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Cold Acclimation and Freezing Tolerance[1]

A Complex Interaction of Light and Temperature

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By comparing growth under five different temperature and irradiance regimes (20°C and 800, 250, and 50 μmol m^{-2} s^{-1} and 5°C and 250 and 50 μmol m^{-2} s^{-1}), we have examined the effects of light, temperature, and the relative reduction state of photosystem II on plant morphology, freezing tolerance (lethal temperature at which freezing injury occurs [LT_{50}]), transcript levels of *Lhcb* and two cold-stimulated genes (*Wcs19* and *Wcs120*), and photosynthetic adjustment in winter rye (*Secale cereale* L. cv Musketeer). We show, for the first time to our knowledge, that in addition to adjustments in photosynthetic capacity, nonphotochemical quenching capacity and tolerance to photoinhibition, the accumulation of the cold-induced transcript *Wcs19*, and the compact plant morphology usually associated with cold-hardening are correlated with the relative reduction state of photosystem II rather than with growth temperature or growth irradiance per se. In contrast, the acquisition of maximal LT_{50}, as well as *Lhcb* and *Wcs120* mRNA accumulation, appears to be dependent on both growth temperature and growth irradiance but in an independent, additive manner. The results are discussed with respect to the possible role of the modulation of chloroplastic redox poise in photosynthetic acclimation to cold-hardening temperatures and the attainment of maximal LT_{50}.

Low temperature is an important environmental factor that limits plant distribution, survival, and crop yields worldwide. Exposure to low, nonfreezing temperatures induces genetic, morphological, and physiological changes in plants, which result in the development of cold hardiness and the acquisition of freezing tolerance (Vasil'yev, 1961; Levitt, 1980; Guy, 1990; Thomashow, 1990; Huner et al., 1993). For example, the development of a prostrate or rosette growth morphology is assumed to be a morphological consequence of development at low temperature (Vasil'yev, 1961; Roberts, 1984) and has been used as a selection criterion for cold hardiness (Fowler et al., 1981; Roberts, 1984; Stushnoff et al., 1984; Blum, 1988).

Biochemical and molecular analyses have demonstrated differential gene expression and the accumulation of specific proteins during the induction of freezing tolerance (Guy, 1990; Howarth and Ougham, 1993; Lee and Chen, 1993; Thomashow, 1990, 1993). Cold-induced genes have been isolated and characterized in many species, such as alfalfa (*Medicago sativa*), *Arabidopsis thaliana*, barley (*Hordeum vulgare*), *Brassica napus*, and wheat (*Triticum aestivum*) (Houde et al., 1992; Chauvin et al., 1993, 1994; Dhindsa et al., 1993; Thomashow et al., 1993; Weretilnyk et al., 1993; Danyluk et al., 1994; Crosatti et al., 1995; Limin et al., 1995). There is a high correlation between the expression of some of these genes, which appear to be up-regulated by low temperature, and the development of freezing tolerance (Guy, 1990; Thomashow, 1990, 1993; Howarth and Ougham, 1993; Lee and Chen, 1993).

In wheat cold acclimation rapidly induces a specific set of *Wcs*, which subsequently disappear upon deacclimation. *Wcs* are regulated by low temperature at the transcriptional level and winter wheat cultivars exhibit higher levels of expression than spring cultivars (Houde et al., 1992; Chauvin et al., 1993; Danyluk et al., 1994; Limin et al., 1995). *Wcs19* is a nuclear gene specifically regulated by low temperature, but it also requires light for maximal induction (Chauvin et al., 1993). In addition, the polypeptide encoded by *Wcs19* has recently been localized to the chloroplast stroma (T. Krause, M. Houde, and F. Sarhan, unpublished results). Since the function of these cold-stimulated genes and their encoded polypeptides have not been demon-

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Abbreviations: Chl, chlorophyll; F_m, F_m', maximum fluorescence yield in the dark-adapted and light-adapted states, respectively; F_o, F_o', minimal fluorescence yields in the dark-adapted and light-adapted states, respectively; F_s, steady-state fluorescence yield; F_v, F_v', variable fluorescence yield in the dark-adapted and light-adapted states, respectively; F_v/F_m, maximal photochemical yield of PSII in the dark-adapted state; *Lhcb*, genes encoding Chl *a*/*b*-binding proteins; LT_{50}, lethal temperature at which freezing injury occurs; P_{max} O_2, maximum light-saturated rate of photosynthesis measured as oxygen evolution; Q_A, primary stable quinone electron acceptor of PSII; q_N, coefficient of nonphotochemical quenching; q_P, coefficient of photochemical quenching; $1 - q_P$, the relative reduction state of PSII; SSC, saline sodium citrate; *Wcs*, wheat cold-stimulated genes.

strated fully, it is difficult to assess their direct relevance to freezing tolerance. Although the polygenic trait of freezing tolerance has been investigated for some time, its regulation by environmental factors has not been elucidated (Guy, 1990; Thomashow, 1990, 1993). In addition, the mechanism by which low-temperature signals are perceived and transduced into specific biochemical responses is largely unknown (Trewavas and Gilroy, 1991; Dhindsa et al., 1993; Bowler and Chua, 1994).

Light, through the process of photosynthesis, provides the energy required for cold acclimation, which leads to the attainment of freezing tolerance (Dexter, 1933; Tysdall, 1933; Steponkus and Lanphear, 1968; Levitt, 1980; Gusta et al., 1982; Griffith and McIntyre, 1993). Huner et al. (1993) and Öquist et al. (1993) have demonstrated that the freezing tolerance of cereals is not only correlated with an increased capacity for q_P but is also correlated with an increased P_{max}. Therefore, any factor that chronically affects photosynthesis may ultimately influence the induction of freezing tolerance.

PSII "excitation pressure" is an estimate of the relative reduction state of PSII and may be calculated as $1 - q_P$, where q_P represents the Chl *a* fluorescence coefficient of photochemical quenching. Thus, conceptually, PSII excitation pressure is analogous to the term "Q_A-mediated redox backpressure" used by Dau (1994). Although $1 - q_P$ may not be related linearly to the absolute reduction state of Q_A (Dau, 1994; Schreiber et al., 1994), $1 - q_P$ does provide a useful estimate of the relative changes in the reduction state of PSII (Adams et al., 1995), and, hence, PSII excitation pressure, for plants exposed to different environmental conditions. PSII excitation pressure can be modulated to a similar extent either by keeping the growth temperature constant and changing irradiance or by keeping growth irradiance constant and changing the temperature (Gray et al., 1996). It has been proposed that modulation of PSII excitation pressure (Maxwell et al., 1995b) or the redox state of plastoquinone (Escoubas et al., 1995) regulates *Lhcb* expression and light-harvesting Chl *a/b* polypeptide accumulation in *Dunaliella* sp. These results are consistent with a recent report regarding light and temperature acclimation in the brown alga *Laminaria saccharina* (Machalek et al., 1996). Furthermore, we have suggested that the low-temperature-induced increase in tolerance to photoinhibitory irradiance in higher plants (Gray et al., 1996) and green algae (Maxwell et al., 1995a) can be explained on the basis of growth under conditions of high PSII excitation pressure. It has been proposed that photosynthetic acclimation associated with growth at high PSII excitation pressure may reflect adjustments to maintain a balance between photochemistry and metabolism (Melis et al., 1985; Horton et al., 1988; Dau, 1994; Anderson et al., 1995; Huner et al., 1996).

Does modulation of PSII excitation pressure affect processes other than photosynthesis? Since low growth temperature modulates PSII excitation pressure and is also required for the development of maximal freezing tolerance, we examined the influence of PSII excitation pressure on the transcript levels of *Lhcb*, the cold-stimulated genes *Wcs19* and *Wcs120*, plant morphology, as well as LT_{50}.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Winter rye (*Secale cereale* L. cv Musketeer) and spinach (*Spinacia oleracea* L. cv Savoy) were germinated from seeds in coarse vermiculite at a temperature of 20/16°C or 5/5°C (day/night) with a 16-h photoperiod in controlled environment chambers (Conviron, Winnipeg, Manitoba, Canada). The PPFD at pot height was adjusted to 50 or 250 μmol m^{-2} s^{-1} at 5°C (5/50 and 5/250, respectively) and 50, 250, or 800 μmol m^{-2} s^{-1} at 20°C (20/50, 20/250, or 20/800, respectively), as previously described (Gray et al., 1994, 1996).

Growth Analysis

Growth parameters of leaf dry weight and leaf area were determined for the second leaves of winter rye plants grown under all temperature/irradiance conditions. Leaves were harvested at various times after planting and the fresh weight was determined immediately. Leaves were dried at 80°C to a constant weight, and were subsequently brought to room temperature in a desiccator and the dry weight was obtained. Leaf area was acquired using a portable leaf area meter (model LI-3000, Li-Cor, Lincoln, NE). In all experiments, fully expanded second or third leaves of similar developmental ages were utilized, as determined by growth kinetic analyses.

Photosynthetic Measurements

CO_2-saturated O_2 evolution of 10-cm^2 leaf discs, composed of segments cut from the middle of several leaves, was measured in the gas phase at 20°C with an O_2 electrode (model LD2, Hansatech Instruments, King's Lynn, UK), as described by Gray et al. (1996).

Susceptibility to photoinhibition was monitored by in vivo room temperature Chl *a* fluorescence using a plant stress meter Chl fluorometer (Biomonitor S.C.I., Umeå, Sweden), as described previously (Gray et al., 1996). Photoinhibition of photosynthesis was induced at 5°C under ambient air conditions using the adaxial sides of leaf segments placed on moist filter paper in aluminum trays, as previously described (Gray et al., 1996). Susceptibility to photoinhibition was quantified by monitoring changes in F_v/F_m as a function of exposure time to an irradiance of 1600 μmol m^{-2} s^{-1}.

q_P and q_N parameters were measured in vivo by Chl *a* steady-state fluorescence under ambient CO_2 conditions using a PAM 101 Chl fluorometer (Heinz Walz, Effeltrich, Germany), as described by Gray et al. (1996). Leaves were dark-adapted at the measuring temperature for 1 h prior to the onset of Chl *a* fluorescence induction. PSII excitation pressure ($1 - q_P$) was measured at the growth temperature and growth irradiance as $(F_s - F_o')/(F_m' - F_o')$ (Adams et al., 1995) after steady-state photosynthesis had been attained. The time required to attain steady-state photosynthesis varied from 20 to 30 min, depending on the measuring temperature and measuring irradiance. q_N, corrected for F_o quenching, was measured after steady-state photosynthesis had been attained and was calcu-

lated as $1 - ([F_m' - F_o']/[\{F_m - F_o\}\{F_o'/F_o\}])$ (Bilger and Schreiber, 1986). F_o' was determined by the application of far-red light (>710 nm; Corning 7–69, Corning Glass Works, Corning, NY) after the actinic light was turned off during steady-state photosynthesis.

RNA Isolation and Gel-Blot Analysis

Plants were harvested 4 h into the photoperiod, immediately frozen in liquid nitrogen, and stored at −80°C until use. Total RNA was extracted essentially as described by Danyluk and Sarhan (1990). Leaf tissue was ground to a fine powder in dry ice using a mortar and pestle. The powder was mixed with equal volumes of the extraction buffer (100 mM Tris-HCl [pH 8.8], 100 mM NaCl, 5 mM EDTA, 1% [w/v] SDS, and 40 units mL^{-1} heparin) and phenol-saturated with 100 mM Tris-HCl (pH 8.8), containing 0.5% (w/v) 8-hydroxyquinoline at 60°C. The homogenate was allowed to cool to room temperature and an equal volume of chloroform:isoamyl alcohol (24:1, v/v) was added. After the sample was centrifuged at 6000*g* for 10 min, the aqueous phase was removed and the organic phase was washed again with the extraction buffer. The aqueous phases were combined and re-extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and once with chloroform:isoamyl alcohol (24:1, v/v). Total nucleic acids were precipitated with 2.5 volumes of cold ethanol and 0.1 volume of 3.0 M sodium acetate (pH 5.5) with 5 mM EDTA overnight at −20°C. Nucleic acids were collected by centrifugation at 6000*g* for 20 min and then washed in an equal volume of 3.0 M sodium acetate (pH 5.5), with 5 mM EDTA. The suspension was frozen at −80°C for 30 min, allowed to thaw at room temperature, and then centrifuged at 6000*g* for 20 min. This was repeated and the subsequent RNA pellet was washed once with cold 70% (v/v) ethanol, dried under a vacuum, dissolved in sterile water, and quantified spectrophotometrically by A_{260}.

Samples (10 μg of total RNA) were denatured in formamide-formaldehyde and mixed with ethidium bromide prior to electrophoresis on 1.5% (w/v) formaldehyde-agarose gels. This allowed for a visual evaluation of RNA quality and equal loading on the gel (Rosen and Villa-Komaroff, 1990). RNA was transferred to a Hybond-C Extra supported nitrocellulose membrane (Amersham) in 20× SSC overnight (1× SSC: 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). The membrane was air-dried and baked at 80°C for 1 h prior to hybridization with the ^{32}P-labeled probes for *Wcs19*, *Wcs120*, and wheat *Lhcb*, as previously described (Houde et al., 1992; Chauvin et al., 1993). The membrane was subjected to washes of decreasing SSC concentrations to a final wash of 0.1× SSC with 0.1% (w/v) SDS at 65°C and then autoradiographed on X-Omat-AR film (Eastman-Kodak) with Cronex Lightning Plus intensifying screens (DuPont) at −80°C. Transcript abundance was quantified by densitometric scanning on a computing densitometer (Molecular Dynamics, Sunnyvale, CA) coupled with ImageQuant software (Molecular Dynamics).

Freezing Tolerance

The ability of leaves to tolerate freezing was determined by changes in electrical conductivity (Dexter et al., 1932). Three leaf sections, approximately 15 mm in length, were wrapped in wet cheesecloth and placed in test tubes in a refrigerated circulating freezing bath (model LT-50DD, Neslab Instruments, Newington, NH). Each tube was seeded with an ice chip and equilibrated at −1°C for 1 h to initiate freezing and then cooled at a rate of 2°C h^{-1}. Tubes were removed at 5°C intervals and allowed to thaw overnight at 7°C. Samples were then equilibrated in deionized water for 24 h at 7°C under constant agitation. Electrical conductivity of the leachate was measured at room temperature, before and after the leaves were boiled, with a conductivity meter (model CDM3, Radiometer, Copenhagen, Denmark). The temperature at which 50% of the total conductivity was measured in the leachate was defined as the LT_{50} (Griffith and McIntyre, 1993).

Cellular Osmolality

Leaves were harvested 4 h into the photoperiod, quickly frozen in liquid nitrogen and ground in a prechilled mortar and pestle. The homogenate was centrifuged at 30,000*g* for 15 min at 4°C. The osmolality of the supernatant was determined with a vapor pressure osmometer (model 5100C, Wescor, Logan, UT), which had been calibrated using sorbitol as a standard.

RESULTS

Growth Kinetics

Leaves of winter rye grown at 20/250 reached full expansion between 15 and 25 d and those grown at 5/250 reached full expansion between 65 and 80 d (Fig. 1A). Leaves grown at 20/250 exhibited a 2.9-fold greater leaf area in comparison to leaves developed at 5/250 (Fig. 1A). However, the specific leaf weight of 5/250-grown leaves

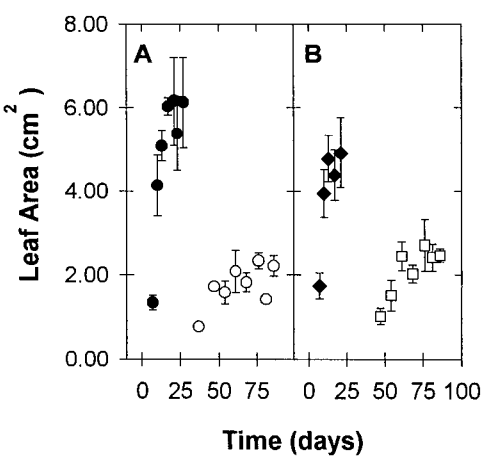

Figure 1. Growth kinetics for second leaves of winter rye developed at the temperature/irradiance regimes indicated. A, Leaf area for growth at 20/250 (●) and 5/250 (○). B, Leaf area for growth at 20/800 (◆) and 5/50 (□). Values represent means ± SE, $n = 3$.

(0.0067 ± 0.0005 g cm^{-2}) was 2.5-fold higher than that of 20/250-grown leaves (0.0027 ± 0.0003 g cm^{-2}). This is consistent with previous results for the development of cold-tolerant plants at low temperatures (Krol et al., 1984). Leaves developed at 20/800 reached full expansion between 10 and 20 d, whereas those developed at 5/50 reached full expansion between 70 and 80 d (Fig. 1B). Growth at 20/800 resulted in a 1.8-fold greater leaf area compared with leaves developed at 5/50 (Fig. 1B). Development at either 20/800 or 5/50 resulted in similar, specific leaf weights (0.0036 ± 0.0005 or 0.0029 ± 0.0001 g cm^{-2}, respectively). The cellular osmolality of rye leaves grown at 5/250 (678 ± 12 mosmol) was 1.5-fold higher than that of rye leaves grown at 20/250 (442 ± 3 mosmol), which is consistent with previous reports (Levitt, 1980). Decreasing growth irradiance at 5°C from 250 to 50 μmol m^{-2} s^{-1} had minimal effects on cellular osmolality (579 ± 43 mosmol), whereas increasing growth irradiance at 20°C from 250 to 800 μmol m^{-2} s^{-1} resulted in a 1.3-fold increase in osmolality (575 ± 6 mosmol). Based on these growth kinetics, we compared all plants in the vegetative state when maximum leaf area and dry weight had been attained, irrespective of growth temperature or irradiance (Krol et al., 1984).

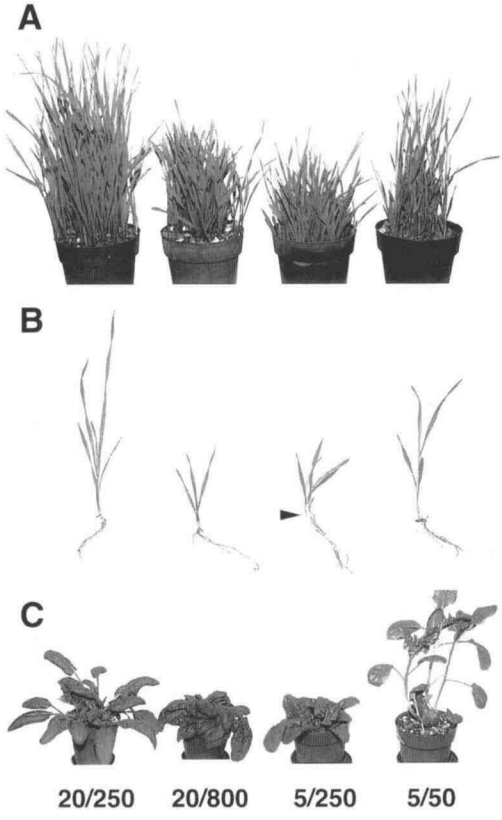

Figure 2. Plant morphology of winter rye and spinach developed at the temperature/irradiance regimes indicated. Plants were photographed at similar developmental ages based on growth kinetic analyses. A, Potted winter rye. B, Individual plants of winter rye; arrowhead indicates the crown. C, Potted spinach.

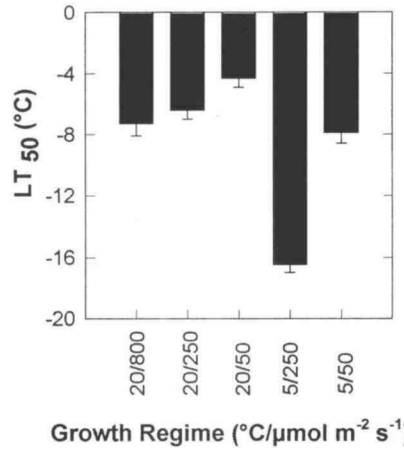

Figure 3. Freezing tolerance of winter rye. Growth occurred at the temperature/irradiance regimes indicated. Freezing tolerance was estimated by electrolyte leakage and expressed as LT_{50} (see "Materials and Methods"). Values represent means ± SE, $n = 3$.

Plant Morphology and Freezing Tolerance

Growth of winter rye at typical nonhardening conditions (20/250) resulted in an elongated plant morphology in contrast to the short, compact morphology associated with growth at cold-hardening temperatures (5/250) (Fig. 2, A and B). However, this difference in plant morphology cannot be due to a simple growth temperature effect, since growth at 20/800 also resulted in a compact morphology typical of plants grown at 5/250 (Fig. 2, A and B). Furthermore, rye plants grown at 5/50 exhibited the elongated plant morphology typical of nonhardened plants grown at 20/250 (Fig. 2, A and B). Although both 20/800- and 5/250-grown plants exhibited a compact growth habit, the crown of the 5/250 plants (Fig. 2B, arrowhead) was considerably larger compared with that of 20/800 plants (Fig. 2B). In addition, the plants grown at 5/50 exhibited greater stem elongation compared with those grown at 20/250 (Fig. 2B). Similar trends in growth habit were observed for the herbaceous cold-tolerant dicot, spinach (Fig. 2C).

Cold-hardened rye plants (5/250) exhibited a 2.6-fold greater freezing tolerance compared with nonhardened controls (20/250; Fig. 3). However, at 5°C, decreasing growth irradiance from 250 to 50 μmol m^{-2} s^{-1} resulted in a 52% decrease in freezing tolerance and an LT_{50} comparable with that of nonhardened control plants (20/250; Fig. 3). In addition, the LT_{50} of plants grown at 20°C decreased by 41%, as a function of varying growth irradiance from 800 to 50 μmol m^{-2} s^{-1} (LT_{50} = −7.3 and −4.3°C, respectively; Fig. 3). Although the induction of freezing tolerance is dependent on exposure to low temperature, the attainment of maximal freezing tolerance is clearly light-dependent. This is consistent with the results of Griffith and McIntyre (1993).

Wcs19 mRNA Accumulation

RNA gel-blot hybridization indicated that *Wcs19* mRNA levels were higher in rye plants grown at 5/250 than those grown at 20/250 (Fig. 4A). Furthermore, the light depen-

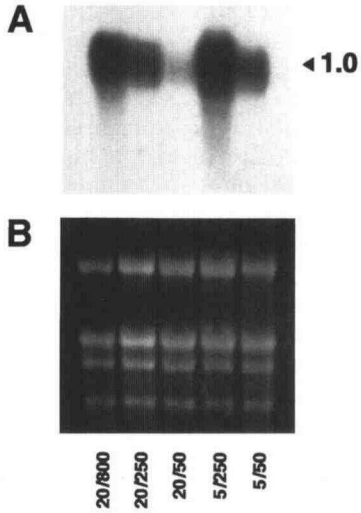

Figure 4. A, Accumulation of *Wcs19* mRNA in winter rye grown at the temperature/irradiance regimes indicated. Equal amounts (10 μg) of total RNA were subjected to formaldehyde-agarose gel electrophoresis and blotted to nitrocellulose membranes. The membranes were then hybridized with the ^{32}P-labeled probe for *Wcs19* (see "Materials and Methods"). A molecular size marker (kb) is indicated at the right. B, Ethidium bromide-stained gel.

dence of *Wcs19* accumulation was demonstrated by decreases in mRNA levels with decreasing irradiance at either 20 or 5°C (Fig. 4A). These data are consistent with previously published results (Chauvin et al., 1993). However, the level of *Wcs19* mRNA in 20/800 plants was similar to that of the 5/250 cold-hardened plants, despite the fact that the former had not been exposed to low temperature. In addition, the accumulation of *Wcs19* mRNA for plants grown at 5/50 was similar to that of plants grown at 20/250 (Fig. 4A).

Wcs120 and *Lhcb* mRNA Accumulation

In contrast to *Wcs19* mRNA, the accumulation of *Wcs120* (Fig. 5A) was greatest in cold-hardened plants (5/250), but this level decreased when rye was grown at 5/50. Lower, but relatively constant, levels of *Wcs120* mRNA were detected at all 20°C growth regimes (Fig. 5A). Maximum *Lhcb* mRNA accumulation (Fig. 5C) appeared to be dependent primarily on growth temperature in winter rye. Although *Lhcb* mRNA levels decreased with increasing growth irradiance at 20°C, rye light-harvesting Chl *a/b* polypeptide levels were similar under all growth conditions (Gray et al., 1996). However, the level of *Wsc120* and *Lhcb* mRNA in rye leaves grown at 5/250 was greater than that observed for leaves grown at 20/800.

Photosynthetic Acclimation to Low Growth Temperature and Irradiance

In experiments designed to examine cold acclimation and freezing tolerance, typical control plants were grown at nonhardening (20°C) and cold-hardening (5°C) temperatures and at a constant irradiance of 250 μmol m^{-2} s^{-1}. The underlying assumption is that any changes observed in these plants are the result of a growth temperature effect (Huner et al., 1993). However, Gray et al. (1996) concluded that the decreased sensitivity to photoinhibition exhibited by cold-hardened plants (5/250), relative to nonhardened controls (20/250), can be accounted for by the fact that cold-hardened plants have acclimated to higher PSII excitation pressure than nonhardened plants. Figure 6A illustrates that sensitivity to photoinhibition, estimated as F_v/F_m as a percentage of nonphotoinhibited controls, P_{max} O_2, as well as q_N are positively and linearly correlated with $1 - q_P$ experienced during growth. Furthermore, Figure 6D illustrates that, in cv Musketeer rye, the transcript levels of a cold-acclimation gene, *Wcs19*, is also correlated positively with $1 - q_P$ (Table I), the relative reduction state of PSII.

DISCUSSION

From the results presented in this report, we suggest that the typical experimental design used to elucidate photosynthetic adjustment to cold-hardening temperatures by comparing 20/250 and 5/250 plants is seriously flawed from a mechanistic point of view. The assumption in this experimental design is that any differences observed are due to growth temperature effects (Huner et al., 1993). However, our previous reports for green algae and higher plants (Maxwell et al., 1995a, 1995b; Gray et al., 1996; Huner et al., 1996), as well as the data presented here, show that this assumption is invalid. Previously, it was assumed that the cold-hardening-induced increase in P_{max} O_2 and the concomitant decreased sensitivity to photoinhibition

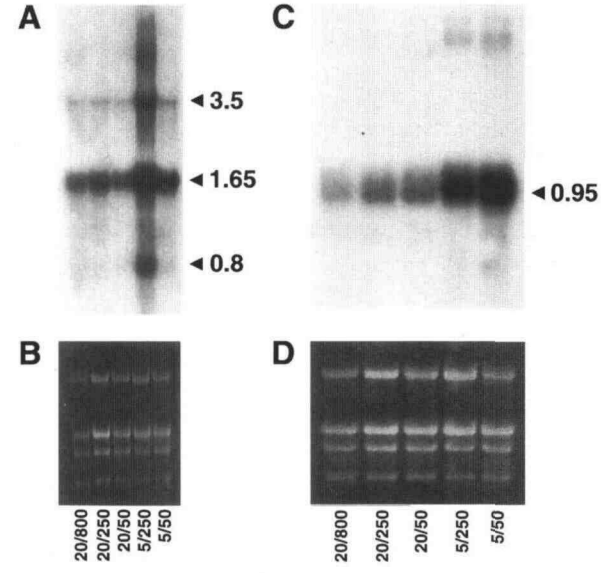

Figure 5. Accumulation of *Wcs120* (A) and *Lhcb* mRNA (C) in winter rye grown at the temperature/irradiance regimes indicated. Equal amounts (10 μg) of total RNA were subjected to formaldehyde-agarose gel electrophoresis and blotted to nitrocellulose membranes. The membranes were then hybridized with either a wheat *Wsc120* or a wheat *Lhcb* ^{32}P-labeled cDNA probe as described in "Materials and Methods." A molecular size marker (kb) is indicated at the right of each panel. B and D represent complementary ethidium bromide-stained gels.

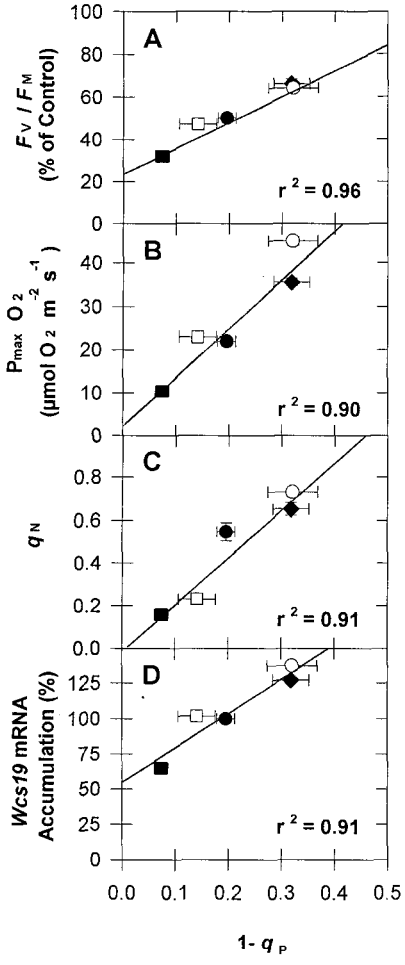

Figure 6. The correlation between susceptibility of rye to photoinhibition (A), P_{max} O_2 (B), q_N (C), and *Wcs19* mRNA accumulation (D) and $1 - q_P$. The relative reduction state of PSII was estimated as $1 - q_P$, as described in "Materials and Methods," and was measured at the growth temperature and growth irradiance. Susceptibility to photoinhibition was estimated by the decrease in the F_v/F_m ratio after a 4-h exposure to 1600 μmol m^{-2} s^{-1} at 5°C and calculated as a percentage of the F_v/F_m ratio prior to photoinhibition. q_N was determined at the growth temperature and irradiance and calculated as described in "Materials and Methods." *Wcs19* mRNA levels were quantified as described in "Materials and Methods" and normalized to that determined for leaves grown at 20/250. ■, 20/50; ●, 20/250; ◆, 20/800; □, 5/50; and ○, 5/250.

was strictly a low growth temperature effect (Huner et al., 1993). However, we have shown that the photosynthetic adjustments exhibited by cold-hardened rye (5/250) mimic those exhibited by high-light rye plants (20/800). Similarly, the photosynthetic response of nonhardened plants (20/250) is mimicked by low-light-grown rye (5/50) (Fig. 6; Gray et al., 1996). We suggest that these results can be explained by the fact that photosynthetic adjustment in rye reflects a response to temperature- or light-induced changes in the relative reduction state of PSII ($1 - q_P$), i.e. changes in PSII excitation pressure (Fig. 6). Thus, cold-hardened rye exhibits comparable sensitivity to photoinhibition to that of high-light-grown rye, because both have grown at similar high PSII excitation pressures. From the data presented in Figure 6, the decreased sensitivity to photoinhibition in rye appears to be correlated with both the P_{max} and also an enhanced q_N. Thus, we suggest that photosynthetic acclimation of rye to high PSII excitation pressure couples an increased photosynthetic capacity with an increased nonphotochemical quenching capacity, which, together, render rye less sensitive to photoinhibition.

As expected, not all low-temperature responses are due to changes in PSII excitation pressure. However, we suggest that by comparing plants grown under a combination of varying temperature and irradiance regimes one can begin to separate effects due to temperature from those due to either light or PSII excitation pressure. For example, in winter rye tolerance to photoinhibition, enhancement of P_{max} O_2, q_N, and *Wcs19* mRNA accumulation (Fig. 6) are correlated with PSII excitation pressure, whereas *Wcs120* mRNA accumulation (Fig. 5A), *Lhcb* mRNA accumulation (Fig. 5B), and the acquisition of maximum LT_{50} (Fig. 3) appear to respond to growth temperature and growth irradiance in an independent, but additive, manner.

Previously published results for higher plants and algae as well as those presented in this report indicate that photosynthetic adjustment to cold-hardening temperatures can be explained in terms of a response to PSII excitation pressure (Maxwell et al., 1995a, 1995b; Gray et al., 1996). Do the effects of PSII excitation pressure extend beyond the photosynthetic apparatus? This report demonstrates, for the first time, that PSII excitation pressure has a significant impact on *Wcs19* mRNA accumulation and plant morphology. Thus, a gene previously associated with cold acclimation appears to respond to the relative reduction state of PSII. Furthermore, we demonstrate that the compact growth morphology usually associated with cold hardening does not represent a response to low temperature per se but, rather, appears to be partly a response to changes in $1 - q_P$ (Fig. 2). The detection of light and signaling events in photomorphogenic development are typically associated with photoreceptors such as phytochrome, as well as the blue light and UV light photoreceptors (von Arnim and Deng, 1996). However, our results for the compact growth morphology cannot be due to phytochrome for the following reasons. First, the compact growth form was observed with no change in photoperiod or light quality. Second, the compact plant morphology was induced in both monocoty-

Table I. *Effect of growth temperature and growth irradiance on* $1 - q_P$

The relative reduction state of PSII, estimated as $1 - q_P$, was determined at the growth temperature and growth irradiance regimes indicated and calculated as described in "Materials and Methods." All values represent means ± SE, $n = 3$.

Growth Regime	$1 - q_P$
°C/μmol m^{-2} s^{-1}	
20/800	0.318 ± 0.034
20/250	0.196 ± 0.017
20/50	0.074 ± 0.013
5/250	0.321 ± 0.047
5/50	0.141 ± 0.035

ledonous and dicotyledonous plants by simply decreasing the growth temperature from 20 to 5°C at a constant irradiance of 250 μmol m^{-2} s^{-1}.

Whereas plant morphology appears to be sensitive to the relative reduction state of PSII, changes in freezing tolerance (LT_{50}) cannot be explained on the basis of changes in $1 - q_P$. Based on the classic morphological selection criterion of short, compact growth morphology (Fowler et al., 1981; Roberts, 1984; Stushnoff et al., 1984; Blum, 1988), one would expect 20/800 plants to be as freezing tolerant as the 5/250 cold-hardened plants (Fig. 2). However, the former were much less freezing tolerant than the latter (Fig. 3). This is consistent with our observation of increased crown size in the 5/250-grown plants in comparison with the 20/800-grown plants, despite the similarity in plant morphology (Fig. 2). Thus, we show that the correlation between plant morphology and freezing tolerance in herbaceous plants is serendipitous and, thus, should not be considered a reliable selection criterion on its own in a breeding program for freezing tolerance.

Overall, our data indicate that the regulation of maximal freezing tolerance in herbaceous plants may be much more complicated than previously assumed. We suggest that this process appears to be a complex interaction of low temperature, light, and chloroplast redox poise, as reflected in the modulation of the relative reduction state of PSII. The transduction pathways associated with each of these signals probably interact not only with each other but also likely act synergistically with other important signal transduction pathways involving phytochrome (Bowler and Chua, 1994), sugar sensing (Jang and Sheen, 1994; Koch, 1996), Ca^{2+} (Trewavas and Gilroy, 1991; Monroy and Dhindsa, 1995; Knight et al., 1996), and plant growth regulators (Bohnert et al., 1995) to elicit the appropriate physiological responses. To elucidate such a complex phenomenon as the acquisition of freezing tolerance, we suggest that the contribution(s) of all three potential signals must be taken into consideration. We believe that a strength of the experimental design illustrated in this report is that it allows one to distinguish between physiological processes, as well as between genes that are regulated by low temperature and those that are either regulated by light alone or by the integration of light and temperature, as estimated by the relative reduction state of PSII, which we suggest reflects alterations in chloroplastic redox poise. Since maximal LT_{50} is dependent on both temperature and light (Fig. 3), we suggest that the physiology of the plant may supersede the genetic potential of the same plant in the ultimate expression of maximal freezing tolerance. We believe that this has very important implications for a breeding program, as well as research into the genetic engineering of freezing-tolerant plants.

In summary, we show, for the first time to our knowledge, that the effects of PSII excitation pressure appear to extend beyond photosynthetic acclimation, to influence plant morphology and expression of a nuclear gene involved in cold acclimation. Thus, we suggest that plant morphology, cold acclimation, and photosynthetic acclimation reflect, in part, a response to a chloroplastic redox signal. Clearly, any attempts to increase levels of freezing tolerance or perhaps the tolerance to any environmental stress must take into account the complex interaction between abiotic stresses such as temperature, nutrient status, water availability, and the fundamental energy source for all photoautotrophs, light.

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