PHOTOSYNTHETICA 45 (3): 409-418, 2007

Chlorophyll *a* fluorescence transient during freezing and recovery in winter wheat

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Abstract

Chlorophyll (Chl) *a* fluorescence measurements as evaluators of plant freezing tolerance are frequently insufficiently sensitive to detect the early metabolic changes that are initiated following exposure to freezing temperatures. Using cold-acclimated winter wheat, I analysed the polyphasic transience (from 50 μs to 1 s) of Chl *a* fluorescence. This enabled detailed studies of the progressive energy flows and efficiencies within the photosystem 2 (PS2) complex that ensue following initial exposure to freezing temperatures right through to the plant recovery stage. The initial consequences of mild frosts that may cause primary damage involve a disturbance to the energy transfer subsequent to Q_A (the primary quinone electron acceptor of PS2). Lower freezing temperatures, on the other hand, may deter energy flow between the PS2 reaction centre (RC), Chl, and Q_A. All primary damage could only be repaired partially. Further freezing-triggered dysfunction of the electron transfer between the PS2 RCs and Q_A was connected with secondary damage that could lead to PS2 deactivation. Both primary and secondary freezing damages were reflected in decreased $PI_{ABS,}$ the Performance Index based on equal absorption that characterizes all energy bifurcations in PS2. PI_{ABS} also differentiated cultivars with contrasting freezing-tolerance either subsequent to the onset of freezing or during the recovery stage. In contrast, the potential quantum yield of PS2 (F_v/F_m), which characterizes efficiency of energy trapping in the PS2 RCs, was only different in cultivars with contrasting freezing-tolerance during the recovery stage.

Additional key words: electrolyte leakage; photosystem 2; *Triticum aestivum*.

Introduction

Changes in the photosynthetic apparatus measured during cold acclimation by means of chlorophyll (Chl) fluorescence studies have been frequently reported and widely discussed, also as an indirect indicator of freezing tolerance of plants (Huner *et al.* 1993, Taulavuori *et al.* 2000, Rapacz *et al.* 2004, Ensminger *et al.* 2006). However, little information is available on the effect of freezing on Chl fluorescence. In a few published reports, measurements of Chl fluorescence on frozen plants have been applied as sensible, non-invasive, early, fast, and easy to use methods for frost-tolerance evaluation (Adams and Perkins 1993, Clement and van Hasselt 1996, Rizza *et al.* 2001, Janowiak *et al.* 2004). These focused on spruce, wheat, oat, and barley, respectively. However, the use of Chl fluorescence for freezing tolerance assessment has also been criticized. Neuner and Buchner (1999) using *Rhododendron ferrugineum* underestimated freezing injuries by employing fluorescence measurements on partially frost injured leaves. In all the works referred to above, no detailed studies on the effect of freezing on photosynthetic apparatus were performed. Steffen *et al.* (1989) reported that decrease

Received 5 October 2006, *accepted* 6 March 2007.
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Abbreviations: ABS – absorption flux; Area – pool size of electron acceptors from PS2 (proportional to the oxidized plastoquinone pool); Chl – chlorophyll; CS – leaf cross-section; DI – dissipation flux; ET – electron transport flux; EL – electrolyte leakage from leaves; F_0 – fluorescence of dark-adapted leaves in time 0; F_m – maximum fluorescence in dark-adapted leaves; F_v – fluorescence change between F_0 and F_m ($F_v = F_m - F_0$); F_v/F_m – quantum efficiency of energy trapping in PS2 reaction centres; PI_{ABS} – performance index based on the equal absorption; PPFD – photosynthetic photon flux density; PS2 – photosystem 2; Q_A – primary quinone electron acceptor of PS2; Q_B – secondary quinone electron acceptor of PS2; RC – reaction centre; RC/CS_0 and RC/CS_m – minimal and maximal densities of active reaction centres per leaf cross-section; TR – energy flux for trapping.
Acknowledgements: The experiments were partially supported by Polish Ministry of Agriculture and Rural Development (Decision no: HORhn 4040/59/2005). I thank Dr M.W. Humphreys from IGER, Aberystwyth, Wales, UK for critical language correction of the manuscript.

in photosynthesis resulting from freezing temperatures and measured as oxygen evolution rate in leaves of two potato species is equivalent to changes in ion leakage from cells, but only at slower cooling rates. As far as Chl fluorescence parameters are concerned, the authors focused mainly on F_v/F_m, but Janowiak *et al.* (2004) measured also effective quantum yield of PS2 (ϕ_{PS2}). More detailed studies of changes in Chl fluorescence parameters caused by freezing were made mainly on conifers using an older equipment for fluorescence measurement (Strand and Öquist 1988, Binder and Fielder 1996).

The aim of my investigations was to check both direct effects and after-effects triggered by freezing of wheat using more informative measurements of Chl fluorescence and to determine the conditions and parameters useful for screening wheat for freezing tolerance. The common approach for fluorescence studies constitutes the measuring of the minimum and maximum fluorescence values, and in calculating parameters such as F_v/F_m ratio. Strasser *et al.* (2000) have developed an analysis of the OJIP fast fluorescence rise, measured from 50 μs to 1 s upon irradiation of the photosynthetic sample, and have also described the linkage between this physical signal and the biological functions. Analysis of fluorescence induction curves measured with fast sampling equipment made it possible to analyse many details of changes in energy transfer within PS2 and the relationships between primary photochemistry and the requirement for electrons in further stages of photosynthetic metabolism (Strasser and Strasser 1995, Strasser *et al.* 2000).

Materials and methods

Plants and experimental design: Two experiments (1 and 2) were performed on winter wheat (*Triticum aestivum* L. emend. Fiori *et* Paol.) cultivar Kobra (*Nasiona Kobierzyc*, Poland). In the third experiment cultivars Tonacja (*HR Strzelce*, Poland) and Clever (*R 2n S.A.S.*, France) were used additionally. Cultivars used in this experiment differ in freezing tolerance. According to the Polish Research Centre for Cultivar Testing (COBORU), freezing tolerance of cvs. Clever, Kobra, and Tonacja was estimated as 1, 4, and 6 in the 1–9 score, respectively (COBORU 2005). In this assessment freezing tolerance was measured three times every winter in standardized conditions by means of field-laboratory method (Koch and Lehman 1969). The results are averaged for five year period and in the score 1 means less than 15 % plants survived tests, 4 means 35–<45 %, 6 means 55–<65 %, 9 means >85 % survival. In all experiments plants were sown in pots containing the mixture of sand, clay soil, and peat (1 : 1 : 1, v:v:v) and placed in growth cabinet. During the first week, temperature +20 °C was maintained during the whole day (photoperiod 12/12 h, 300 μmol m^{-2} s^{-1} PPFD; *Philips AGRO* sodium lamps). Next the temperature was decreased to +15 °C and day length was shortened to 10 h (no change in PPFD and light sources). After 3 weeks the plants were cold acclimated for three weeks at +2/5 °C (day/night); photoperiod, PPFD, and light sources as before. After completion of cold acclimation freezing tests were performed. In Exps. 2 and 3 plants were recovered at +15 °C after freezing (details as before cold acclimation) for 7 and 5 d, respectively. All experiments were repeated in 2 or 3 (Exp. 1) independent series.

Freezing tests:
Exp. 1: The youngest, but fully expanded leaves were cut from plants (10 for each freezing temperature: –6, –9, –12, and –15 °C), divided into 2-cm long segments, and placed on ice (5 cm^3 of frozen deionised water) in transparent conductivity vessels. Next vessels were put into programmed freezer with the temperature of 0±0.5 °C. A freezing–thawing cycle was performed separately for each freezing temperature, in darkness. The temperature was decreased with the initial rate of 3 K per h from 0 to –6 °C and next, in the case of lower freezing temperatures, with the rate of 10 K per h down to the desired temperature. Freezing temperature was kept for 90 min and next temperature was increased up to 0 °C with the rate of approximately 3 K per h.

Exps. 2 and 3: In comparison to Exp. 1, whole pots with plants and non-detached leaves were put into the freezer, freezing temperature was maintained for 150 (instead 90) min, and all rates of temperature change were about 3 K per h. In Exp. 3 plants were frozen at –12 °C only.

Chl *a* fluorescence: Polyphasic Chl *a* fluorescence transients (Strasser *et al.* 1995) were measured by means of Handy PEA fluorometer (*Hansatech*, Kings Lynn, UK). All fluorescence measurements were taken on middle part of the youngest, but fully expanded leaf, in 10 replications for each experimental series/freezing temperature/measuring condition/cultivar. Before measurements, the LED source of the fluorometer was calibrated using an *SQS* light meter (*Hansatech*, Kings Lynn, UK). All measurements were taken using a saturating pulse of 3 000 μmol m^{-2} s^{-1}, pulse duration of 1 s, and fixed gain (1×). The effectiveness of fluorescence saturation during measurement was always controlled by induction curve analysis (Handy PEA software, v. *1.30*). Single measurements, which are too short to observe saturation of fluorescence, were not taken into consideration. Before all measurements, leaves were dark-adapted for 15 min in a leaf clip (*Hansatech*). In Exp. 1, measurements were taken also on pre-irradiated leaves, *i.e.* after dark adaptation and measurement of F_0 (pulse intensity as above, duration of 100 ms) the leaves were irradiated in leaf clip

with measuring LED's of Handy PEA (800 μmol m^{-2} s^{-1}) for 30 s and the fluorescence induction curve was measured again (saturating pulse of 3 000 μmol m^{-2} s^{-1}, pulse duration of 1 s, and fixed gain 1.5×).

In direct measurements after freezing (Exps. 1 and 3), leaf clips were attached after 30 min of de-freezing at +5 °C in the dark, or after 90 min of following irradiation (300 μmol m^{-2} s^{-1}) in vessels. In the recovery period (Exps. 2 and 3) and control measurements of non-frozen plants in Exp. 3, clips were put on leaves always between 09:00 and 10:00, it means during the 2nd h of the day.

Calculation of JIP-test parameters: Dark-adapted photosynthetic samples exhibit upon irradiation a fast fluorescence rise from initial fluorescence intensity F_0 to a maximal intensity F_P. The latter depends on the irradiance and becomes highest under saturating irradiance, denoted then as F_m. Between these two extremes the fluorescence intensity showed intermediate steps F_J at about 2 ms and F_I at about 30 ms, while F_m was reached after about 300 ms (Neubauer and Schreiber 1987, Strasser *et al*. 1995). Fluorometer used in presented experiments (Handy PEA, *Hansatech*, Kings Lynn, UK) measures fluorescence rise of high time resolution (10 μs), which allows detailed analysis of fluorescence transients. Due to the typical shape of the fluorescence rise which shows the steps 0, J, I, P (Fig. 1) the following information was collected and used in further calculations (Strasser *et al*. 2000): fluorescence levels $F_{50\mu s}$, $F_{100\mu s}$, $F_{300\mu s}$, F_{2ms} (F_J), F_{30ms} (F_I), and F_m, t_{Fm}, *i.e*. mean time to reach F_m, and Area, *i.e*. the area above induction curve from F_0 to F_m which is proportional to the pool size of electron acceptors (Q_A and finally plastoquinone) on the reducing side of photosystem 2, PS2 (Strasser and Strasser 1995) were recorded. F_0, initial (minimal) fluorescence was calculated by Handy PEA internal software as an interpolation of induction curve to time 0 (Strasser and Strasser 1995). On the basis of the theory of energy flow in PS2 (Strasser and Tsimilli-Michael 2001), further parameters were calculated according to the equations of the JIP-test both in dark-adapted and pre-irradiated leaves. In pre-irradiated leaves the parameters TR_0/CS, ET_0/CS, DI_0/CS, M_0 were not marked with the subscript 0, and F_0 and F_m values refer to initial and maximum fluorescence under pre-irradiation. For simplification in this section this detail was omitted. The first group of parameters represents the phenomenological fluxes of energy: absorbed (ABS/CS), trapped in PS2 reaction centres, RCs (TR_0/CS), used for electron transport (ET_0/CS) and dissipated (DI_0/CS), where CS stands for the excited cross-section of the tested sample. The value of the initial fluorescence in dark-adapted leaves (F_0) serves as a measure (in arbitrary units) of the phenomenological absorption flux ABS/CS (Strasser and Strasser 1995) and was used in calculation both in dark-adapted and pre-irradiated samples. The use of arbitrary units in calculation of JIP test imposes the use of fixed gain during measurements of parameters which will be directly compared. Another phenomenological fluxes of energy can be calculated in the same arbitrary units:

$$TR_0/CS = (F_v/F_m)\ ABS/CS$$
$$ET_0/CS = (F_v/F_m)\ [1 - (F_J - F_0)/(F_m - F_0)]\ ABS/CS$$
$$DI_0/CS = ABS/CS - TR_0/CS$$

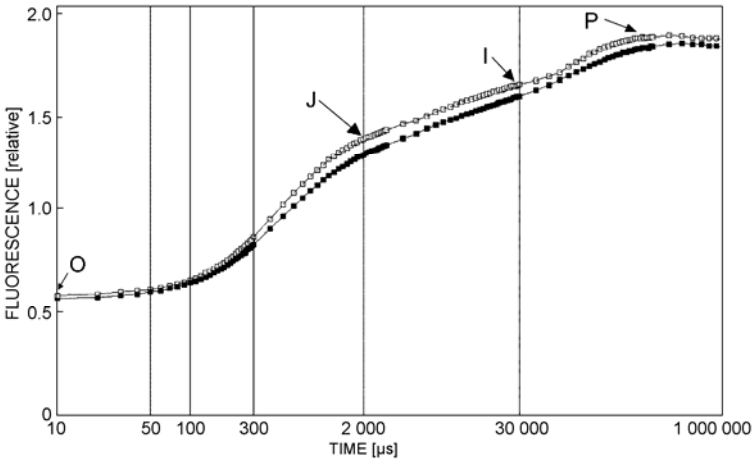

Fig. 1. Typical curves of chlorophyll fluorescence transient in frozen winter wheat leaves with the indication of the steps 0, J, I, and P. *In time axis* (logarithmic) start and end points of the measurements as well as time-points of fluorescence measurements [$F_{50\mu s}$, $F_{100\mu s}$, $F_{300\mu s}$, F_{2ms} (F_J), F_{30ms} (F_I)] used in calculations are indicated. *Open symbols* indicated a leaf with F_v/F_m = 0.693 and PI_{ABS} = 0.863 while *closed ones* a leaf with F_v/F_m = 0.696 and PI_{ABS} = 0.744. F_0 was an estimation of the curve to 0 ms and F_m was the maximal fluorescence recorded during measurements.

Another parameter used in calculations of JIP test is M_0 the normalized value of the initial increment of variable fluorescence (M_0 = dV/dt). Variable fluorescence $V = (F - F_0)/(F_m - F_0)$, where F is actual fluorescence intensity at any time. This can be calculated as 4 $(F_{300\mu s} - F_{50\mu s})/(F_m - F_{50\mu s})$ (Tsimilli–Michael *et al.* 2000). M_0 was used for calculation of further parameters.

The expression RC/CS_0 (active RCs per excited cross-section) for the concentration of the RCs was derived as follows:

$$RC/CS_0 = [(ABS/CS)/(F_v/F_m)]/\{M_0/[(F_J - F_0)/(F_m - F_0)]\}$$

For calculating maximal number of active RCs (in initial state during measurement) is RC/CS_m, ABS/CS was replaced by F_m.

The last JIP-test parameter calculated in this paper is

a so called performance index for equal absorption, PI_{ABS} (Strasser and Tsimilli-Michael 2001). This index groups the bifurcations of the energy cascades based on the equal absorption and is calculated from the yield of energy trapping (TR_0/ABS) and the yield of transfer of trapped energy into electron transport (ET_0/TR_0) normalized for the amount of energy absorbed by single RC (ABS/RC):

$$PI_{ABS} = \{[(TR_0/ABS)/[1 - (TR_0/ABS)]\}\ \{[(ET_0/TR_0)/[1 - (ET_0/TR_0)]\}\ [1/(ABS/RC)],$$

where

$$TR_0/ABS = F_v/F_m$$

$$ET_0/TR_0 = 1 - [(F_J - F_0)/(F_m - F_0)]$$

$$ABS/RC = M_0\ \{1/[(F_J - F_0)/(F_m - F_0)]\}\ (F_m/F_v)$$

Electrolyte leakage: Measurements of plasma membrane injuries were done as described in Rapacz (1999) and the index of injuries (%EL) was calculated according to Flint *et al.* (1967). Measurements were done in 10 replications in each experimental series after taking measurements of Chl fluorescence.

Statistical treatment: All data were analysed using *Statistica 6.1* software (*Statsoft*, Tulsa, OK, USA).

Results

Direct effects of freezing (Exp. 1): When leaf damage was studied using electrolyte leakage, an initial increase in %EL was visible after freezing at –6 °C with damage enlarged almost linearly with the decreases of freezing temperatures (Fig. 2*A*). %EL was independent of irradiation applied to samples for 90 min after de-freezing. Different results were observed for F_v/F_m, Chl *a* fluorescence, the parameter used commonly for

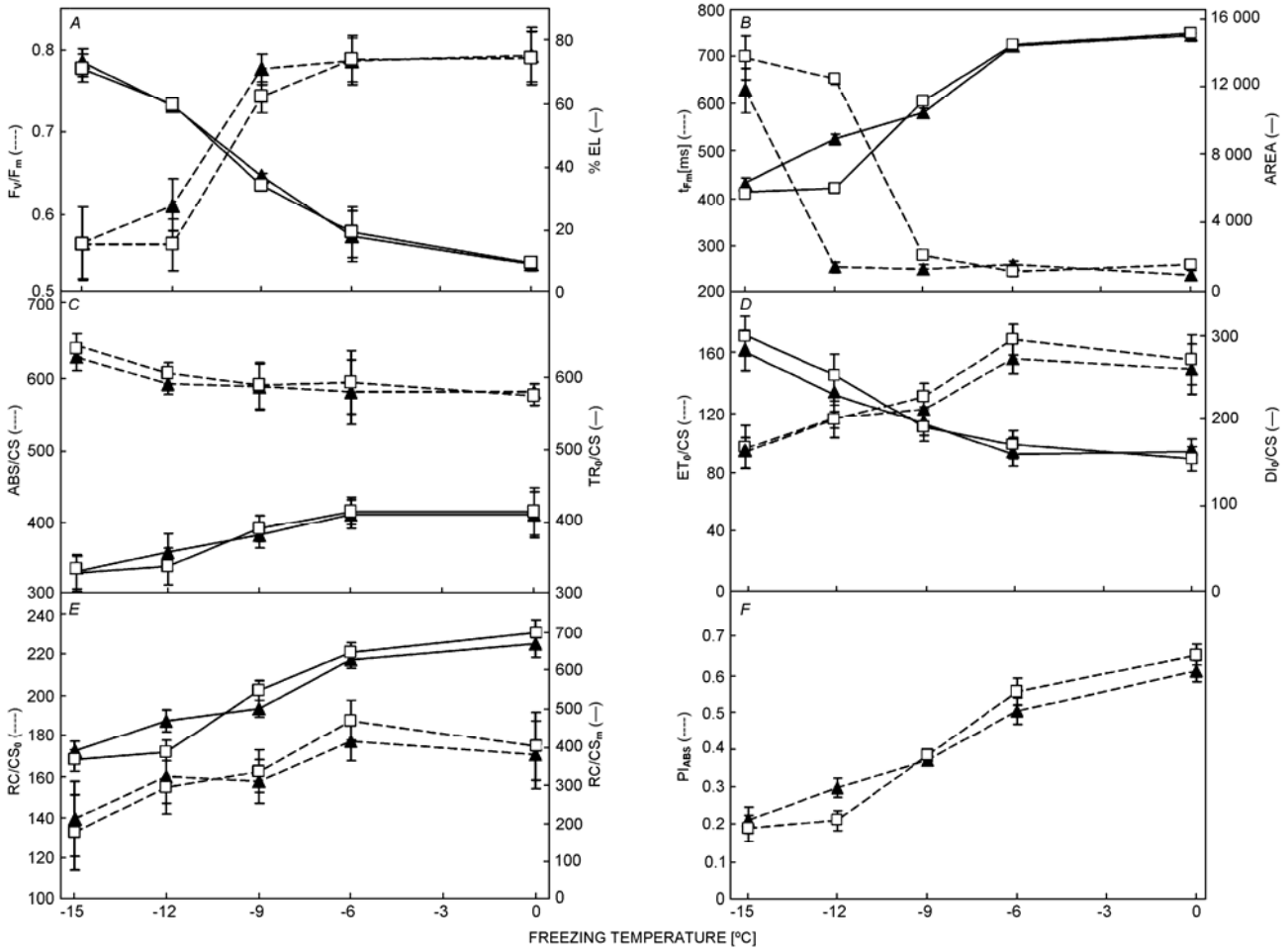

Fig. 2. Changes in electrolyte leakage (% EL) and chlorophyll *a* fluorescence induction parameters measured after freezing in five temperatures on dark-adapted leaves of winter wheat cv. Kobra (Exp. 1). Each parameter was measured either after 30 min of de-freezing at +5 °C in the dark (*closed triangles*) or after 30 min of de-freezing in the dark and following 90 min of irradiation at +5 °C and 300 μmol m^{-2} s^{-1} (*open squares*). Means of 2 independent experiments ± standard errors, $n = 20$.

estimation of freezing damage. F_v/F_m was not affected by freezing at –6 and –9 °C, but was clearly reduced after freezing at –12 °C, and this effect was enhanced to some extent by irradiating after de-freezing (Fig. 2*A*). In the case of Area, which represents the pool size of acceptors available for PS2, the changes observed were very similar for those for %EL, with one exception: the detrimental effect of freezing at –12 °C was enhanced by irradiating the leaves (Fig. 2*B*). On the other hand, considerable increase in t_{Fm}, which represents the acceptor activity rather than the acceptors pool, was observed in samples irradiated for 90 min after freezing at –12 and –15 °C, which implies a decreased activity by the acceptors (Fig. 2*B*). Without irradiation, the same effect was visible only after freezing at –15 °C (Fig. 2*B*). In the case of phenomenological energy fluxes within PS2, almost no negative effects of freezing were observed for photon absorption (ABS/CS). Only after freezing at –15 °C, a little increase was observed. Trapping efficiency (TR_0/CS) decreased slightly and gradually after freezing at –12 °C without considerable effect of irradiation, whereas electron transport activity (ET_0/CS) clearly decreased soon after freezing at –9 °C (Fig. 2*C,D*). Activity of energy dissipation (DI_0/CS) increased significantly starting from –12 °C (Fig. 2*D*). Densities of active PS2 RCs per leaf cross section (RC/CS_0, RC/CS_m) decreased just after freezing at –9 °C, and the irradiation had high influence on maximal activity (RC/CS_m), especially after freezing at –12 °C (Fig. 2*E*). Changes in performance index (PI_{ABS}) corresponded very well with changes in %EL (Fig. 2*F*). Some decrease was observed even after freezing at –6 °C. The effects of irradiation after freezing were visible only for –12 °C, and in the irradiated leaves no changes were observed for the samples frozen to temperatures between –12 and –15 °C.

Freezing triggered changes in the photosynthetic apparatus following leaf pre-irradiation for 30 s directly prior to measurements, which affected Q_A redox state during the measurements of transient fluorescence, were similar to those observed in dark-adapted leaves (Fig. 3). But in contrast to dark-adapted leaves, the decreases in TR/CS and ET/CS were similar (after freezing at –15 °C, they achieved only about 45 % of the value at 0 °C), whereas in dark-adapted leaves TR_0/CS and ET_0/CS was reduced to 85 and 60 %, respectively (Figs. 2*C,D* and 3*B,C*). Also a trend towards an increase in initial fluorescence, which depended on absorption and on the redox state of Q_A after pre-irradiation, after freezing was more distinct than ABS/CS in dark-adapted leaves and observed both after freezing at –12 and –15 °C (Fig. 3*B*).

After-effects of freezing observed during recovery (Exp. 2): Changes in photosynthetic apparatus observed during recovery were dependent on the levels of damage observed directly after freezing (Fig. 4). Photon absorption (ABS/CS) remained unaffected when measured directly after freezing (Figs. 2*C* and 3*B*), but the decrease in ABS/CS was visible just one day after freezing for plants frozen at –15 °C and declined dramatically at the 7th day of recovery. RCs remained undamaged in plants

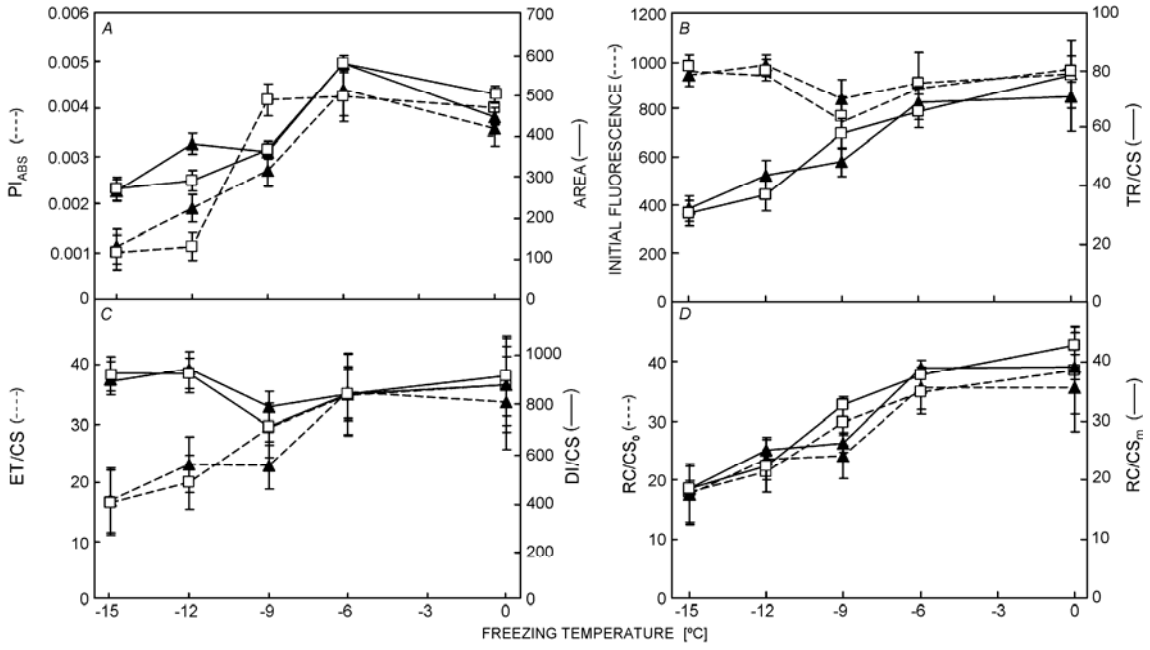

Fig. 3. Changes in chlorophyll *a* fluorescence induction parameters measured after freezing in five temperatures on pre-irradiated (30 s, PPFD = 800 μmol m^{-2} s^{-1}) leaves of winter wheat cv. Kobra (Exp. 1). Each parameter was measured either after 30 min of de-freezing at +5 °C in the dark (*closed triangles*) or after 30 min of de-freezing in the dark and following 90 min of irradiation at +5 °C and PPFD = 300 μmol m^{-2} s^{-1} (*open squares*). Means of 2 independent experiments ± standard errors, *n* = 20.

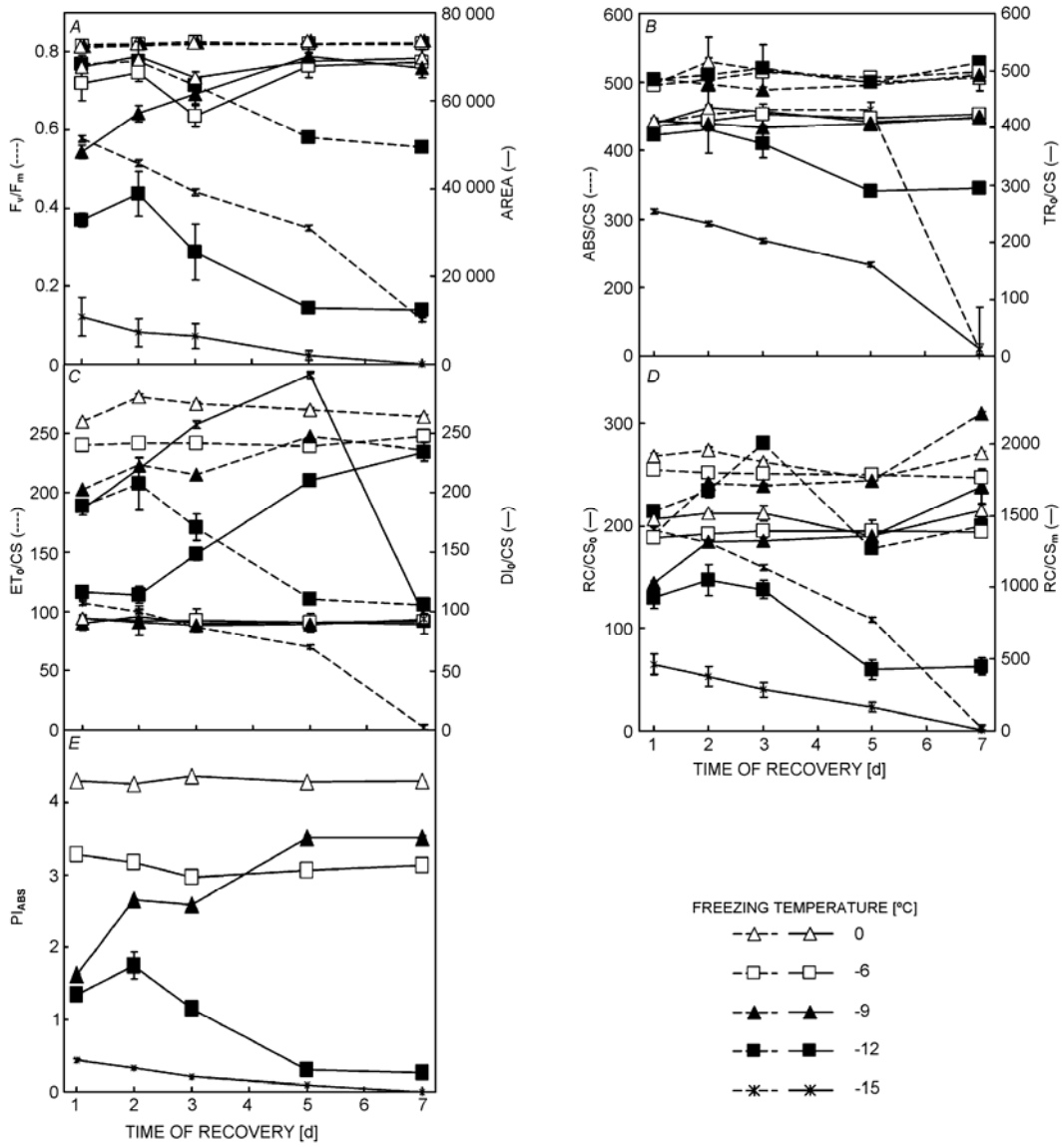

Fig. 4. Changes in chlorophyll *a* fluorescence induction parameters measured during recovery of winter wheat cv. Kobra plants frozen at different temperatures (Exp. 2). Measurements were taken always during the second hour of the day on dark-adapted leaves. Means of 2 independent experiments ± standard errors, $n = 20$.

treated with 0 (control), –6 °C, and –9 °C (F_v/F_m, Fig. 4*A*). For plants frozen at –12 and –15 °C, F_v/F_m was lower at the first day of recovery and decreased gradually during the experiment. Very similar changes were observed also for TR_0/CS (Fig. 4*B*). Area values for plants frozen at –9 °C increased during recovery, achieving the level of the control plants and the plants frozen at –6 °C by the end of the experiment (Fig. 4*A*). In the –12 °C frozen plants, Area increased on the 2nd d of recovery and thereafter decreased again. For –15 °C frozen plants Area was very low at the beginning and gradually decreased during the experiment. ET_0/CS was highest in the control plants during the whole period of recovery (Fig. 4*C*). On the 1st d of recovery the values of ET_0/CS were different for all freezing temperatures, but during recovery an increase in electron transport activity was observed in plants frozen at –6 and –9 °C. At the 5th d of recovery, values of ET_0/CS remained similar, but failed to reach the values found for the control plants. Plants frozen at –12 °C initially improved ET_0/CS, but from the third day decreases in ET_0/CS were observed. In the –15 ºC frozen plants, a gradual decrease of ET_0/CS was observed during the whole recovery period. Very similar results were obtained for the performance index PI_{ABS}, but in this case higher values at the end of the recovery period were observed in –9 ºC than –6 ºC frozen plants (Fig. 3*E*). DI_0/CS was similar and stable during the whole experiment for control, –6 ºC, and –9 °C frozen plants (Fig. 4*C*). In –12 ºC frozen plants DI_0/CS increased gradually, and in –15 ºC frozen plants an initial increase

followed by a decrease was observed. This fall was induced by destruction of the PS2 RCs and antennas in dying leaves (see also TR_0/CS and ABS/CS: if less energy was available in the PS2 RCs, then less energy was available for dissipation). The number of active RCs (RC/CS_0 and RC/CS_m) decreased gradually during the recovery of –15 ºC frozen plants (Fig. 3*D*). The –12 ºC frozen plants demonstrated a temporary increase in values of both parameters, that was particularly high in RC/CS_0 at day 3 (RC/CS_0 was even greater than in the control plants). Similar effects of increasing minimal (RC/CS_m) density of active RCs were visible also on the 7th d of the recovery period in plants frozen at –9 °C. The control and –6 ºC frozen plants showed similar and stable activities of the RCs.

Table 1. Changes in chlorophyll *a* fluorescence transients measured in three winter wheat cultivars of different freezing tolerance: Clever (C, with freezing tolerance of 1 in 1–9 score, as described in the text), Kobra (K, freezing tolerance 4), Tonacja (T, freezing tolerance 6). Measurements were taken on cold acclimated plants (3 weeks at +5/2 °C, day/night, photoperiod 10/14 h, PPFD = 300 μmol m^{-2} s^{-1}) before freezing (Control), after 5 h of freezing at –12 °C in the dark and 1 h of thawing at +2 °C also in the dark (Freezing), and after 5 d of recovery at +15 °C, (photoperiod 12/12 h, PPFD = 300 μmol m^{-2} s^{-1}, Recovery), all measurements according the Exp. 3 conditions. Means ± standard errors, n = 10. Values of the same parameter marked with *different letters* are significantly different (p<0.05, Duncan's test).

		Control	Freezing	Recovery
F_v/F_m	C	0.824 ± 0.010 a	0.770 ± 0.019 b	0.451 ± 0.023 d
	K	0.817 ± 0.011 a	0.767 ± 0.024 b	0.593 ± 0.023 c
	T	0.814 ± 0.011 a	0.771 ± 0.014 b	0.806 ± 0.012 a
Area	C	66400 ± 2205 b	38800 ± 2598 d	9400 ± 1322 e
	K	86600 ± 3640 a	48800 ± 2705 c	12800 ± 1528 e
	T	84400 ± 2674 a	70200 ± 3117 b	82400 ± 3006 a
PI_{ABS}	C	3.88 ± 0.18 a	1.15 ± 0.10 d	0.15 ± 0.03 f
	K	3.88 ± 0.15 a	1.61 ± 0.23 c	0.38 ± 0.08 e
	T	3.66 ± 0.15 a	2.74 ± 0.18 b	3.69 ± 0.20 a
ABS/CS	C	508 ± 31 a	501 ± 38 a	556 ± 24 a
	K	503 ± 15 a	514 ± 55 a	500 ± 19 a
	T	527 ± 24 a	522 ± 43 a	512 ± 38 a
TR_0/CS	C	419 ± 25 ab	386 ± 27 b	251 ± 21 d
	K	411 ± 13 ab	394 ± 37 ab	297 ± 24 c
	T	429 ± 23 a	403 ± 35 ab	413 ± 30 ab
ET_0/CS	C	257 ± 16 a	159 ± 12 c	87 ± 18 d
	K	254 ± 7 a	211 ± 18 b	123 ± 15 c
	T	251 ± 14 a	248 ± 11 a	262 ± 17 a
DI_0/CS	C	89 ± 5 d	115 ± 12 c	305 ± 21 a
	K	92 ± 2 d	120 ± 22 c	203 ± 23 b
	T	98 ± 7 cd	119 ± 8 c	99 ± 6 cd
RC/CS_0	C	264 ± 16 b	244 ± 29 bc	190 ± 68 d
	K	271 ± 7 b	219 ± 47 cd	182 ± 55 d
	T	314 ± 26 a	266 ± 16 b	263 ± 18 b
RC/CS_m	C	1502 ± 50 a	939 ± 49 d	347 ± 26 e
	K	1478 ± 40 ab	1064 ± 39 c	449 ± 57 e
	T	1687 ± 35 a	1164 ± 42 c	1354 ± 88 b

Effects of freezing on Chl *a* fluorescence transient in wheat cultivars contrasting in their freezing tolerance (Exp. 3): Before freezing, all cultivars showed only slight differences in PS2 activities (Table 1). Freezing tolerant Tonacja (T) showed a somewhat higher RC/CS_0 and freezing susceptible Clever (C) lower Area than the remaining cultivars. Directly after freezing, differences in some parameters of Chl *a* fluorescence between contrasting cultivars were higher and well-matched to the level of freezing tolerance. These concerned the parameters that characterized electron transfer after Q_A or overall performance of PS2 (Area, ET_0/CS, and PI_{ABS}). The highest values were observed in the freezing tolerant cv. T, intermediate in the less tolerant Kobra (K), and lowest in the freezing susceptible cv. C. At the same time, no differences between cultivars were observed in F_v/F_m, ABS/CS, TR_0/CS, and DI_0/CS. Differences in RC/CS_0 and RC/CS_m were only partially compatible with the cultivars' freezing tolerance. After 5 d of recovery, differences between contrasting cultivars were visible in

values of F_v/F_m, ET_0/CS, TR_0/CS, DI_0/CS, and PI_{ABS}. This implies that after recovery, contrasting cultivars were characterized by different degree of damage over the entire PS2. Additionally, the photosynthetic apparatus of the most tolerant cv. T almost recovered fully equivalent to that found for the controls, with the exception of the active RCs density, RC/CS_0, and RC/CS_m. Thus despite the recovery of function of the photosynthetic apparatus in the whole leaf (*e.g.* by the increase in mesophyll cell or chloroplasts number), a part of the RCs may still remain damaged.

Discussion

My results demonstrate that measurements of Chl fluorescence transient can be a very fast and objective method for estimation of freezing injuries. However, measuring protocols should be chosen very carefully taking into account events occurring in photosynthesizing cells during freezing and recovery.

The effectiveness of Chl *a* fluorescence measurements in freezing tolerance assessment depends on the correct adjustment of parameters and evaluating conditions (freezing temperature, time distance from freezing to measurement, irradiance after freezing, *etc*.). Similar conclusions were also made by Binder and Fielder (1996) who used variable Chl fluorescence techniques for studying changes in white spruce needles 24 h after freezing. They found that the effects of freezing on Chl fluorescence may depend on the cold acclimation stage and freezing temperature. No correlation between visible needle damage and Chl fluorescence attributes was observed for well acclimated plants (from October to December), whereas all studied fluorescence parameters correlated well with injuries in September. They observed a decrease in absorbed energy with increasing freezing stress whereas such a decline was not observed by Adams and Perkins (1993) in the same species. As demonstrated in the present paper a possible reason seems to be different terms of measurements. Adams and Perkins (1993) measured Chl fluorescence almost directly after freezing.

Thylakoid membranes were usually reported as the primary site of chloroplast freezing damages (Thebud and Santarius 1981, Griffith *et al.* 1982, Krause *et al.* 1988, Sror *et al.* 2003). Thylakoid membranes lose their ability for electron transport and photophosphorylation. Transient rupture of membranes is accompanied by the loss of internal soluble proteins and osmotically active solutes, leading to vesicle collapse. Strand and Öquist (1988) reported that in Scots pine damages in thylakoid structures lead to inhibition of the electron flow from Q_A, and they believed that irreversible freezing injuries are the consequence of damage to the Q_B protein. Similarly in my experiments early changes in Chl fluorescence observed directly after freezing developed from disturbances in energy flow from Q_A. However, changes observed initially in ET_0/CS, Area, and PI_{ABS} may be explained not only as an effect of minor membrane injuries. Krause *et al.* (1988) reported that during moderate freezing stress CO_2 assimilation is more readily affected by freezing than the activity of the thylakoids. In their experiment CO_2 assimilation and related fluorescence changes were the most freezing-sensitive parameters and the inhibition of CO_2 fixation in initial stages of damage was independent of thylakoid inactivation. Experimental data indicated that freeze-thaw treatment affected the light-regulated enzymes of the carbon reduction cycle. Inhibition of photon activation of these enzymes may in turn be based on altered properties of the chloroplast envelope.

Differences in the decrease in electron transfer after Q_A measured directly after freezing may be related to genetically based freezing tolerance in wheat cultivars. The only report concerning possibility of direct estimation of freezing injuries after freezing by means of Chl fluorescence measurements concerned the use of current photochemical efficiency of PS2 as an indicator (Janowiak *et al.* 2004). This parameter reflects changes in electron acceptors' activity, but its measurement is more complicated (during measurements PAR should be always the same) and takes more time than analysis of fluorescence transient.

In contrast to mild freezing injuries, damage caused by lower temperature induced changes in energy transfer from PS2 RCs to Q_A. Moreover, in this case damages were enhanced by short irradiation after freezing and were also better visible in pre-irradiated leaves. Irradiation for 90 min after freezing at –12 °C increased damage to the level observed without irradiation in leaves frozen at –15 °C. This was most clearly indicated by the increase of t_{Fm}. Such abrupt changes in electron acceptors' activity may be a consequence of damage in thylakoid structure. Sudden decrease in electron acceptors' activity was observed as secondary photoinhibitory injuries after freezing at –15 °C probably even after irradiation by pulses during measurements. In leaves frozen at –12 °C, photoinhibitory damage was enhanced by longer exposure during pre-irradiation before measurement or irradiation after freezing. These photoinhibitory changes include a decrease in electron transfer inside PS2 (TR_0/CS, F_v/F_m), an increase in energy dissipation (DI_0/CS), and an increase in initial fluorescence (ABS/CS = F_0 in dark-adapted samples). Increases in ABS/CS were observed clearly after freezing at –15 ºC and after 90 min of irradiation at –12 °C. According to Krause and Weis (1984) such a change should be considered as an effect of photoinhibition and not as a direct result of freezing.

Changes observed during recovery were distinct from those observed directly after freezing. During the recovery stage and dependent on the degree of injuries,

either regeneration or intensifying of the dysfunction of cells and tissues leading to plant death is observed (Levitt 1972). The increase in cellular dysfunction during this period, called secondary injuries, is connected with irreversible damage of the plasma membranes. Irreversible injured plasma membranes undergo a cytological aberration, namely, 'protoplasmic swelling' (Arora and Palta 1988). This cellular symptom is thought to be caused by replacement of Ca^{2+} from the membrane by extracellular K^+, and subsequent perturbation of K^+ transport properties of the plasma membrane. This is connected also with decreasing plasma membrane ATPase activity (Arora and Palta 1991). Further dysfunction of cellular functions after freezing may be due both to desiccation (Levitt 1972), increase in reactive oxygen species formation (McKersie *et al.* 1997), and photoinhibition in photosynthetically active cells (Lovelock *et al.* 1995, Verhoeven *et al.* 1996).

My results demonstrate that the photosynthetic apparatus of the plants frozen at –6 and –9 °C was gradually recovered. However, after 7 d of recovery, energy transfer after Q_A was still lower there than in control plants. On the other hand, after freezing at –12 and –15 °C the photosynthetic apparatus was gradually destroyed during recovery. In the plants frozen at –9 and –12 °C a transient increase in photochemical activity (ET_0/CS, Area, and PI_{ABS}) was observed on the second day of recovery. Similar activation was reported after mechanical wounding by Quilliam *et al.* (2006) and explained by the increasing demands for assimilates necessary for regeneration (sink activity). This may also explain the higher ET_0/CS and PI_{ABS} observed after freezing at –9 °C as compared to –6 °C. After 7 d of recovery, plants frozen at –15 °C showed almost complete inactivation of PS2 with F_v/F_m close to 0.

Gradual decrease in F_v/F_m observed during recovery in plants frozen at –12 °C indicates that F_v/F_m is a good indicator of secondary freezing injuries. After freezing at –12 ºC and 5 d of recovery different F_v/F_m values were also measured in wheat cultivars of diverse freezing tolerance. The usefulness of F_v/F_m measured during recovery was reported by Clement and van Hasselt (1996) and Rizza *et al.* (2001). However, F_v/F_m represents in fact only amount of energy trapped in PS2 RCs in relation to energy absorbed (Strasser *et al.* 2000). So, if damage of photosynthetic apparatus is triggered by disorder in energy flow from Q_A (caused for instance by the decrease in carboxylation or in pool size of acceptors due to mild damages of thylakoid membranes) F_v/F_m remains unaffected. Hence F_v/F_m is not a general indicator of freezing injuries.

The parameter which may be recommended as fitting the degree of freezing damage both directly after freezing and during recovery is PI_{ABS}. It may be also more sensitive to any change in photosynthetic apparatus as shown in Fig. 3. In this example, values of PI_{ABS} of samples which did not vary in F_v/F_m differed by 16 %. Performance Index, keeping a general overview of the photochemical events, was also successfully applied for determining condition of urban trees (Hermans *et al.* 2003) and has permitted precise documentation of the vitality decrease of beech fumigated with O_3 (Clark *et al.* 2000).

My results give an opportunity for the development of new, very fast, and simple methods for freezing tolerance screening by freezing of detached leaves and direct measuring of Chl fluorescence parameters such as PI_{ABS}, Area, or ET_0/CS. This should be verified in further experiments.

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