased significantly thereafter and they were the highest after 4–6 h of illumination (Fig. 5). This suggests that during the greening of the wheat seedlings, indirect light signalling (nonphotoreceptor-mediated) mechanisms associated with activation of photosynthetic metabolism could function and contribute to the regulation of both respiration and the genes encoding respiratory enzymes including AOX. In particular, potential roles of both carbohydrates affecting AOX transcript levels and posttranslational regulation of the AOX protein by redox status can be considered (Rasmusson and Escobar 2007).

The increase of V_{alt} during the later period of greening is likely to be connected with oxidation of NADH, which could be imported to mitochondria from chloroplasts *via* malate/oxaloacetate shuttle (Padmasree and Raghavendra 1999). Feng *et al.* (2007) revealed the increased *AOX1c* expression during greening of etiolated rice seedlings only after 4 h of continuous illumination. When etiolated *Arabidopsis aox1a* mutant seedlings were exposed to illumination, the reduction of the photosynthetic electron transport chain and the accumulation of reducing equivalents was greater in the mutant during de-etiolation (Zhang *et al.* 2010). All this suggests that AOX activation in light is associated with photosynthetic metabolites. The increase of mitochondria adjacent location to chloroplasts during the wheat seedlings greening (Fig. 1*C–E*) indicated possible metabolites exchange between these organelles (Logan 2006).

AOX may be considered as an antioxidant enzyme regulating the redox state of mitochondrial ubiquinone pool and hence preventing the formation of ROS (Maxwell *et al.* 1999, Millenaar and Lambers 2003), especially under stressful conditions including excessive light (Noguchi and Yoshida 2008). According to our data, the dependence of V_{alt} on the level of lipid peroxidation fitted the positive linear function ($y = 0.06\ x - 0.26$) during the greening and it had low coefficient of determination ($R^2 = 0.38$). These results suggest that AP might not function exclusively to prevent excessive formation of ROS (Umbach *et al.* 2005, Feng *et al.* 2007), in particular, during the greening.

In the etiolated, starch-accumulating leaves (Fig. 1*A*, Table 1), the alternative pathway could work as an overflow for cytochrome pathway according to the often-discussed "energy overflow" hypothesis (Lambers 1982). Such situation could be expedient for the tissues with a large pool of carbohydrates. In this case, AP oxidizing the excess of carbohydrates is necessary for prevention of the ubiquinone pool over-reduction in mitochondrial ETC and for the TCA continuation, when cytochrome pathway is restricted by a low ADP concentration (Vanlerberghe and McIntosh 1997, Millenaar and Lambers 2003). A more detailed study is needed to reveal mechanisms responsible for the light regulation of respiration.

Conclusion: Clearly, de-etiolation as a light-stimulated chloroplast development from etioplasts is a complex and highly regulated process. We found that the dramatic physiological changes in the greening wheat seedlings occurred during the first 12 h of continuous illumination. During the first 2 h of greening, the level of lipoperoxidation activity was low, and etiolated leaves are thought to avoid ROS formation due to functioning of LPOR in PLBs as shown by Schoefs and Franck (2003). After 4 h of illumination, lipoperoxidation processes were intensified, and the developing photosynthetic machinery needed protection against irreversible damage caused by ROS. During this period, the energy dissipation processes in chloroplasts and mitochondria increased significantly. It was found that the increase in VXC activity was accompanied by the fast rate of induction and relaxation of nonphotochemical quenching of Chl fluorescence. The values of these parameters were the highest during the later phase of de-etiolation (between the 4^{th} and the 12^{th} h of illumination). Respiration activity and alternative respiratory pathway capacity also increased during the

later phase of de-etiolation (4–6 h). The time course of the changes in respiration activity and AP capacity was accompanied by dark heat emission. With maturation of chloroplasts, the activity of the energy dissipation and lipoperoxidation processes decreased. In general, our results showed that the induction of the energy-dissipating systems was regulated by the level of photosynthetic machinery development and light stress susceptibility of de-etiolating leaves.

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