

# Non-Invasive Monitoring of the Light-Induced Cyclic Photosynthetic Electron Flow during Cold Hardening in Wheat Leaves

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The effect of irradiance during low temperature hardening was studied in a winter wheat variety. Ten-day-old winter wheat plants were cold-hardened at 5 °C for 11 days under light (250  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) or dark (20  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) conditions. The effectiveness of hardening was significantly lower in the dark, in spite of a slight decrease in the  $F_v/F_m$  chlorophyll fluorescence induction parameter, indicating the occurrence of photoinhibition during the hardening period in the light. Hardening in the light caused a downshift in the far-red induced AG (afterglow) thermoluminescence band. The faster dark re-reduction of  $P700^+$ , monitored by 820-nm absorbance, could also be observed in these plants. These results suggest that the induction of cyclic photosynthetic electron flow may also contribute to the advantage of frost hardening under light conditions in wheat plants.

**Key words:** Frost Tolerance, Photosynthesis, *Triticum aestivum* L.

## Introduction

Low temperature is one of the most important factors in limiting the growth and distribution of plants. Living organisms have evolved mechanisms which enable them to adapt to daily and seasonal fluctuations in temperature. Even in frost-tolerant species a certain period of growth at low, but non-freezing temperature is required for the development of frost hardiness. This phenomenon is called frost hardening. This cold acclimation includes several physical and biochemical processes including changes in the membrane composition (Szalai *et al.*, 2001; Wang *et al.*, 2006) and the accumulation of protective compounds (Galiba *et al.*, 1993; Kaplan *et al.*, 2006), including antioxidants (Kocsy *et al.*, 2001; Suzuki and Mittler, 2006) generated by a complex signal transduction network (Van Buskirk and Thomashow, 2006).

Winter cereals need not only to grow but also to develop at low temperature to achieve maximum cold tolerance to survive the winter, when the temperature is generally well below 0 °C. Pho-

tosynthesis provides the energy necessary for the attainment of a cold-acclimated state and may contribute to achieving the highest level of freezing tolerance. Thus, any environmental factor that has a negative impact on photosynthesis may ultimately influence the induction of freezing tolerance (Ensminger *et al.*, 2006). Due to its complexity, photosynthesis is one of the main targets of several types of stress. The potential for an energy imbalance between the photochemical processes, electron transport and the metabolism is exacerbated under conditions of either high light or low temperatures, leading to increased excitation pressure in Photosystem 2 (PS2). In an attempt to compensate for exposure to high PS2 excitation pressure, plants can acclimate by reducing energy transfer efficiency to PS2, either by diverting energy from PS2 to Photosystem 1 (PS1) through state transitions or by dissipating excess energy as heat by non-photochemical quenching (Huner *et al.*, 1998). Changes in PS2 excitation pressure are reflected in alterations in the redox state of PS2, which can be monitored *in vivo* by exploiting chlo-

rophyll-*a* fluorescence as a non-invasive probe. Photochemical fluorescence quenching (qP) can be used to estimate the proportion of open PS2 reaction centres, which reflects the relative oxidation state of  $Q_A$ , the primary quinone electron acceptor of PS2, providing a useful estimate of relative changes in the PS2 excitation pressure of organisms exposed to changing environmental conditions.

Thermoluminescence (TL) emission is a widespread phenomenon in various (mineral or organic) materials, which can be concisely described as an emission of light at characteristic temperatures from samples exposed to electromagnetic or particle radiation prior to being warmed up in the dark. A common feature of all TL phenomena is the storage of radiant energy in metastable trap states, which can be released via thermally stimulated radiative detrapping. [For details of the history and practical applications of photosynthetic thermoluminescence see Vass (2003); Ducruet (2003).] In all the higher plant species assayed so far, dark-adapted leaves exposed to far-red (FR) light exhibit both a TL B band, due to a charge recombination between the  $S_2/S_3$  states of the water splitting system and the secondary quinone acceptor,  $Q_B$ , downshifted by lumen acidification, and a band at about 40–45 °C that behaves similarly to the afterglow (AG) superimposed on luminescence decay at constant temperatures (Miranda and Ducruet, 1995a). Uncouplers, freezing and diuron-like inhibitors suppress the afterglow emission.

It has been shown that white light modifies the afterglow emission, which reflects the dark reduction of  $Q_B$  (Ducruet *et al.*, 2005a), in the same way as it triggers the cyclic pathway, as monitored both by the dark re-reduction kinetics of  $P700^+$  after FR illumination and by photoacoustic spectroscopy. Furthermore, the relaxation of the AG band towards its dark-adapted location after white light illumination occurs much faster in chilling-sensitive than in chilling-tolerant maize inbred lines, indicating that cyclic and/or chlororespiratory pathways might play a role in the tolerance of low temperature stress (Ducruet *et al.*, 2005b).

It was shown in winter rye plants that the induction of freezing tolerance during the frost hardening period is dependent not only on temperature but also on light. Hardening under low light conditions was much less effective than under normal light conditions (Gray *et al.*, 1997).

In this study it is shown that the induction of cyclic photosynthetic electron flow may also contribute to the advantage of frost hardening under light conditions in wheat plants.

## Materials and Methods

### *Plant material*

Seeds of winter wheat (variety Mv Emese; Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvásár, Hungary) were sown in plastic pots containing 3:1 (v,v) loamy soil and sand. The plants were grown for 10 d in a Conviron PGR 15 growth chamber with a 16 h/8 h light/dark period (Tischner *et al.*, 1997) at 20/18 °C (day/night) with 75% relative humidity and  $250 \mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetic photon flux density (PPFD) at the leaf level. Low temperature hardening was carried out in a chamber of the same type at a constant temperature of 5 °C either under the light conditions of normal growth (light-hardened plants) or at  $20 \mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD (dark-hardened plants). The measurements were carried out between the 10th and 12th day of hardening, assuming that no significant sudden changes took place during this period.

### *Determination of freezing tolerance*

To determine the ability of plants to tolerate freezing the pots were put in a frost chamber for 1 d at –10 or –12 °C in the dark. Then the frozen seedlings were cut off at ground level and the regrowth of the plants was evaluated after 2 weeks.

### *Chlorophyll-*a* fluorescence induction measurements*

The chlorophyll fluorescence induction parameters of the youngest fully expanded leaves were determined at ambient temperature using a pulse amplitude modulated fluorometer (PAM-2000; Walz, Effeltrich, Germany) as described earlier (Janda *et al.*, 1994). Before the measurements, the plants were dark-adapted for 30 min. Quenching parameters were calculated according to Schreiber *et al.* (1986). The nomenclature of van Kooten and Snel (1990) was used.

### *Thermoluminescence measurements*

TL measurements were performed using the laboratory-made apparatus and software described earlier (Ducruet and Miranda, 1992; Du-

cruet *et al.*, 1998; Janda *et al.*, 2000). Luminescence was detected with a Hamamatsu H5701-50 photomultiplier linked to an amplifier. A 4 × 4 cm Pel-tier element (Marlow Instruments, Dallas, Texas, USA) was used for temperature control. The leaf sample was gently pressed against the plate using a rubber ring and a Pyrex window, with the addition of 100  $\mu$ l water for better thermal conduction. The plants were dark-adapted for 2 h before the measurements. The samples were cooled to 0.1 °C within a few seconds and illuminated via a fiberoptic by far-red light provided by a PAM 102-FR light source (Walz) for 30 s (setting: 11) prior to the measurement. The rate of heating during measurement was 0.5 °C s<sup>-1</sup>. The TL measurements were repeated several times and representative curves are presented.

#### P700 measurements

A modulated PAM-100 fluorometer (Walz) was used for monitoring the kinetics of P700<sup>+</sup> reduction (Schreiber *et al.*, 1988; Ducruet *et al.*, 2005b). The redox state of P700 in leaves was measured as absorption at 820 nm using a dual-wavelength detector Walz ED-P700DW-E at room temperature. In both types of measurements, the detached leaf was approx. 1–2 mm from the light guide. The decomposition of re-reduction kinetics into exponentials was carried out with MATLAB software (MATLAB, 2006). First the records were low-pass filtered in order to suppress the high measurement noise, then decaying periods were selected. Each decay period was approximated by a second-order exponential curve. Curve fitting was carried out using the Steiglitz-McBride method (Steiglitz and McBride, 1965).

#### Statistical analysis

The results were the means of at least 5 measurements and were statistically evaluated using the standard deviation and T-test methods. Representative curves are shown for thermoluminescence.

## Results

### *Survival of freezing after hardening under different light conditions*

Ten-day-old winter wheat plants were cold-hardened at 5 °C for 11 days under different light conditions. The effectiveness of hardening was tested by freezing plants at –10 and –12 °C. All the unhardened plants died after 1 day of freezing even at –10 °C. Plants hardened under normal light conditions (250  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>) had a survival rate of 100% even after freezing at –12 °C. However, dark-hardened plants (at 20  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>) only partially survived both freezing temperatures (1% and 35% after freezing for 1 day at –12 and –10 °C, respectively).

### *Chlorophyll-a fluorescence induction measurements*

Several chlorophyll-a fluorescence induction parameters were measured to follow changes in the electron transport chain within PS2 (Table I). The  $F_v/F_m$  parameter, representing the maximum photochemical efficiency of PS2, slightly decreased in light-hardened plants, while there was no change in those hardened in the dark. Similar tendencies could be observed in  $\Delta F/F_m'$ , which represents the actual photochemical efficiency (Genty *et al.*, 1989), and photochemical quenching (qP): both were lower when hardening took place in the light and slightly higher in the dark compared with the unhardened control plants. Non-photochemical quenching (qN) significantly increased after hardening in the light.

### *Effect of hardening on the AG TL band*

The far-red illumination of leaves at low but non-freezing temperatures induces a thermoluminescence (TL) band peaking at around 40–45 °C (afterglow or AG band), together with a B band peaking below 30 °C. In unhardened control wheat plants grown for 22 days at 20/18 °C this

	Unhardened (control)	Light-hardened	Dark-hardened
$F_v/F_m$	0.813 ( $\pm$ 0.003)	0.757 ( $\pm$ 0.029)**	0.817 ( $\pm$ 0.004) <sup>ns</sup>
$\Delta F/F_m'$	0.611 ( $\pm$ 0.051)	0.429 ( $\pm$ 0.074)***	0.741 ( $\pm$ 0.020)***
qP	0.824 ( $\pm$ 0.033)	0.698 ( $\pm$ 0.066)**	0.948 ( $\pm$ 0.019)***
qN	0.187 ( $\pm$ 0.148)	0.536 ( $\pm$ 0.144)***	0.297 ( $\pm$ 0.085) <sup>ns</sup>

\*\*, \*\*\*, Significant differences compared to control plants at the 0.01 and 0.001 levels, respectively; ns, not significant.

Table I. Chlorophyll-a fluorescence induction parameters in the control and in plants cold-hardened under normal light (250  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>) or dark (20  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>) conditions.

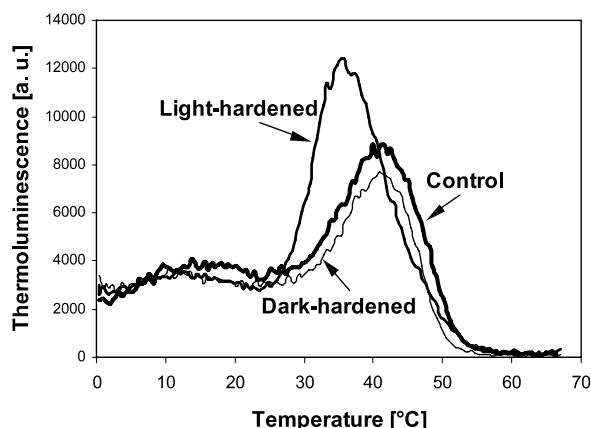


Fig. 1. Effects of cold hardening under different light conditions on the TL curve induced by 30 s FR light in winter wheat plants. TL was recorded at a heating rate of  $0.5^{\circ}\text{C s}^{-1}$ .

AG peak position was around  $41^{\circ}\text{C}$  (Fig. 1). In plants which were cold-hardened for 12 days in the light at  $250\ \mu\text{mol m}^{-2}\text{ s}^{-1}$  this peak position downshifted to  $36^{\circ}\text{C}$ . Dark-hardened plants did not show this effect (Fig. 1).

#### *Changes in the kinetics of $P700^{+}$ reduction after cold hardening*

Dark-adapted leaves of wheat plants were first illuminated for 50 s with FR light to oxidize  $P700$ , then the dark reduction kinetics was determined. Preliminary experiments showed that this period of FR illumination is sufficient to stabilize the reduction state of  $P700$ . Two exponential components could be fitted with the dark decay of the signal after 50 s FR illumination. The data in Table II show that hardening in the light enhanced the re-reduction of  $P700$  compared to the unhardened control plants. When hardening was carried out in the dark, there was no significant change in the time constant of the fast component; however, the second phase was even slower than in unhardened plants.

## Discussion

Using the electrolyte leakage method it has been reported that in rye plants low temperature hardening in the light is significantly more efficient than hardening under dark conditions (Gray *et al.*, 1997). Using a more direct test to determine the rate of survival of frozen plants, similar results were obtained in another cereal species, wheat. Even hardening in the dark increased the survival of winter wheat plants, but its effectiveness was substantially lower than hardening in the light. In winter rye tolerance to photoinhibition, the enhancement of maximum light-saturated photosynthesis, and the mRNA accumulation of certain cold-induced genes are correlated with PS2 excitation pressure (Gray *et al.*, 1997). The chlorophyll-*a* fluorescence induction method is widely used to detect the effects of low temperature in plants (Janda, 1998). The  $F_v/F_m$  parameter, which represents the maximum quantum efficiency of PS2, indicates that wheat plants suffered slight photoinhibition during low temperature hardening at  $5^{\circ}\text{C}$  in the light. Since the temperature dependence of the  $F_v/F_m$  parameter in the range of  $5\text{--}20^{\circ}\text{C}$  is negligible in wheat leaves (Janda *et al.*, 1994), this parameter did not show any difference after low temperature hardening in the dark. Both the actual quantum yield of PS2 and the  $qP$  are temperature- and light-dependent, which may explain the lower and higher values in light-hardened and dark-hardened plants, respectively. In accordance with the theory of Gray *et al.* (1997), this also means that the excitation pressure represented by the  $1-qP$  parameter was highest under light hardening conditions in wheat. Earlier results showed that cereals responded by increasing their capacity to keep the primary quinone acceptor of PS2 oxidized (Huner *et al.*, 1993; Janda *et al.*, 1994). Although it was suggested that this capacity was the main mechanism leading to enhanced tolerance of photoinhibition in cereals during low temperature hardening (Huner *et al.*, 1998), the decreased sensitivity to photoinhibition in rye also correlated

	Unhardened (control)	Light-hardened	Dark-hardened
t-fast	$1.41 (\pm 0.21)$	$0.86 (\pm 0.03)^*$	$1.34 (\pm 0.11)^{\text{ns}}$
t-slow	$6.27 (\pm 1.41)$	$4.44 (\pm 0.12)^*$	$17.9 (\pm 1.14)^{***}$

\*, \*\*\*, Significant differences compared to control plants at the 0.05 and 0.001 levels, respectively; ns, not significant.

Table II. Time constant (s) of the fast and slow exponential phases (t-fast and t-slow) of  $P700$  dark re-reduction after the oxidation of unhardened and hardened wheat plants by 50 s far-red light.

with enhanced qN (Gray *et al.*, 1997). The present results also suggest that the photosynthetic acclimation of wheat plants to high PS2 excitation pressure is coupled with increased non-photochemical quenching capacity, which may contribute to acclimation to low growth temperatures.

Due to the progress in the instrumentation of the TL technique, it has been more frequently used in stress physiological research in recent years, including investigations on the effects of low temperature stress (Janda *et al.*, 1999, 2000, 2004; Ducruet, 2003). Recently a decline was observed in the B band induced by two single turnover flashes in cold-hardened Scots pine needles, together with a downshift of its peak position, suggesting a narrowed redox potential gap between the primary quinone acceptor,  $Q_A$ , and the secondary quinone acceptor,  $Q_B$  (Sveshnikov *et al.*, 2006). It was shown in an earlier work that low growth temperature also caused a slight downshift in the  $T_{max}$  value of the B band induced by one flash in chilling-sensitive young maize plants (Janda *et al.*, 2000). In green algae the downshift in the B band after photoinhibitory treatment was assumed to be the consequence of either a conformational change in the D1 protein of PS2 (Ohad *et al.*, 1988) or the different extent of reduction in the Q and B bands (Janda *et al.*, 1992). However, it has also been shown that freezing leaf samples prior to TL detection may significantly alter the behaviour of the TL bands, so this must be considered in the evaluation of the results (Miranda and Ducruet, 1995a; Homann, 1999; Janda *et al.*, 2004). Acidification in the lumen of the thylakoid membrane may also lead to a downshift of the TL B band (Miranda and Ducruet, 1995b).

Due to the increased demand for ATP, cyclic electron flow is often triggered under stress conditions in plants (Manuel *et al.*, 1999). The induction of cyclic electron flow also contributes to pumping protons into the lumen, thus producing stronger non-photochemical quenching to dissipate the excess light energy. Such a role has also been considered for chlororespiration, which would maintain an acidic lumen pH value to protect the oxygen-evolving complex in the dark and may modify the activity of the cyclic electron flow (Peltier and Cournac, 2002). The FR illumination of unfrozen wheat leaves at low but non-freezing temperatures induces a TL B band peaking below 30 °C and an AG band peaking at around 40–45 °C (Janda

*et al.*, 1999). The  $T_{max}$  value of this B band is lower than that of the B band induced by a single white turnover flash excitation, which peaks at around 30–35 °C. In unhardened control winter wheat (variety Mv Emese) plants grown for 22 days at 20/18 °C this AG peak position was around 41 °C. Hardening at 5 °C in the light for 12 days caused an approx. 5 °C downshift in the  $T_{max}$  value of the AG band. This was not observed for dark-hardened plants. The  $T_{max}$  value of the TL AG band was usually lower in cold-tolerant maize genotypes than in sensitive ones and relaxed more slowly towards its dark-adapted location (Ducruet *et al.*, 1997, 2005b). The investigation of the re-reduction kinetics of P700<sup>+</sup> gives an indirect measurement of the cyclic electron flow (Schreiber *et al.*, 1988). The decay of the dark re-reduction of P700 can usually be decomposed into two exponential components representing two pathways of electron donations from stromal reductants to PS1 (Bukhov *et al.*, 2002). Luminescence, P700 and photoacoustic measurements consistently demonstrate that both cyclic and plastoquinone-reducing electron pathways are induced when plants are submitted to white light and that this induction lasts longer in cold-tolerant maize lines than in sensitive ones (Ducruet *et al.*, 2005b).

The hardening of wheat plants in the light, but not in the dark, also enhanced the re-reduction of P700 compared to the unhardened control plants. Similar observations were made in *Cucumis sativus* leaves under chilling conditions (Bukhov *et al.*, 2004). These results support the idea that the downshift in the AG band in cold-hardened plants is the result of the enhanced cyclic electron transport rate.

In conclusion, measurements on thermoluminescence and P700 relaxation kinetics suggest that growth at low, hardening temperatures may increase the activity of the cyclic photosynthetic electron transport chain, which may contribute to an optimal energy balance during periods of low temperature stress.

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