

The Effects of Cold Acclimation on Photosynthetic Apparatus and the Expression of *COR14b* in Four Genotypes of Barley (*Hordeum vulgare*) Contrasting in their Tolerance to Freezing and High-light Treatment in Cold Conditions

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Received: 30 October 2007 Returned for revision: 4 December 2007 Accepted: 17 December 2007 Published electronically: 2 February 2008

- **Background and Aims** Cold acclimation modifies the balance of the energy absorbed and metabolized in the dark processes of photosynthesis, which may affect the expression of cold-regulated (COR) genes. At the same time, a gradual acclimation to the relatively high light conditions is observed, thereby minimizing the potential for photo-oxidative damage. As a result, the resistance to photoinhibition in the cold has often been identified as a trait closely related to freezing tolerance. Using four barley genotypes that differentially express both traits, the effect of cold acclimation on freezing tolerance and high-light tolerance was studied together with the expression of *COR14b*, one of the best-characterized barley COR genes.
- **Methods** Plants were cold acclimated for 2 weeks at 2 °C. Freezing tolerance was studied by means of electrolyte leakage. Changes in photosynthetic apparatus and high-light tolerance were monitored by means of chlorophyll fluorescence. Accumulation of *COR14b* and some proteins important in photosynthetic acclimation to cold were studied with western analysis. *COR14b* transcript accumulation during cold acclimation was assessed with real-time PCR.
- **Key Results** Cold acclimation increased both freezing tolerance and high-light tolerance, especially when plants were treated with high light after non-lethal freezing. In all plants, cold acclimation triggered the increase in photosynthetic capacity during high-light treatment. In two plants that were characterized by higher high-light tolerance but lower freezing tolerance, higher accumulation of *COR14b* transcript and protein was observed after 7 d and 14 d of cold acclimation, while a higher transient induction of *COR14b* expression was observed in freezing-tolerant plants during the first day of cold acclimation. High-light tolerant plants were also characterized with a higher level of PsbS accumulation and more efficient dissipation of excess light energy.
- **Conclusions** Accumulation of *COR14b* in barley seems to be important for resistance to combined freezing and high-light tolerance, but not for freezing tolerance *per se*.

Key words: Chlorophyll *a* fluorescence, cold acclimation, *COR14b*, non-photochemical energy quenching, freezing tolerance, gene expression, *Hordeum vulgare*, photoinhibition, photosynthetic acclimation to cold, photosynthetic capacity.

INTRODUCTION

Temperature changes that trigger cold acclimation may be analysed by studying the photosynthetic apparatus (Ensminger *et al.*, 2006). Changes of temperature and light intensity modify the balance of energy absorbed during the light phase of photosynthesis and the energy metabolized in dark processes. These changes influence the relative state of photosystem II (PSII) oxidation (Huner *et al.*, 1998). Such changes can modify the balance between energy utilization in the processes of growth and cold acclimation (Rapacz, 1998*a, b*), as well as modifying expression of some genes directly involved in cold acclimation (Ndong *et al.*, 2001).

Among the cold-regulated genes, *COR14b* is one of the best-characterized in cereals (Crosatti *et al.*, 2003). This gene is differentially expressed in freezing-sensitive and freezing-tolerant plants under both laboratory and field conditions (Stanca *et al.*, 1996; Giorni *et al.*, 1999; Crosatti *et al.*, 2003). Accumulation of this protein is evidence of

a direct interaction between the cold-regulated (COR) gene expression originating from a cold-induced signalling pathway and the redox state of the chloroplast (Ensminger *et al.*, 2006). The redox state of chloroplast plastoquinone promotes the accumulation of *COR14b* protein in the light, while the level of *COR14* mRNA depends on cold treatment only (Dal Bosco *et al.*, 2003). *COR14b* protein is accumulated in the stromatal component of the chloroplast (Crosatti *et al.*, 1999).

On the other hand, the relative excess of light energy can lead to increased production of damaging reactive oxygen species (Müller *et al.*, 2001). In such circumstances, regulation of the balance between absorption and utilization of light energy is necessary, thereby minimizing the potential for photo-oxidative damage. To regulate and protect photosynthesis against this stress event, so-called low-temperature photoinhibition – a gradual acclimation to the conditions in which light-energy absorption exceeds the capacity for light utilization – has been observed during low-temperature growth (Huner *et al.*, 1993). Two general strategies of photosynthetic apparatus acclimation to relative excess of light can be observed in higher

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plants during cold hardening (Huner *et al.*, 1993). The first strategy is that of increasing light-energy consumption in photochemical processes by increasing photosynthetic capacity. This strategy is observed mainly in over-wintering herbaceous plants, which must accumulate high amounts of photoassimilates before and during winter. The second strategy relies on an intensification of protective non-photochemical mechanisms that harmlessly dissipate excess excitation energy as heat, which is more typical of evergreen trees and shrubs. However, it is hard to assume that non-photochemical mechanisms, which are the most important in equilibration of the absorption and utilization of light energy (Müller *et al.*, 2001) are not involved, partially at least, in any case of photosynthetic acclimation to cold. In herbaceous plants the development of a non-photochemical mechanism has been observed, depending on genotype (Rapacz *et al.* 2004; Humphreys *et al.*, 2007) as well as environmental factors (Adams *et al.*, 2002).

Changes in the relative redox state of PSII affect both increasing freezing tolerance and acclimation of photosynthetic apparatus to avoid low-temperature photoinhibition. Therefore, the resistance to photoinhibition in cold has often been identified as a trait closely related to cold tolerance (Huner *et al.*, 1998; Pockock *et al.*, 2001; Rapacz *et al.*, 2004) and plants that were not able to increase the resistance to photoinhibition were also not able to increase freezing tolerance during cold acclimation (Rapacz *et al.*, 2007). However, data obtained previously has indicated that the approximate correlation between winter hardiness (or freezing tolerance) and the ability of photosynthetic acclimation to cold was about 0.7, which means that, in the case of some plants, freezing tolerance and the tolerance to cold-induced photoinhibition are, at least partially, independent (Rapacz *et al.*, 2004). The aim of the present experiment was to characterize freezing tolerance and photosynthetic acclimation to cold in barley plants that differentially expressed both traits. Most attention was paid to the possible connections between *COR14b* expression, freezing tolerance and photosynthetic acclimation to cold.

MATERIALS AND METHODS

Plant material

The experiments were performed on four winter barley *Hordeum vulgare* L. varieties and advanced breeding lines of generations F_6 – F_8 ('Carola' and lines POA4006, POA4044 and POA4784) supplied by Dr W. Mikulski (Szelejewo Plant Breeding, Poland) and R. Madejewski (Piastr Plant Breeding, Poland), selected from about 60 breeding lines during three winters. These selected two- (POA4006 and POA4044) and six-rowed genotypes represented a high spectra of variation in freezing tolerance (as measured under field-laboratory and laboratory conditions), de-acclimation ability and also changes in photosynthetic apparatus observed during cold acclimation, which could suggest opposing abilities to adjust to cold and photosynthetic acclimation to cold (Rapacz *et al.*, 2008).

Plant growth and cold acclimation

Seeds of the plants studied were sown in 20-cm pots containing a sand : peat mixture (1 : 1, v/v) and placed in an air-conditioned greenhouse (+21/ +17 °C, day/night, with a natural photoperiod of about 12/12 h). After 7 d, plants were moved to a growth chamber [1 week at +20 °C, photoperiod 10/14 h, 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (PPFD); Philips AGRO sodium light source] and then cold acclimated (2 weeks at +2 °C, with the same PPFD and photoperiod). The experiment was carried out twice, in autumn 2006 and spring 2007. High-light treatment studies were repeated twice on plants growing in the spring, sown in two series at 1-week intervals. In this case, plants of particular genotypes were sown on different days to maintain the same length of cold acclimation period. Real-time PCR studies were done for the first time during the main experiment in the spring and were repeated in June 2007. All data presented in this paper are means from two independent experiments.

Measurements of chlorophyll fluorescence parameters before and during cold acclimation

All PSII chlorophyll *a* fluorescence measurements were made at room temperature with a FMS2 fluorometer (Hansatech, Kings Lynn, UK) on the middle section of the youngest, fully expanded leaves. Before measurements of minimum and maximum fluorescence of dark-adapted leaves (F_0 and F_m , respectively), leaves were dark adapted for at least 15 min in leaf-clips. Values of steady-state fluorescence of light-adapted leaves (F_s) and maximum fluorescence of light-adapted leaves (F'_m) were recorded sequentially when F_s became stable after re-exposure to actinic light (250 $\mu\text{mol m}^{-2} \text{s}^{-1}$). In the case of F_m and F'_m measurements, the saturating pulse intensity was about 10 000 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Next, minimal fluorescence of light-adapted leaves (F'_0) was measured on removal of the actinic light but with the addition of far-red light to ensure rapid opening of PSII reaction centres. Photochemical quenching coefficient (q_p) was calculated according to Schreiber *et al.* (1994): $q_p = (F'_m - F_s)/(F'_m - F'_0)$. Non-photochemical quenching (NPQ) was calculated as: $\text{NPQ} = (F_m - F'_m)/F'_m$ (Bilger and Bjorkman, 1991). Measurements were made in five replications of each experimental series, 1 d before cold acclimation started (day 0) and on the 7th and 14th days of cold acclimation.

Estimation of frost resistance

The youngest (but fully expanded) leaves were cut from plants (ten for each freezing temperature: -3, -6, -9, -12 and -15 °C), divided into 2-cm-long segments, and placed on ice (5 cm³ of frozen deionized water) in conductivity vessels. Then vessels were put into a programmed freezer with the temperature of 0 ± 0.5 °C. A freezing–thawing cycle was performed in darkness, separately for each freezing temperature. The temperature was decreased at a rate of 2 °C h⁻¹. Freezing temperature was kept for 90 min and then the temperature was increased up to 0 °C at a rate of approx. 3 °C h⁻¹. 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). The temperature causing a 50 % electrolyte leakage (T_{EL50}) was estimated from a linear regression fitted to the central part of the sigmoid relationship between the freezing temperature and electrolyte leakage, using at least three temperatures.

Responses of chlorophyll fluorescence in hardened and non-hardened plants exposed to high light

Plant response to photoinhibitory treatment was studied 1 d before cold acclimation started (non-acclimated plants) and on 14th day of cold acclimation (cold-acclimated plants). Chlorophyll fluorescence was measured using a pulse-amplitude-modulated fluorometer FMS2 (Hansatech) with saturating light flashes of $10\,000\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$. Measurements were done in an OxyLab leaf chamber (Hansatech) cooled with a water bath at $+5\ ^\circ\text{C}$. The temperature was controlled by an internal sensor (Hansatech). The initial measurements (F_0 , F_m) were made in dark-adapted (20 min) middle fragments of the youngest fully expanded leaves detached immediately before measurements were taken. Samples were subsequently exposed for 20 min to high light of $1200\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$, which was measured close to the leaf with an internal sensor (Hansatech), and provided by an LS2H lamp (Hansatech), supplied with a halogen light source, Decostar 51S 50W (Osram, Germany). After the exposure, F'_m and F'_0 were measured as described above and the next leaves were darkened. After 10 min of dark relaxation, maximum fluorescence (F_{m10}) was recorded and, after a subsequent 20 min of relaxation, minimum chlorophyll fluorescence after high-light treatment (F_{0HL}) was measured. On the basis of these measurements, q_p and the kinetics of relaxation of non-photochemical fluorescence quenching were calculated. The fast-relaxing (within the first 10 min of dark relaxation after light treatment) component of fluorescence quenching (qE) was assigned to the energy-dependent mechanism connected with photoprotection and the slowly relaxing (after 10 min of dark relaxation) component of fluorescence quenching (qI) was assigned to a mix of photoprotection and photodamages (Müller *et al.*, 2001). Quenching parameters of the Stern–Volmer type were calculated according to Thiele *et al.* (1997) as follows:

$$qE = (F_m/F'_m - F_m/F_{m10})$$

and

$$qI = F_m/F_{m10} - 1$$

To distinguish between photoprotective mechanisms and photoinhibition inside the qI component $(F_0 - F_{0HL})/F_0$ was proposed as the indicator of photoinhibitory damages. According to Gilmore *et al.* (1996), F_0 is decreased in direct proportion to F_m by the photoprotective quenching, whereas photoinhibition normally increases the F_0 level while decreasing F_m .

All measurements were done with eight replications (four for each experimental series).

The effect of non-lethal freezing on the response to high light

After 14 d of cold acclimation, plants were subjected during the night to non-lethal freezing ($-6\ ^\circ\text{C}$) for 4 h. Freezing and de-frosting rates were about $1.5\ ^\circ\text{C h}^{-1}$. After defrosting, plants were transferred back to the conditions of cold acclimation and reaction to the high-light treatment was measured as described above. Measurements were made on six replicates (three for each experimental series).

Protein accumulation

The western experiment was repeated twice with three replications (leaves) in each experiment. Samples were always collected at the same time of the day (between the 6th and 7th hour of the day), a day before cold acclimation and on the 1st, 7th and 14th day of hardening. Basic characteristics of the proteins and antibodies used in the experiment are presented in Table 1. Frozen leaves (one for each sample – the youngest and fully expanded) were ground to a fine powder in liquid nitrogen. Total leaf proteins were precipitated with 10 % (w/v) trichloro-acetic acid and 0.07 % (v/v) β -mercaptoethanol dissolved in acetone (5 mL for 1 g of the leaf). Proteins were separated by SDS–PAGE using the buffer system of Laemmli (1970). Exactly 5 mg of lyophilized protein powder were dissolved in 280 μL of loading buffer (4 % w/v SDS, 12 % w/v glycerol, 2 % v/v β -mercaptoethanol, 0.01 % w/v bromophenol blue in 50 mM Tris, pH 6.8), boiled for 2 min and

TABLE 1. Characteristics of detected proteins and antibodies used in the experiment, together with the dilutions applied

Protein	Mol. weight (kD)	Primary antibody	Secondary antibody (alkaline-phosphatase conjugated)
COR14b	14.4	Rabbit anti-COR14b raised against a synthetic polypeptide, AgriSera, Vännäs, Sweden 1 : 20 000	Goat anti-rabbit IgG (whole molecule), Sigma 1 : 50 000
SPS (sucrose phosphate synthase)	130	Chicken anti-SPS, AgriSera 1 : 40 000	Rabbit anti-chicken IgY (IgG) (whole molecule), Sigma 1 : 50 000
RbcL (RuBisCO large subunit)	58	Chicken anti-RbcL (global antibody), AgriSera 1 : 20 000	
PsbS	22	Chicken anti-PsbS (global antibody), AgriSera 1 : 40 000	
PsbA (D1)	32	Chicken anti-PsbA (global antibody), AgriSera 1 : 20 000	

centrifuged (7 min, 12000 *g*). Thirty microlitres of supernatant were loaded into a 12 % polyacrylamide gel with a 5 % stacking gel. Together with samples, molecular weight markers (Sigma) were loaded into the gel. Electrophoresis was run in Mini-Protean III apparatus (Bio-Rad, Hercules, CA, USA) as follows: 195 V, 45 min in the case of higher-molecular-weight proteins [Rubisco large subunit (RbcL) and sucrose phosphate synthase (SPS) together with high-molecular-weight range SigmaMarker] and 165 V, 55 min in the case of COR14b, PsbA and PsbS with low-molecular-weight range SigmaMarkers. In addition, a control sample from 14-d cold-acclimated 'Carola' plants was loaded on every gel. This enabled an indirect comparison of protein accumulation from different blots (different times of cold acclimation). In the cases in which observed accumulation of target protein in control samples was clearly different between membranes, such membranes were discarded.

Polypeptides from freshly electrophoresed gels were electrophoretically transferred (Semi-Dry Electrophoretic Transfer Cell, Bio-Rad) to ImmunBlot PVDF-membranes (0.2 μm pore size; Bio-Rad) by applying changing voltage (10 V, 15 min; 13 V, 15 min; 15 V, 15 min) and non-limiting current in transfer buffer (Tris-glycine, pH 9.0) as described by Towbin *et al.* (1979) and Kurien and Scofield (2003). The quality of the transfer and the position of the desired protein bands were checked by stacking the gel with 0.2 % (w/v) Coomassie brilliant blue R-250 (Sigma) and the membrane with ATX Ponceau S red staining solution (Sigma). Both gels and membranes were photographed with fixed settings (EOS 300D, Canon). After overnight blocking of the membrane with 2 % (w/v) fat-free milk powder in TBS and washing in TBS, the membrane was incubated with a primary antibody (Table 1) for 1 h. After the samples had been washed with TBS, polypeptide–primary antibody complexes were incubated for 1 h with a secondary antibody conjugated with alkaline phosphatase (Table 1) and washed in TBS and AP-buffer. The complexes were visualized using SIGMAFAST BCIP/NBT alkaline phosphatase activity detection kit. After staining, membranes were photographed with fixed settings (EOS 300D, Canon).

The COR14b antibody used in the experiments showed some cross-reaction with the second cold-induced band (of higher molecular weight), which has been reported before by Crosatti *et al.* (1999) for a different antibody, but, as was suggested there, only the lower molecular weight band was taken into consideration.

Analysis of COR14b transcript accumulation during the first day of cold acclimation

Plants were transferred at time 0 from darkness (+20 °C) to the light (250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD). For cold treatment, plants were transferred from +20 °C to +2 °C. The experiment was repeated twice. Each time, two samples were collected from each genotype.

Samples (approx. 0.04 g from the middle part of the youngest, fully expanded leaf) were collected for both control and cold-acclimated plants just before the transfer,

after 3 h and 6 h of the first day of cold acclimation, as well as in the 6th hour of the 7th and the 14th days of cold acclimation (150th and 318th hours of cold acclimation, respectively) and immediately frozen in liquid nitrogen. To analyse the expression of the *COR14b* gene by real-time RT PCR (Higuchi *et al.*, 1993), total RNA was isolated using RNeasy Plant Mini Kit (Qiagen) and then subjected to reverse transcription reaction integrated with removal of genomic DNA contamination (QuantiTect Reverse Transcription Kit, Qiagen). The final concentration and quality of cDNA was determined spectrophotometrically (Ultrospec 2100 Pro, supplied with ultramicrovolume cell; Amersham Biosciences, Buckinghamshire, UK).

PCR amplification was performed using a 7500 real-time PCR System (Applied Biosystems, Foster City, CA, USA) in 96-well plates using TaqMan MGB probes to detect dsDNA synthesis (Livak *et al.*, 1995). Each PCR reaction was done in triplicate, in 25 μL volume containing 900 nM of each primer, 250 nM of TaqMan MBG probe, 2.5 μL of cDNA (corresponding to approx. 38 ng), 12.5 μL of TaqMan Universal PCR Master Mix and 2.5 μL of RNase-free water. Reactions were run using default cycling parameters (initial denaturation at 95 °C for 10 min followed by 40 cycles of incubation at 95 °C for 15 s, and 60 °C for 1 min). In every cDNA expression of both *COR14b* and *actin* genes, a reference housekeeping gene (An *et al.*, 1996) was studied separately using: for *COR14b*, specific forward primer (AGACCCAGATCG ATGGCTTCT), specific reverse primer (GCACGGCCTG GGAAGAG) and specific probe (6-FAM-TCGGAGG AGGCGCG-MGB); and for *actin*, specific forward primer (TCCTCTGTGGAGAAGAGCT ACGA), specific reverse primer (TCAGCCCCAATGGTGATCA) and specific probe (6-FAM-TGCCCGATGGGCAG-MGB). Primers and probes were designed using Primer Express 3.0 software (Applied Biosystems) on the basis of *Hordeum vulgare* sequences published in the NCBI database [public domain information on the US National Library of Medicine web pages (<http://www.ncbi.nlm.nih.gov>); accession numbers: AJ512944, *COR14b*; AY145451, *actin*] and supplied by Applied Biosystems. Data were analysed using 7500 real-time PCR Sequence Detection Software v1.3 (Applied Biosystems). C_T values were determined as the numbers of amplification cycles needed to reach a fixed threshold in the exponential growth region of the amplification curve (representing fluorescence emission intensity due to production of a particular amplicon).

Expression of the *COR14b* gene in cold relative to the control was calculated with the $2^{-\Delta\Delta C_T}$ method as described by Livak and Schmittgen (2001).

RESULTS

Resistance to freezing and photoinhibition

Genotypes of winter barley showed different freezing tolerance (Fig. 1). The temperature in which 50 % of electrolyte leakage from leaf tissue was observed decreased clearly during cold acclimation in every genotype and this decrease was statistically significant only during the first week of

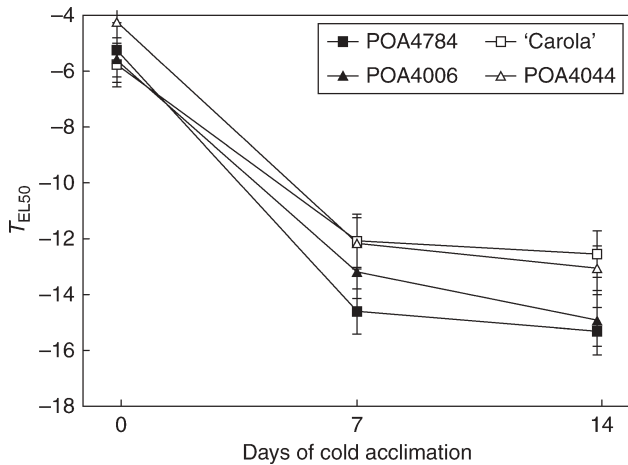


FIG. 1. Changes in freezing tolerance of winter barley genotypes during cold acclimation (2 °C, photoperiod 10/14 h, PAR = 250 μmol m⁻² s⁻¹). T_{EL50} means the temperature at which 50 % of total electrolytes were released from leaf tissues. Values of T_{EL50} and coefficient intervals for P = 0.05 were calculated on the basis of freezing tests made in two separate experiments at five freezing temperatures: -3, -6, -9, -12 and -15 °C; in total 20 replicates for each freezing temperature.

acclimation. During the second week, freezing tolerance increased only slightly and, on the basis of the measurements taken at this time, two groups of genotypes could be identified according to their freezing tolerance: POA4044 and 'Carola' were less freezing tolerant than POA4784 and POA4006, by about 2.5 °C on average. The results obtained confirmed previous data obtained during 3 years of field-laboratory studies (Rapacz *et al.*, 2008).

Changes in photosynthetic apparatus during cold acclimation were studied by means of chlorophyll fluorescence parameters (Table 2). A progressive decrease in apparent (maximum) quantum yield of PSII (F_v/F_m , where $F_v = F_m - F_0$) together with an increase in minimum fluorescence (F_0) was observed in every plant except 'Carola', which may suggest photodamage occurred during growth in cold (2 °C) and relatively high (250 μmol m⁻² s⁻¹) light intensity. Between 0 and the 14th day of cold acclimation a decrease in both F_v/F_m and F_0 was observed in 'Carola' (by 6 % and 4 %, respectively), while the photosynthetic apparatus of POA4784 and POA4006 can be

classified as the most cold sensitive (19 % decrease in F_v/F_m and 17 % increase in F_0 for POA4784 and 13 % and 32 % for POA4006). Changes observed in POA4044 were rather small (8 % decrease in F_v/F_m and 12 % increase in F_0). On the 14th day of cold acclimation, genotypes were characterized with diverse F_v/F_m values (in descending order: 'Carola', POA4044, POA4006 and POA4784).

A decrease in the photochemical chlorophyll fluorescence quenching coefficient (q_p) during cold acclimation was observed in every genotype and the highest decrease (by 37 %) was seen in POA4784. The coefficient of non-photochemical chlorophyll fluorescence quenching (NPQ) increased during cold acclimation in every plant and the highest increase was observed in POA4044 (by 28 %). The changes described are in fact a sum of two effects: cold-induced photodamage and photosynthetic acclimation to cold. A more detailed fluorescence study was performed under conditions of controlled high-light treatment. Short-term exposure of plants to very high light intensity in cold (20 min of 1200 μmol m⁻² s⁻¹ at 5 °C) gave an opportunity to indicate both the different sensitivities to photoinhibition of the genotypes studied as well as the presence of mechanisms of the photosynthetic acclimation triggered by 14 d of cold hardening (Table 3). Cold-acclimated plants showed higher values of q_p during high-light treatment compared with non-acclimated plants. The highest values of q_p , indicating the higher photosynthetic capacity of cold-acclimated plants were noticed in every plant, and the highest values were observed in the most freezing-tolerant POA4006 and POA4784.

An alternative mechanism of excess light utilization depends on excess light energy dissipation and is evident in higher values of the fast-relaxing component of NPQ, qE . Before cold acclimation the highest value of qE was measured in POA4006. During cold acclimation, qE increased in every genotype and the observed increase was lowest in POA4784 (5 %), resulting in the lowest values of qE observed in this genotype after cold acclimation and the highest in 'Carola' and POA4044 (by 10 % and 8 %, respectively). Although the increase in the slow-relaxing component of NPQ, qI , may sometimes be an effect of some acclimatory mechanisms, together with the increase in F_0 observed during high-light treatment, it can be evidence of a photoinhibitory event (Müller *et al.*, 2001). Before cold acclimation, POA4006 was less

TABLE 2. Changes in chlorophyll fluorescence parameters of four winter barley genotypes during cold acclimation at +2 °C

Genotype	Day of cold acclimation											
	0				7				14			
	F_0	F_v/F_m	q_p	NPQ	F_0	F_v/F_m	q_p	NPQ	F_0	F_v/F_m	q_p	NPQ
'Carola'	253 c	0.822 a	0.842 a	0.852 e	248 c	0.803 a	0.780 b	0.883 de	242 cd	0.775 b	0.694 c	0.930 cd
POA4006	273 bc	0.801 a	0.736 b	0.971 bc	318 b	0.752 bc	0.686 c	1.015 b	360 a	0.694 d	0.619 d	1.093 a
POA4044	291 b	0.805 a	0.780 b	0.864 e	310 b	0.777 b	0.738 b	0.958 bcd	325 b	0.743 c	0.680 c	1.107 a
POA4784	235 d	0.817 a	0.876 a	0.853 e	257 c	0.757 bc	0.785 b	0.910 cde	276 bc	0.659 e	0.548 e	0.992 bc

Means of ten replications from two independent experiments.

Values of the same parameter with the same letter did not differ significantly at P = 0.05 (Duncan's multiple range test).

TABLE 3. The response of winter barley plants non-acclimated, cold-acclimated (14 d at 2 °C) and cold-acclimated + subjected to non-lethal freezing temperatures (−5 °C during the night) to the high-light treatment (20 min of 1200 μmol m^{−2} s^{−1} at 5 °C)

Genotype	Plants											
	Non-acclimated				Cold acclimated				Cold acclimated and frozen			
	q_P	q_E	q_I	$(F_0 - F_{0HL})/F_0$	q_P	q_E	q_I	$(F_0 - F_{0HL})/F_0$	q_P	q_E	q_I	$(F_0 - F_{0HL})/F_0$
'Carola'	0.079 ^c	1.832 ^g	0.261 ^b	0.104 ^{ab}	0.103 ^b	2.010 ^{de}	0.196 ^c	0.025 ^e	0.103 ^b	2.011 ^{de}	0.195 ^c	0.030 ^{de}
POA4006	0.081 ^c	2.064 ^{cd}	0.198 ^c	0.045 ^{cde}	0.136 ^a	2.209 ^a	0.199 ^c	0.046 ^{cde}	0.135 ^a	2.136 ^b	0.238 ^b	0.059 ^{cd}
POA4044	0.078 ^c	1.937 ^{ef}	0.283 ^a	0.072 ^{bc}	0.105 ^b	2.093 ^{bc}	0.199 ^c	0.065 ^c	0.102 ^b	2.081 ^{bc}	0.200 ^c	0.069 ^c
POA4784	0.074 ^c	1.870 ^{fg}	0.273 ^{ab}	0.098 ^b	0.131 ^a	1.971 ^c	0.268 ^b	0.101 ^b	0.131 ^a	1.911 ^f	0.296 ^a	0.129 ^a

Photochemical quenching of chlorophyll fluorescence (q_P), components of non-photochemical quenching—fast-relaxating q_E and slow relaxating q_I , as well as the change in F_0 observed during high-light treatment: $(F_0 - F_{0HL})/F_0$ was measured in two independent experiments, total in eight or six (frozen plants) replications.

Values of the same parameter with the same letter did not differ significantly at $P = 0.05$ (Duncan's multiple range test).

susceptible to photoinhibition, while after cold acclimation POA4784 suffered most from high light, having the highest q_I and increasing F_0 by 10 % during high-light treatment. When cold-acclimated plants were subjected to non-lethal freezing (−5 °C) during the night before high-light treatment, susceptibility to photoinhibition and q_E was strongly influenced, while q_P remained almost the same (Table 3). The ability to dissipate energy, as indicated by q_E , decreased in POA4784 and POA4006 and probably was responsible for the increase in photoinhibitory damage observed in these two genotypes (q_I increased after freezing by 10 % and by 19 % in POA4784 and POA4006, respectively), but the increase in F_0 was statistically significant only in POA4784. In the case of 'Carola' and POA4044 both parameters remained unaffected.

Accumulation of COR14b and selected proteins from photosynthetic apparatus

Analysis of western blots showed some differences in protein accumulation between genotypes (Fig. 2). COR14b was below detection levels before cold acclimation (Fig. 2A). At the end of the first day of cold acclimation, the highest amount of protein was recorded in freezing-tolerant and susceptible-to-photoinhibition

POA4784, medium amounts were recorded in freezing-susceptible and tolerant-to-photoinhibition POA4044 and 'Carola', and the lowest amount was in POA4006. On the 7th day of cold acclimation, the picture was quite different – tolerant-to-photoinhibition POA4044 and 'Carola' accumulated more COR14b than susceptible-to-photoinhibition POA4784 and, especially, POA4006. On the 14th day the differences between genotypes were very similar but the accumulation level was lower.

The accumulation of D1, the core protein of PSII, was similar in all genotypes before cold acclimation and after the first day of cold acclimation (Fig. 2B). A transient increase of D1 accumulation was visible during the first day of cold acclimation. During the following days the level of accumulation was lower in POA4784 than in other plants.

On days 0 and 14, PsbS protein had accumulated in amounts proportional to the q_E values presented above (Fig. 2C and Table 3). Prior to cold acclimation, the highest accumulation of PsbS was observed in POA4006, which was also less sensitive to high-light treatment at that time. An induction of PsbS synthesis was observed on the 7th day of hardening and, during the 2nd week of cold acclimation, PsbS accumulation probably slightly decreased. In both terms, POA4784, which showed the

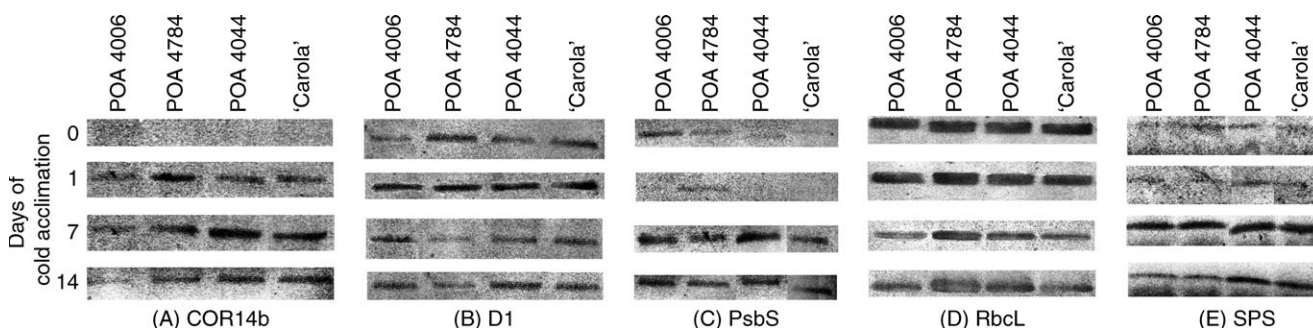


FIG. 2. Western blots of changes in the accumulation of (A) COR14b, (B) D1, (C) PsbS, (D) RbcL and (E) SPS proteins during cold acclimation (2 °C, photoperiod 10/14 h, PAR = 250 μmol m^{−2} s^{−1}) of winter barley genotypes. Representative scans were chosen from six replicated bands (three in each independent experiment).

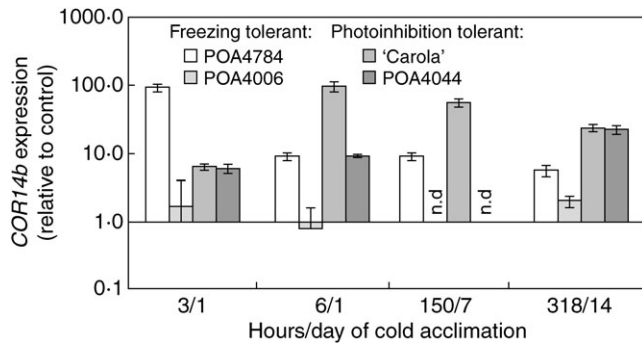


FIG. 3. *COR14b* transcript accumulation during cold acclimation at +2 °C, relative to non-acclimated plants. Both cold-acclimated and non-acclimated plants were transferred from +20 °C at time 0 from darkness to the light (250 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Means and errors for four leaves (cDNAs) from two independent series (12 real-time PCR reactions in total). n.d., No data.

lowest *qE* and was less susceptible to photoinhibition after cold acclimation, accumulated less PsbS than remaining genotypes.

Differences in the accumulation of RbcL during cold acclimation can be attributed neither to freezing- nor to photoinhibition-tolerance of plants (Fig. 2D). The highest accumulation during the first day of cold acclimation was observed in POA4784. In the following days in cold the accumulation level decreased.

On the 7th day of cold acclimation, a clear increase in SPS protein accumulation was observed (Fig. 2E). The accumulation seemed to decrease again on the 14th day, when the lowest accumulation was observed in freezing-tolerant genotypes POA4006 and POA4784.

COR14b transcript accumulation during the first day of cold acclimation

Real-time RT-PCR measurements showed that *COR14b* transcript levels were already affected by low temperature after 3 h (Fig. 3). At this time, differences in transcript accumulation between plants were very similar to those observed in *COR14b* protein accumulation 3–4 h later. The highest *COR14b* expression was evident for POA4784, a medium level was seen for POA4044 and 'Carola', whereas induction of transcript accumulation in POA4006 was within the range of measurement error. By the 6th hour of low-temperature treatment, a decrease in relative expression level was observed in freezing-tolerant POA4784 and POA4006, while in 'Carola' and POA4044, which are insensitive to low-temperature photoinhibition and susceptible to freezing, a further increase in transcript accumulation was observed. *COR14b* transcript accumulation was also higher in 'Carola' and POA4044 than in plants sensitive to photoinhibition after 7 d and 14 d of cold acclimation (6th hour of the day). Although data for POA4006 and POA4044 after 7 d of cold acclimation are missing, in the remaining plants the decrease in the transcript level was observed between the 7th and the 14th

day of cold acclimation, which was also visible in the case of protein accumulation (Fig. 2A).

DISCUSSION

The results presented here indicate that the genotypes studied showed different degrees of resistance to freezing and low-temperature photoinhibition. POA4006 and particularly POA4784 were freezing tolerant but susceptible to photoinhibition, while 'Carola' and POA4044 were freezing susceptible but tolerant to photoinhibition. Differences in sensitivity to photoinhibition were more visible when non-lethal freezing treatment preceded high-light treatment. POA4784 showed a similar reaction in previous experiments, having a very high survival rate after controlled freezing of plants cold-acclimated in the field (a 3-year experiment) and low freezing tolerance measured as the decrease in F_v/F_m after freezing (Rapacz *et al.*, 2008).

All genotypes studied in the present paper showed an increasing photosynthetic capacity during cold acclimation. This increase was connected with freezing tolerance rather than with resistance against photoinhibition in cold, which was in turn connected mainly with the increased ability to dissipate energy during high-light treatment. Previous studies conducted in other Poaceae (wheat and rye) and Brassicaceae showed that cold acclimation in these plants was accompanied by increased photosynthetic capacity (Huner *et al.*, 1998; Pocock *et al.*, 2001; Savitch *et al.*, 2002). However, studies on the *npq1* mutant of *Arabidopsis thaliana* revealed that the xanthophyll cycle and the associated NPQ seem to be relevant to the protection of photosynthesis against sudden increases in light intensity during cold treatment (Havaux and Kloppstech, 2001).

PSII protein, PsbS, belonging to the LHC protein superfamily but different in structure and pigment-binding characteristics (Funk *et al.*, 1995) is essential for *qE* (Li *et al.*, 2000). In the present experiment, accumulation of PsbS protein was associated with higher *qE* values both before and after 14 d of cold acclimation. Savitch *et al.* (2002) observed similar changes in pine (*Pinus contorta*) but not in winter wheat, indicating differences in excess light utilization strategies during cold acclimation between evergreen and herbaceous plants. In the present paper, changes in *qE* and PsbS accumulation were responsible for the different sensitivity to high-light treatment observed between genotypes. Three reasons are possible. (1) Savitch *et al.* (2002) studied wheat plants cold acclimated for a longer time (68 d). After such a long cold treatment, mechanisms different from those connected with PsbS and the xanthophyll cycle may be involved in photoprotection (Havaux and Kloppstech, 2001). In addition, in the present experiment some decrease in PsbS accumulation was probably visible between the 7th and the 14th days of cold acclimation. (2) The authors did not distinguish between *qE* and *qI*. (3) In that experiment plants were not affected by a sudden increase in high-light treatment when changes in *qE* are most important for photoprotection (Havaux and Kloppstech, 2001).

One of the first sites of photoinhibitory damage in thylakoids is the D1 polypeptide of the PSII reaction centre. The damaged D1 protein is degraded, and the recovery of PSII activity needs *de novo* synthesis of D1 (Aro *et al.*, 1993). Studies by Schäfer *et al.* (1993) showed that the actual level of D1 reflected the balance between D1 degradation and resynthesis. In addition, the changes in D1 accumulation did not depend on a pronounced increase of the slowly reversible non-photochemical quenching *q_L*. Cleland *et al.* (1990) also suggested that the loss of photochemical activity during photoinhibition can be uncoupled from D1 degradation. Savitch *et al.* (2002) showed a decrease in D1 accumulation 68 d after wheat was transferred to cold. The sudden increase in *PsbA* (D1-coding gene) transcript accumulation was observed in freezing- and photoinhibition-tolerant *Festuca pratensis*, which may suggest that the resistance to photoinhibition during the first days of cold treatment may be connected with activation of D1 resynthesis (Canter *et al.*, 2000). Comprehensive results were obtained in the present paper: D1 accumulation increased initially and then gradually decreased.

Freezing-tolerant genotypes of winter barley studied in the present experiment showed increasing capacity for photochemical quenching of excess light after cold acclimation, which suggests changes occurring in photosynthetic carbon metabolism. Changes in the accumulation of RbcL, ribulose-1,5-carboxylase-oxygenase, a main enzyme of the Calvin cycle, were not connected with either freezing tolerance or the tolerance against photoinhibition of the genotypes studied. A transient increase in RbcL accumulation was observed in POA4784 during the first day of cold acclimation. An increase in many Calvin cycle enzymes, including Rubisco activity, during cold acclimation has been observed repeatedly (Hurry *et al.*, 1994, 1995a, b). After long-term cold treatment the increase in Rubisco activity was higher in freezing-tolerant plants (Huner *et al.*, 1993; Hurry *et al.*, 1995b), which was attributed to increasing specific activity rather than increased accumulation.

In comparison to Rubisco, accumulation of SPS protein was associated with freezing tolerance of the genotypes of barley studied. After clear induction of SPS accumulation observed after 7 d of cold acclimation, a further decrease was observed and this decrease was higher in freezing-tolerant plants. This observation is in agreement with the results obtained by Bascunan-Godoy *et al.* (2006) for *Colobanthus quitensis*, an Antarctic plant with high freezing tolerance. Some increase in SPS accumulation was observed there after 7 d of cold treatment while, after 21 d, SPS protein levels returned to those of the control. It is probable that in freezing-tolerant genotypes an increase in SPS-specific activity, which has been reported many times for prolonged cold acclimation of freezing-tolerant plants (Hurry *et al.*, 1994; Savitch *et al.*, 2000; Bascunan-Godoy *et al.*, 2006), can compensate for a lower level of the protein.

The most interesting question that should be asked on the basis of the results presented here is about the role of COR14b protein during cold acclimation. The present

results indicate that accumulation of both *COR14b* transcript and protein after 1 and 2 weeks of cold acclimation is connected to the differences in photoinhibition tolerance, but not with freezing tolerance *per se*. In previous reports, observed accumulation of COR14b protein was higher in winter cultivars, while some variations for this character were detected in less-freezing-tolerant spring cultivars (Stanca *et al.*, 1996; Giorni *et al.*, 1999; Crosatti *et al.*, 2003). As in the case of the relationship between COR14b accumulation and freezing tolerance, the relationship between freezing tolerance and the tolerance of low-temperature-induced photoinhibition has been reported as a particular discriminant between spring- and winter-type plants, with spring plants, in general, being deficient in freezing and photoinhibition tolerance (Huner *et al.*, 1993). In addition, winter plants lose the ability to avoid photoinhibition after completing vernalization, or when the intense elongation growth starts (Rapacz, 2002; Gusta, 2006), and COR14b accumulation decreases in winter barley at later developmental stages (Crosatti *et al.*, 2008).

Although many advanced molecular studies have been performed on *COR14b*, little is certain about the function of the final protein produced. *COR14b* is a member of a small family of COR genes widespread in cereals and including wheat *WCS19* (Chauvin *et al.*, 1993), *WCOR14* (Tsvetanov *et al.*, 2000) and *WCOR15* (Takumi *et al.*, 2003), all of which encode chloroplast-targeted COR proteins analogous to the *Arabidopsis* protein COR15a (Lin and Thomashow, 1992; Thomashow, 1994). In addition, COR14b protein is targeted to chloroplast stroma (Crosatti *et al.*, 1999). The main function of COR15a protein in *Arabidopsis thaliana* seems to be *in vivo* stabilization of thylakoid membranes against freezing-induced damage, which was demonstrated in transgenic plants overexpressing this gene (Artus *et al.*, 1996). On the other hand, in the same paper some protective effects on isolated protoplasts were also observed, but this did not significantly affect the LT₅₀ values of the protoplast survival curves. Over-expression of *COR15a* increased the freezing tolerance of protoplasts frozen at temperatures between -4°C and -8°C (the protective effect on thylakoids was the highest over the same range), and decreased survival over the range of -2°C to -4°C . According to Uemura *et al.* (1995), the predominant form of membrane injury during freezing between -2°C to -4°C is expansion-induced lysis, while over the range of -4°C to -8°C membrane damage resulted from freeze-induced lamellar-to-hexagonal II phase transitions, which were observed both in the plasma membrane and various endomembranes, mainly in the chloroplast envelope. Thus, it seems possible that constitutive expression of *COR15a* might decrease the incidence of freeze-induced formation of hexagonal II phase lipids, which should be associated with increasing membrane fluidity (Uemura *et al.*, 1995). High membrane fluidity seems to be crucial not only for membrane protection from low-temperature stress but also for resistance to photoinhibition (Vijayan and Browse, 2002). Although an indirect photoprotective effect of COR14b via modification of thylakoid membrane

properties seems to be possible, the exact role of COR14b in photoprotection needs to be verified in future studies.

The decrease in *COR14b* transcript and protein accumulation observed in the present experiment after longer period of cold acclimation confirms the previous reports of Stanca *et al.* (1996) and Giorni *et al.* (1999). This effect may be connected with the fact that *qE* quenching of excess light energy in herbaceous plants is more important during the first few days after a low-temperature shift, and afterwards different protective mechanisms are developed (Havaux and Kloppstech, 2001; Müller *et al.*, 2001). One of the possible mechanisms of the down-regulation of COR14b may be higher photosynthetic capacity, which is fully developed after longer low-temperature growth (Huner *et al.*, 1993).

The results presented here showed that high levels of COR14b accumulation and tolerance to photoinhibition may not be necessary during the development of freezing tolerance. However, the differences between photoinhibition-susceptible and -tolerant genotypes were recorded under a very high-light treatment that triggers photoinhibition on a scale that usually does not apply under field conditions. Cold acclimation is also a very complex trait with a number of genes involved and the lowered expression of just one of them (with the exception of some regulatory genes) cannot easily decrease freezing tolerance (Fowler *et al.*, 1999).

The induction of COR14b expression may be assumed to be the symptom of co-ordinated gene expression regulated by the CBF family of transcription factors in the most important and best-recognized gene expression regulation network acting in cold acclimation (Chinnusamy *et al.*, 2006). COR14b contains a single CRT motif controlling the LT-responsiveness of the gene (Dal Bosco *et al.*, 2003). CBFs bind CRT elements and barley *HvCBF* products bind the COR14b CRT motif, implicating a direct interaction between the HvCBFs and *COR14b* gene regulation (Skinner *et al.*, 2005, 2006). During the first 3 h of cold acclimation, the highest (but transient) increase in *COR14b* expression was observed in the most freezing-tolerant genotype POA4784, but not in the second-most freezing-tolerant POA4006. The barley *CBF3* gene is transiently up-regulated within 15 min of exposure to cold (Choi *et al.*, 2002), thus an increase in *COR14b* expression, followed by down-regulation, could occur in POA4006 before the 3rd hour of cold acclimation. This hypothesis can be additionally supported by the observation that at the 6th hour of cold acclimation the accumulation of COR14b protein in POA4006 was at the level as in POA4784 and 'Carola', while the transcript level was lower both at that time and at the 3rd hour after transfer to cold; thus protein must have been accumulated earlier.

Finally, it can be concluded that increasing expression of COR14b may, on the one hand, lead to increasing light-energy dissipation via the *qE* component of NPQ and, on the other hand, may be a symptom of a more 'productive' gene expression network that runs during cold acclimation. As a result, the increase in energy dissipation during cold acclimation may, in average genotypes and conditions, reflect in fact the cold-acclimation ability of plants, as was observed by Rapacz *et al.* (2004) in the *Lolium-Festuca*

complex. Moreover, the increased ability to dissipate excess light observed in freezing-tolerant *Lolium-Festuca* hybrids as an effect of gene transfer from the more-freezing-tolerant *Festuca* to *Lolium* (Humphreys *et al.*, 2007) may indicate the transfer of some components of mechanisms controlling gene expression during cold acclimation and not the exigency of NPQ mechanism for the development of freezing tolerance.

ACKNOWLEDGEMENTS

Funding was provided by the Polish Ministry of Science and Higher Education (2 P06A 002 28).

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