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Survey of gene expression in winter rye during changes in growth temperature, irradiance or excitation pressure

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Abstract

Previous comparisons of winter rye plants (*Secale cereale* L. cv. Musketeer) grown in a combination of specific temperature (°C)/irradiance (μmol m^{-2} s^{-1}) regimes (20/50; 20/250; 20/800; 5/50; 5/250) revealed (1) that photosynthetic acclimation to low temperature mimics photosynthetic acclimation to high light because both conditions result in comparable reduction states of photosystem II (PSII), that is, comparable PSII excitation pressure; (2) that the relative redox state of PSII also appears to regulate a specific cold acclimation gene, *Wcs19*. In order to identify additional genes regulated differentially by either low temperature, irradiance or excitation pressure, we initiated a detailed analysis of gene expression. We identified and characterized 42 differentially expressed genes from wheat and rye. Based on their patterns of regulation under the five growth conditions employed, 37 of the cDNAs could be classified into four groups: genes regulated by PSII excitation pressure, low temperature, growth irradiance and interaction between growth temperature and irradiance. Partial sequence analyses revealed that several of these genes encode known chloroplastic proteins such as ELIPs, transketolase, carbonic anhydrase and Mg-chelatase. However, five of the genes could not be classified unambiguously into any one of these four categories. The implications of these results and the limitations of the experimental design are discussed in terms of larger-scale genomic studies designed to understand the interactions of multiple abiotic stresses to which a plant may be exposed when examining regulation of gene expression.

Abbreviations: Chl, chlorophyll; F_m, F'_m, maximum fluorescence in the dark- and light-adapted state respectively; F'_o, minimal fluorescence yield in the light-adapted state; LI, light intensity; LT, low temperature; PAR, photosynthetic active radiation; PPFD, photosynthetic photon flux density; Q_A, primary stable quinone electron acceptor of PSII; qp, coefficient of photochemical quenching; 1–qp, the relative reduction state of PSII; Rep, Wep, rye and wheat excitation pressure respectively

Introduction

Acclimation of cold-hardy plant species to low, non-freezing temperatures induces specific morphological, physiological and molecular changes that result in the development of cold hardiness, which is associated with the acquisition of maximum freezing tolerance (Levitt, 1980; Steponkus, 1984; Guy, 1990; Thomashow, 1998). In winter-hardy cultivars of *Triticum aestivum* L., cold acclimation up-regulates the expression of a set of wheat cold-regulated (*Wcor*) genes to higher levels than in less hardy spring cultivars (Houde *et al.*, 1992; Chauvin *et al.*, 1993; Ouellet *et al.*, 1993; Limin *et al.*, 1995; Danyluk *et al.*, 1998). Although freezing tolerance is clearly temperature-dependent, the attainment of maximum freezing tolerance is light-dependent (Dexter, 1933; Gray *et al.*, 1997). Morphologically, the development of a compact, rosette growth habit, which is associated with cold acclimation of winter cereals, is correlated

with freezing tolerance and has been used as a selection criterion in breeding for cold hardiness (Fowler and Carles 1979; Levitt, 1980). Levitt (1980) assumed that this growth habit was solely a consequence of growth and development at low temperatures.

Recently, it was reported that photosynthetic acclimation to low temperature mimics photosynthetic acclimation to high light in winter cereals such as rye and wheat (Gray *et al.*, 1996, 1997, 1998), green algae (Maxwell *et al.*, 1994, 1995a, 1995b) and cyanobacteria (Miskiewicz *et al.*, 2000). These results have been explained on the basis of the notion that plants do not necessarily respond to absolute growth irradiance or absolute growth temperature per se but, rather, respond to PSII excitation pressure, that is the relative reduction state of Q_A, the first stable electron acceptor of PSII (Huner *et al.*, 1998). Thus, cold-acclimated rye and wheat grown at 5 °C/250 μmol m^{-2} s^{-1} (5/250) exhibit a similar tolerance to photoinhibition as plants grown at high light (20/800) because both cold-acclimated and high-light plants are exposed to comparable high excitation pressure measured as 1–qp (Adams *et al.*, 1995; Gray *et al.*, 1996). Similarly, non-acclimated rye and wheat grown at 20/250 exhibit a similar sensitivity to photoinhibition as plants grown at low temperature but low light (5/50) because both non-acclimated plants and plants grown at 5/50 are exposed to comparable low excitation pressure. Further, Gray *et al.* (1997) showed that excitation pressure rather than low temperature regulates the short, compact growth habit exhibited by cold-acclimated wheat, rye and spinach as well as the expression of the nuclear-encoded *Wcs19* gene. Thus, regulation by chloroplastic redox poise extends beyond photosynthetic acclimation and influences plant morphology through some unknown long-distance signalling process. Recently, it was suggested that H_2O_2 may be involved in this long-distance signalling process regulated by excitation pressure (Karpinski *et al.*, 1999). Although the morphology of wheat and rye grown at 20/800 mimicked that of cold-acclimated plants, growth at high light was not correlated with development of freezing tolerance. However, maximum freezing tolerance was dependent on both low temperature and light in an independent but additive manner (Gray *et al.*, 1997).

It appears that specific aspects of photosynthetic acclimation as well as cold acclimation are not regulated by either absolute growth irradiance or the absolute growth temperature but, rather, PSII excitation pressure which reflects the relative redox state of Q_A. Thus, we maintain that the typical experimental design used to elucidate the mechanisms of cold acclimation by comparing non-acclimated plants grown at 20/250 with cold-acclimated plants grown at 5/250 is flawed. The underlying assumption is that differences observed at the morphological, physiological, biochemical and molecular levels between plants grown at 20/250 and 5/250 are due to differences in growth temperature only. However, this assumption is invalid since plants grown at 20/250 and 5/250 differ significantly with respect to excitation pressure. To separate low-temperature effects from the effects of excitation pressure, the experimental design must include the following comparisons: for the effects of low excitation pressure, plants grown at 20/250 must be compared to plants grown at 5/50; for the effects of high excitation pressure, plants grown at 5/250 must be compared to plants grown at 20/800 (Gray *et al.*, 1997, 1998). In addition, by employing an experimental design which includes plants grown at 20/50, 20/250, 20/800, 5/50 and 5/250 one is able to separate the effects due to temperature alone and from those due to light alone from those due to excitation pressure. The present study was undertaken to determine if our experimental design can be employed as a general tool to identify new plant genes whose expression is regulated by excitation pressure as well as to separate those genes whose expression is under the control of either light alone, temperature alone or light plus temperature in an independent but additive manner. Detailed analysis of gene expression revealed that 37 genes could be classified into four broad groups based on the type of regulation whereas five genes could not be classified unambiguously. The implications of our results and the limitations of our experimental design are discussed in terms of larger-scale genomic studies designed to test environmental regulation of gene expression.

Materials and methods

Plant material and growth conditions

Seeds of winter rye (*Secale cereale* L. cv. Musketeer) were germinated 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, Manitoba, Canada). Fluorescent tubes (cool white, 160 W, F72T12/CW/VHO Sylvania) provided a photosynthetic active radiation (PAR) which was adjusted

to a photosynthetic photon flux density (PPFD) of 50 and 250 μmol m^{-2} s^{-1} at 5 °C (5/50 and 5/250 respectively) and either 50, 250 or 800 μmol m^{-2} s^{-1} at 20 °C (20/50, 20/250 or 20/800 respectively). Supplemental lighting was provided in the 20/800 growth chamber by a metal halide lamp (MS400-HOR, 400 W; Venture Lighting, Cleveland, OH). The PPFD was measured at pot height with a Li-Cor (Lincoln, NE) quantum/radiometer/photometer (model LI-189) equipped with a model LI-190SA quantum sensor (Li-Cor). Hoagland nutrient solution was supplied to all plants as required.

PSII excitation pressure measurement

For each measurement, a leaf disc was constructed from the mid portions of four leaves from four different plants grown at one of the five regimes. Chlorophyll (Chl) *a* fluorescence induction curves were collected with a pulse-modulated Chl fluorescence system (PAM 101, Heinz Walz, Effeltrich, Germany) employing the leaf discs dark-adapted for 30 min under ambient CO_2 conditions. Minimum PSII fluorescence in the dark-adapted state was excited by a non-actinic, modulated measuring beam (0.12 μmol m^{-2} s^{-1}) at 1.6 kHz. Maximum fluorescence (F_m) was induced by saturating white-light pulses (800 ms, 5700 μmol m^{-2} s^{-1}: KL 1500, Schott Glaswerke, Mainz, Germany) and controlled by a trigger control unit (PAM 103, Heinz Walz). Leaves were infiltrated with 10 μM DCMU to ensure that the white light pulses were saturating. The actinic light corresponded to a growth irradiance of either 50, 250 or 800 μmol m^{-2} s^{-1}. F'_m was calculated with the extrapolation method of Markgraf and Berry (1990). Three flashes of decreasing intensity (5700, 2850 and 1400 μmol m^{-2} s^{-1}) were applied after steady photosynthesis had been attained. F'_o was determined immediately after turning off the actinic light in the presence of far-red light (>715 nm; Corning 7-69; Corning Glass Works, Corning, NY) to ensure maximal oxidation of Q_A. All measurements were made at the corresponding growth temperature of either 5 or 20 °C. PSII excitation pressure was estimated as 1–qp, where qp is the Chl *a* fluorescence photochemical quenching parameter (Schreiber *et al.*, 1986), and was measured at the growth temperature and irradiance described in detail previously (Gray *et al.*, 1996). All fluorescence parameters obtained from leaves exposed to actinic light were calculated after steady-state photosynthesis had been attained. All data are the averages of three or four separate experiments.

Growth kinetic analyses indicated that 6–8-week old leaves from plants germinated and grown at 5 °C were at a developmental age comparable to that of 2–3-week old leaves from plants germinated and grown at 20 °C. For all five conditions, fully expanded second, third and fourth leaves were used in all experiments. Leaves were harvested 3 h after the beginning of the light and stored at −80 °C until analyses.

Purification of poly(A)$^+$ RNA, in vitro *translation and 2D polyacrylamide gel electrophoresis*

Total RNA and poly(A)$^+$ RNA were prepared as previously described (Danyluk and Sarhan, 1990). Poly(A)$^+$ RNA was translated *in vitro* with a wheat germ extract from Promega in the presence of ^{35}S-methionine and proteins were prepared for 2D polyacrylamide gel electrophoresis by the phenol extraction procedure described by Hurkman and Tanaka (1986). The *in vitro* translation products were separated by IEF/SDS-PAGE (Danyluk and Sarhan, 1990) with the following modifications. The gels in the first dimension (IEF) contained 2% w/v ampholines pH 5–8 (BioRad), 1% w/v ampholines pH 3–10 and 1% w/v ampholines pH 3–5. Equal levels of radioactivity (5×10^6 cpm) were loaded on each gel, and the gels were focused for 14 h at 800 V. Two-dimensional protein separation was performed in triplicate for each *in vitro* translation reaction. After the second dimension the gels were fixed, stained with 0.1% w/v Coomassie blue, destained, dried and autoradiographed with Kodak XAR-5 film at −80 °C for two weeks.

Construction and screening of the cDNA library

Poly(A)$^+$ RNA from plants grown at 20 °C/800 μmol m^{-2} s^{-1} were used to synthesize double-stranded cDNAs (Amersham kit) and ligated to *Xho*I-*Eco*RI adaptors. The cDNAs were purified in a Sephacryl S-300 column (Pharmacia) and then ligated to the vector Uni-ZAP (Stratagene). The library was screened with ^{32}P-labelled cDNA probes prepared from poly(A)$^+$ RNA isolated from plants grown at 20 °C/800 μmol m^{-2} s^{-1} (high light excitation pressure plants) and at 20 °C/250 μmol m^{-2} s^{-1} (control plants). The plaques showing a differential hybridization signal with these probes were selected and purified with standard molecular biology techniques (Sambrook *et al.*, 1989). ESTs used in this study were

Table 1. The effect of growth temperature and irradiance on 1–qp. All data represent the means ± SD from three independent experiments.

Growth regime	1–qp
20/50	0.031 ± 0.009
20/250	0.134 ± 0.057
2/800	0.325 ± 0.062
5/50	0.143 ± 0.025
5/250	0.352 ± 0.023

randomly chosen from this rye high-light cDNA library and the wheat cold-acclimated cDNA library described previously (Houde *et al.*, 1992).

Northern blots and DNA sequencing

Total RNA (5 μg) samples were separated on formaldehyde agarose gels as described by Rosen and Villa-Komaroff (1990). After electrophoresis, RNA was transferred onto nitrocellulose membranes and hybridized with the different ^{32}P-labelled cDNA inserts. Filters were washed at 65 °C with several buffer changes of decreasing SSC concentration (5–0.1×) and autoradiographed on Kodak X-Omat-RP films with intensifying screens at −80 °C.

The cDNAs were sequenced at their termini with the T7 sequencing kit (Pharmacia). A computer-aided search of protein and DNA sequences was carried out with the FASTA and TFASTA programs in the Genetic Computer Group's sequence analysis software Wisconsin package, version 10.0.

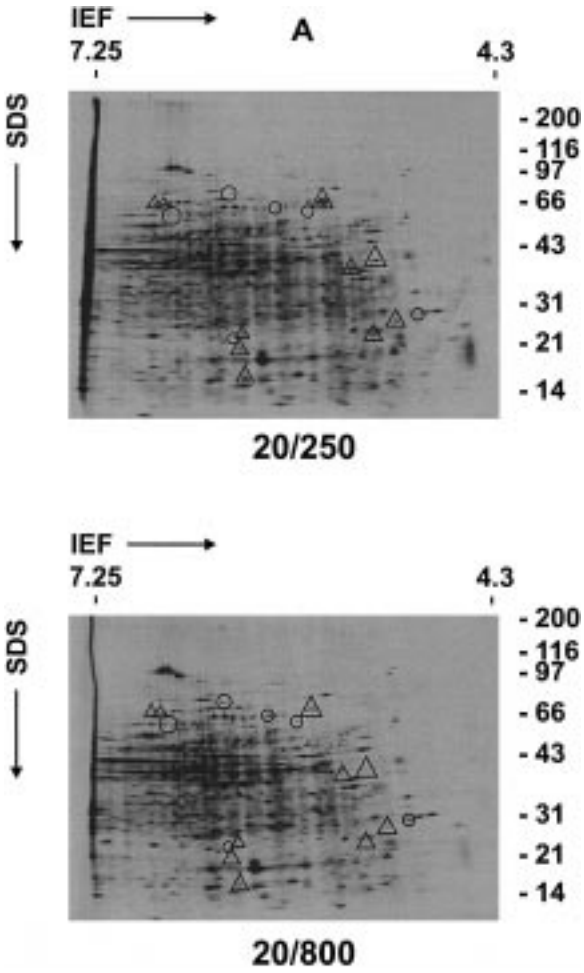

Figure 1. Patterns of *in vitro* translation products synthesized from mRNA isolated from rye plants exposed to 20 °C and 250 μmol m^{-2}s^{-1} (20/250) (panel A) and from plants exposed to 20 °C and 800 μmol m^{-2} s^{-1} (20/800) (panel B). Circles indicate translation products or groups of products that increased during high-light growth while triangles indicate products that decreased during high-light growth. $M_r \times 10^{-3}$ of standard proteins are indicated on the right-hand side.

Results and discussion

Effects of growth temperature and growth irradiance on PSII excitation pressure

Cold acclimation in cereals is a complex interaction of low temperature (LT), irradiance and excitation pressure, which reflects the relative redox poise of the photosynthetic electron transport system (Gray *et al.*, 1997; Huner *et al.*, 1998). Table 1 summarizes the values for PSII excitation pressure estimated as 1–qp and measured for rye plants used in this study that were grown at either 20 °C or 5 °C and increasing irradiance. The results show that increasing irradiance resulted in increased 1–qp at both 20 and 5 °C. More importantly, plants grown at either 20/800 or 5/250 not only exhibited a comparable 1–qp but also exhibited the highest values of 1–qp. Thus, these plants are considered to be grown under high excitation pressure. In contrast, plants grown at either 20/250 or 5/50 exhibited a comparable low 1–qp. These plants are considered to be grown at low excitation pressure relative to plants grown at either 20/800 or 5/250. As expected, plants grown at 20/50 exhibited the lowest values of 1–qp and are considered to be grown at a very low PSII excitation pressure relative to plants grown at 20/250 or 5/50. These results for effects

of growth temperature and irradiance on PSII excitation pressure in winter rye are consistent with those published previously (Gray *et al.*, 1997, 1998).

These results indicate that whenever one manipulates either temperature or irradiance, one also modulates excitation pressure (Huner *et al.*, 1998). Thus, photoautotrophs do not necessarily respond either to absolute growth temperature or to absolute growth irradiance (Huner *et al.*, 1998). Furthermore, there is no single growth condition that can serve as a control to complement the complex interactions among irradiance, temperature and excitation pressure. It is important to note that the modulation of excitation pressure is not restricted to temperature and light but that the combination of light and other environmental stress conditions such as drought, nutrient availability and salinity have the potential to modulate excitation pressure (Huner *et al.*, 1998). These considerations of experimental design and proper controls will be essential in functional genomic studies surveying environmental stress regulation of gene expression.

Gene expression during growth of rye at two light intensities

To determine if growth of rye plants at two different light intensities or levels of excitation pressure is associated with a differential gene expression, we analysed the translation products encoded by mRNAs isolated from control (20/250) and high-light-grown (20/800) plants. A comparison of the electrophoretic patterns showed that growth under high light leads to quantitative changes in at least 16 translatable mRNAs (Figure 1). Of these, six mRNAs were found to increase and encode polypeptides between 21 and 70 kDa (pI ranging from 4.8 to 7) while ten mRNAs were found to decrease and encode polypeptides between 15 and 70 kDa (pI 5–7.1). Overall, these results reveal that growth of rye plants at two light intensities or PSII excitation pressures is associated with a differential expression of nuclear genes. In an effort to determine the nature of these genes, we differentially screened a high-light rye cDNA library and identified 11 clones. However, in our detailed analysis of gene expression, we also included 31 ESTs from rye and wheat to evaluate the capacity of our system to distinguish between different types of regulation. Because the ESTs were isolated from libraries prepared from mostly leaf tissue, they represent good tools to study the regulation by such factors as growth irradiance, PSII excitation pressure and growth temperature. Characterization of these clones by northern blot analysis with our experimental design revealed different but reproducible patterns of regulation. Based on these results, we classified these genes into 4 groups: (1) genes regulated by PSII excitation pressure (this broad group was further separated into three subgroups based on specific expression characteristics); (2) genes regulated by low temperature; (3) genes regulated by growth irradiance; (4) genes regulated by the interaction between growth temperature and irradiance.

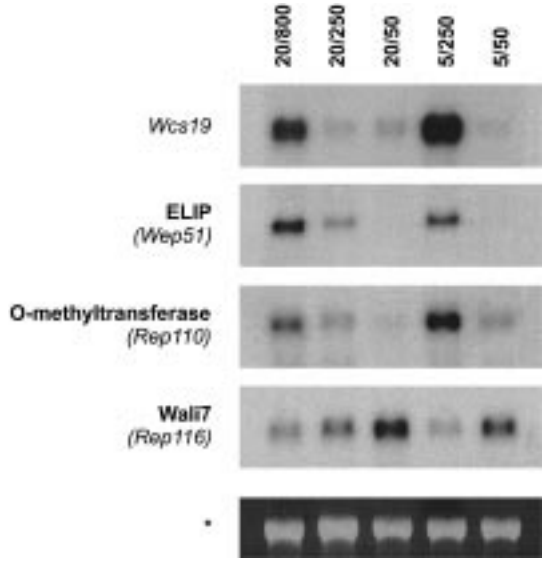

Figure 2. Expression analysis of cDNAs regulated by the level of PSII excitation pressure. Equal amounts of total RNA (5 μg) were separated by agarose gel electrophoresis in the presence of formaldehyde and transferred onto nitrocellulose membranes. The blots were probed with the ^{32}P-labelled cDNA insert corresponding to each clone. The final wash was at 65 °C in 0.1× SSC containing 0.1% SDS. The bands were visualized by autoradiography. 20/800, 20/250 and 20/50 represent plants grown at 20 °C and at 800, 250 or 50 μmol m^{-2} s^{-1} respectively. 5/250 and 5/50 represent plants grown at 5 °C and at 250 or 50 μmol m^{-2} s^{-1} respectively. * represents an ethidium bromide-stained 28S ribosomal band included to show RNA loading.

Regulation of transcript levels by PSII excitation pressure

Genes regulated by the level of PSII excitation pressure

Figure 2 illustrates the RNA hybridization blots for 4 transcripts corresponding to *Wcs19*, *Wep51*, *Rep110* and *Rep116* (*Wep* and *Rep* for wheat or rye excitation pressure). As reported previously (Gray *et al.*, 1997), *Wcs19* exhibited higher expression in plants grown at 20/800 or 5/250 than in plants grown at 20/250 or

5/50 (Figure 2). The levels of expression exhibited by *Wcs19* under the various growth conditions cannot be explained as responses to either growth temperature or growth irradiance. For example, if we compare plants grown at either 20/250 or 5/250, we may conclude that the expression of *Wcs19* is regulated by low growth temperature. This clearly is not the case for two reasons. First, plants grown at 20/800 exhibit a level of expression of *Wcs19* that is comparable to rye grown at 5/250. Second, rye grown at 5 °C but low light (5/50) exhibited minimal expression of *Wcs19* (Figure 2). However, the results for *Wcs19* expression are consistent with previous reports and were included in this study as a benchmark control for positive regulation by the level of excitation pressure (Gray *et al.*, 1997). Similar trends were observed for both *Wep51* and *Rep110* (Figure 2). Sequence analyses revealed that *Rep110* shows a homology with a barley catechol *O*-methyltransferase (Table 2) whereas *Wep51* is the rye homologue of the barley early light-inducible protein (ELIP). The up-regulation of *Wep51* is consistent with recent reports which showed that the accumulation of ELIP polypeptides is regulated by excitation pressure rather than either LT or high light per se (Krol *et al.*, 1999; Montane *et al.*, 1999). It has been suggested that ELIPs may be zeaxanthin-binding proteins that protect the photosynthetic apparatus from over-excitation (Adamska *et al.*, 1997; Krol *et al.*, 1999). High levels of excitation pressure induced by high light and LT can lead to oxidative damage due to a higher level of unutilized electrons resulting in greater production of O_2^- and H_2O_2. Thus it is possible that ELIPs could have a prolonged protective function during growth conditions inducing high PSII excitation pressures.

In contrast to *Wcs19*, *Wep51* and *Rep110* which appeared to be up-regulated by high excitation pressure, *Rep116* appeared to be down-regulated by high excitation pressure. Rye grown at either 20/800 or 5/250 exhibited a comparable low level of expression. Rye grown at either 20/250 or 5/50 exhibited a comparable intermediate level of expression whereas plants grown at the lowest excitation pressure (20/50) exhibited the highest level of expression (Figure 2). These results cannot be explained based on simple growth irradiance since rye grown at 250 μmol m^{-2} s^{-1} exhibited higher levels of expression at 20 °C than at 5 °C. Furthermore, the results for *Rep116* cannot be explained as a simple low growth temperature effect since rye grown at 5/50 exhibited a higher level of expression than plants grown at 5/250. Sequence analysis indicated that *Rep116* is the homologue of the aluminium-induced wheat gene, *Wali7* (Table 2). The fact that *Rep116* is the rye homologue of *Wali7* supports the thesis that regulation by excitation pressure probably extends beyond photosynthetic acclimation to include nutrient stress (Huner *et al.*, 1998). Moreover, recent studies have shown a genetic evidence supporting the fact that aluminium and oxidative stresses are strongly linked in plants (Richards *et al.*, 1998; Esaki *et al.*, 2000). It has been shown that the over-expression of an *Arabidopsis* blue copper-binding protein gene (*AtBCB*) and a tobacco glutathione *S*-transferase gene (*ParB*) conferred a degree of resistance to aluminium and increased tolerance to oxidative stresses induced by diamide (Esaki *et al.*, 2000). Although *Lhcb* transcript and polypeptide levels are inhibited by high excitation pressure in green algae (Maxwell *et al.*, 1995a), *Wali7* is the first reported example of a higher-plant gene that appears to be negatively regulated by excitation pressure.

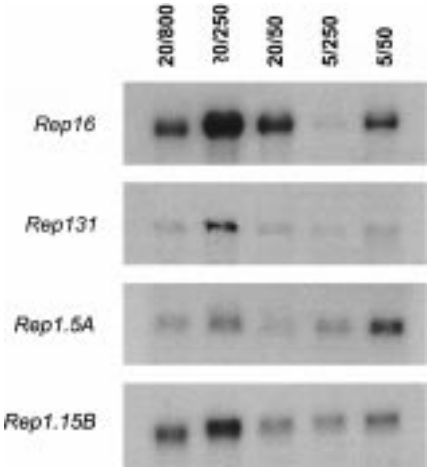

Figure 3. Expression analysis of cDNAs regulated partially by the level of PSII excitation pressure. Methods and abbreviations as in Figure 2.

Genes partially regulated by PSII excitation pressure

This group consists of four genes (*Rep16*, *Rep131*, *Rep1.5A* and *Rep1.15B*) which were expressed to higher levels during growth at low excitation pressures (20/250 and 5/50) than at high excitation pressures (20/800 and 5/250) (Figure 3). These results tend to suggest that these genes are negatively regulated during growth at higher excitation pressures. However, the expression pattern of this group differs from the previous group on two aspects. First, the low level of expression of these genes during growth at 20/50

Table 2. Properties and regulation of the cDNAs.

Regulation[a]	Clone identity[b]	Insert size (bp)	Homology[c]	GenBank accession number
PSII excitation pressure				
a	*Wcs19*	900	barley cor14a	L13437
	Wep51[3]	600	barley ELIP	BE899643
	Rep110[1]	1500	barley catechol *O*-methyltransferase (OMT)	BE213680
	Rep116[1]	1200	wheat aluminium-induced protein *wali7*	BE213681
b	*Rep16*[1]	1100	*Arabidopsis* hypothetical protein	BE213682
	Rep131[1]	2300	?	BE213683
	Rep1.5A[1]	600	?	BE213684
	Rep1.5B[1]	1500	?	BE213685
c	*Rep141*[2]	1100	barley carbonic anhydrase	BE899637
	Rep1.8B[2]	900	maize OEC 17 kDa protein precursor	BE231461
	Rep3[2]	1300	wheat PsbP OEC 23 kDa protein precursor	BE231462
	Rep4B[2]	800	barley plastocyanin	BE231463
	Rep1.15A[2]	1600	wheat ribulose 5-phosphate kinase (RPK)	BE231463
	Rep13[2]	800	?	BE231465
	Rep21A[2]	900	rice ferredoxin NADP reductase	BE231466
	Rep312[2]	800	Rubisco small subunit	BE231478
	Rep351[2]	1000	barley chlorophyll *a/b*-binding protein	BE231467
	Rep410.1[2]	500	?	BE231468
	Rep411.1[2]	700	maize chlorophyll *a/b*-binding protein	BE899638
	Rep412.1[2]	900	Rubisco small subunit	BE899639
	Rep641[2]	1000	wheat 23 kDa OEC	BE899640
	WepA26[3]	750	?	BE231479
	WepA56[3]	800	wheat acetyl-CoA carboxylase	BE940823
	WepA79[3]	700	?	BE231480
	WepA9[3]	500	?	BE231485
	WepA61[3]	1000	?	BE231481
	WepA39[3]	700	rice ribulose-5-phosphate-5-epimerase	BE231482
	WepA40[3]	600	*Arabidopsis* nitrilase-associated protein (NAP) 16 kDa	BE231483
	WepA54[3]	1000	?	BE231484
Growth temperature				
	LT4a[2]	700	CP12 protein	BE231469
	LT15a[2]	400	barley Mg-chetalase subunit	BE231470
	LT23[2]	800	?	BE231471
	LT621[2]	2000	barley and *Craterostigma plantagineum* transketolase Tk7-Tk10	BE899641
Growth irradiance				
	Li2A[2]	1200	?	BE231473
Growth temperature and irradiance				
	1.5B[1]	900	wheat proline-rich protein Wcor518	BE231474
	171[1]	1000	?	BE231475
	1.17[1]	600	?	BE231476
	A32[3]	400	barley cp31 BHv protein	BE231487
?				
	20A[1]	1600	tomato ATP-binding subunit	BE899642
	22[1]	1600	?	BE231477
	A7[3]	1000	?	BE231488
	1.2a[2]	2500	oat glycine decarboxylase P protein	BE231472
	A62[2]	1200	rice EST	BE231486

[a]Regulation by PSII excitation pressure: **a**, level; **b**, partially; **c**, threshold.
[b]Clone identity: [1]cDNAs identified by screening; [2]cDNAs from rye ESTs; [3]cDNAs from wheat ESTs.
[c] ?, unknown regulation or homology.

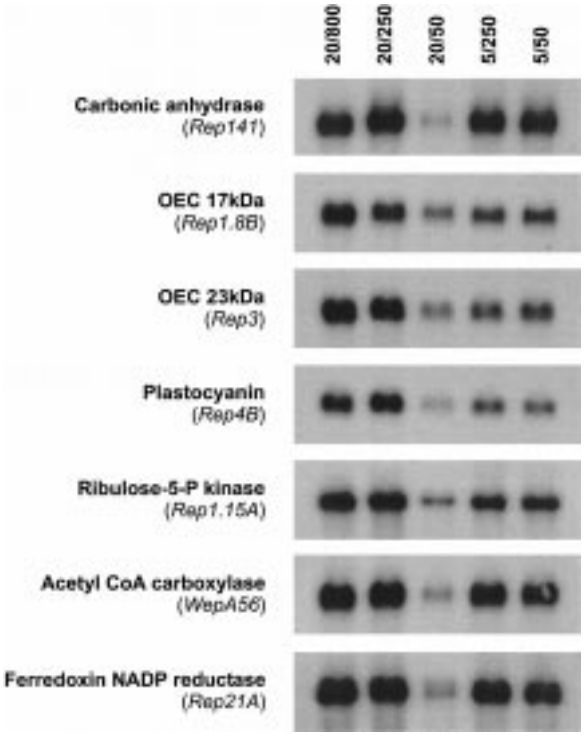

Figure 4. Expression analysis of cDNAs regulated by a threshold PSII excitation pressure. Methods and abbreviations as in Figure 2.

compared to 20/250 contradicts a strong correlation of a down-regulation at progressively higher excitation pressures. In fact, the results suggest that the genes are regulated positively only during growth at low excitation pressures (20/250 and 5/50). Whether this type of regulation is maintained in plants during growth at 5 °C under an excitation pressure equivalent to the 20/50 growth regime will have to be confirmed in a future study. Second, the comparison of the level of expression of these genes during growth at identical low excitation pressures (20/250 and 5/50) or high excitation pressures (20/800 and 5/250) reveals that they are not similar. In fact, when the expression of these genes is compared under identical excitation pressures, low temperature appears to negatively affect the expression of the *Rep16*, *Rep131*, and *Rep1.15B* genes whereas it positively affects the expression of the *Rep1.5A* gene. Sequence analysis reveals that *Rep16* shows a homology with an *Arabidopsis* hypothetical isoprenylated protein. A recent report (Dykema *et al.*, 1999) has shown that isoprenylated proteins are capable of binding transition metal ions. Ion uptake, transport and sequestration are essential to meet the nutritional requirements for plant growth and development. On the other hand, *Rep131*, *Rep1.5A* and *Rep1.15B* did not show any significant homology with known genes in the database.

Genes regulated by a threshold PSII excitation pressure

The detailed northern analyses of the 21 genes in this group (Table 2) revealed an interesting expression profile. Figure 4 shows an example of this type of regulation. Transcripts corresponding to all genes showed their lowest levels of expression when rye plants were grown under a very low excitation pressure growth regime (20/50). However, at 5 °C and under the same light irradiance (5/50), their expression increased significantly. Under low (20/250 or 5/50) or high (20/800 or 5/250) PSII excitation pressure, their transcripts accumulation is similar. These results suggest that a minimum threshold PSII excitation pressure close to the one produced during growth at 20/250 or 5/50 is needed for maximal induction of these genes. To confirm this observation, a 5 °C growth regime corresponding to the very low excitation pressure (20/50) will be included in future experimental designs. Terminal sequencing of these genes revealed that five of them encode proteins involved in photosynthetic sugar biosynthesis such as carbonic anhydrase, epimerase, ribulose 5-phosphate kinase and rubisco small subunit (rbcS) (Table 2). Seven other genes encode proteins involved in the electron transport chain such as chlorophyll *a/b*-binding protein (Cab), plastocyanin, ferredoxin NADP reductase and the OEC (oxygen-evolving complex) 17 and 23 kDa protein precursors. The latter two genes were identified as acetyl-CoA carboxylase and nitrilase-binding protein (Table 2).

All identified genes except Cab, plastocyanin, ferredoxin NADP reductase, OEC 17 and 23 kDa protein precursors and nitrilase-associated protein (NAP) are involved in carbon fixation and biosynthesis of sugars and lipids. Because these functions require reducing power and energy, growth at 20/50 results in a lower level of expression of these enzymes. Finally, the involvement of Cab, plastocyanin, ferredoxin NADP reductase and OEC 17 and 23 kDa protein precursors in the photosynthetic electron transport suggests that at 20/50 the lower level of electron flow leads to lower expression of these genes. However, at 20/800 or 5/250, these genes are highly expressed because it is always essential for the plant that all free electrons be transferred to downstream acceptors to avoid over-excitation that leads to pho-

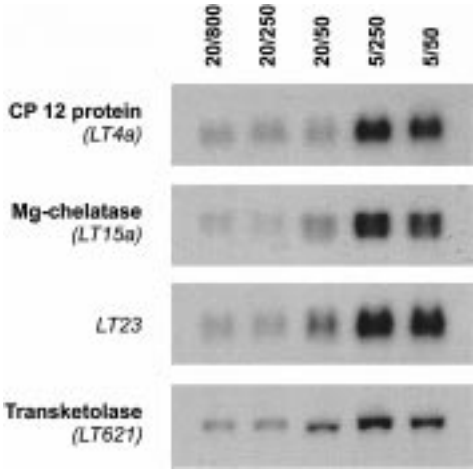

Figure 5. Expression analysis of cDNAs regulated by low temperature. Methods and abbreviations as in Figure 2.

toinhibition. Although the mechanism regulating the expression of this group of genes is unknown, an interesting possibility lies in the demonstration that levels of ferredoxin-1 (*Fed-1*) transcripts in pea and *Arabidopsis* (Elliot *et al.*, 1989; Dickey *et al.*, 1992, 1994), the small subunit of ribulose-bisphosphate carboxylase (rbcS) transcripts in petunia (Thompson and Meagher, 1990) and the light-harvesting chlorophyll-binding protein (Lhcb) transcripts in pea (Warpeha and Kaufman, 1990; Marrs and Kaufman, 1991; Kaufman, 1993) can be regulated by light-mediated changes in transcript stability. It has been proposed that at a low light intensity condition, transcripts corresponding to these genes are destabilized due to the lack of stability elements which have been reported to occur within the 5′-untranslated region (5′-UTR) of transcripts encoding chloroplast-targeted proteins (Anderson *et al.*, 1999).

Regulation of transcript levels by low growth temperature

This group consists of four genes (*LT4a*, *LT15a*, *LT23* and *LT621*; *LT* for low temperature). Detailed characterization revealed that these genes were expressed to higher levels during growth at both LT regimes (5/250 and 5/50) compared to all 20 °C growth regimes (Figure 5). In addition, the similar expression levels of these genes within the 20 or 5 °C temperature regimes indicates that they are not regulated by factors such as growth irradiance or PSII excitation pressure. Together these results indicate that these genes are regulated only by LT. Sequence analysis has allowed the identification of *LT621* as a transketolase (Table 2) which is known to be involved in both the reductive pentose phosphate cycle (RPPC) and the oxidative pentose phosphate cycle (OPPC). The former occurs in the chloroplast only whereas the latter occurs in both the chloroplast and the cytosol (Heldt, 1997). This is consistent with the reports that growth and development of wheat, rye and *Arabidopsis* at LT induces a general increase in the activities and levels of specific enzymes of the RPPC and sucrose biosynthesis pathway (Hurry *et al.*, 1998; Strand *et al.*, 1999). Furthermore, it has been proposed that the transketolase reaction may be considered near-limiting in the homeostatic regulation of carbon flux through the RPPC (Fridlyand *et al.*, 1999). In addition, partial sequencing revealed the identity of *LT15a* as the Mg-chelatase involved in chlorophyll biosynthesis (Table 2). It catalyses the first step unique to chlorophyll synthesis, namely the insertion of Mg^{2+} into protoporphyrin IX (Walker and Weinstein, 1994). This is consistent with the observations that growth of winter wheat and winter rye at LT results in an increase in leaf chlorophyll content (Huner *et al.*, 1984). LT4a has been identified as a homologue of the *Arabidopsis* CP12 protein. CP12 is a small nuclear-encoded chloroplast protein that interacts with the NAD(P)H glyceraldehyde-3-phosphate dehydrogenase, a key enzyme of the reductive pentose phosphate cycle (Calvin cycle) (Wedel *et al.*, 1997). Thus, beside a proposed role in regulating the activity of the Calvin cycle, one can assume that *CP12* has also a similar function during low-temperature growth. Analysis of the partial sequence of *LT23* shows no homology with known genes in the database.

Regulation of transcript levels by growth irradiance

The *Li2a* gene (*Li* for light intensity) (Figure 6A) is an example of a gene whose expression appears to be regulated by growth irradiance rather than either growth temperature or excitation pressure for the following reasons. First, regardless of growth temperature, increasing growth irradiance resulted in an increased level of expression with the highest transcript levels observed in rye grown at 20/800. Second, transcript levels of the *2a* gene in rye grown at an irradiance of 250 μmol m^{-2} s^{-1} were comparable regardless of whether plants were grown at either 20 °C or 5 °C (Figure 6A). Third, transcript levels of the *2a* gene in plants grown at low light (50 μmol m^{-2} s^{-1}) were similar whether rye was grown at 20 °C or

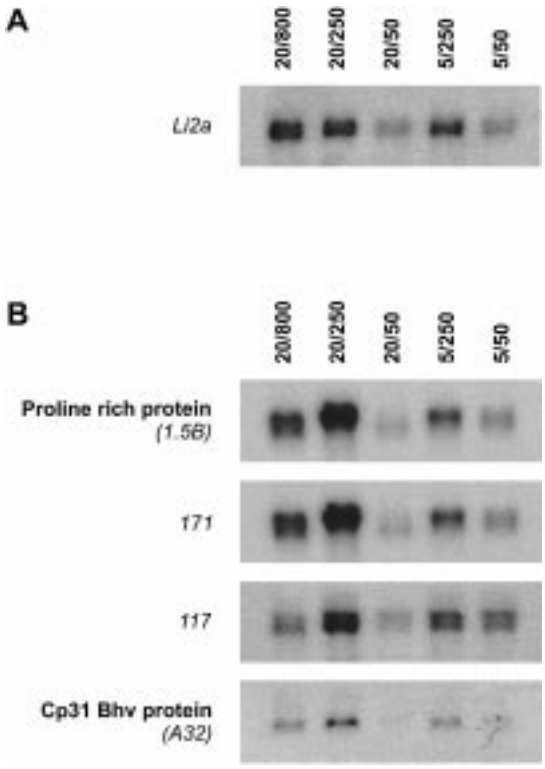

Figure 6. Expression analysis of cDNAs regulated by growth irradiance (A) or by the interaction between growth temperature and irradiance (B). Methods and abbreviations as in Figure 2.

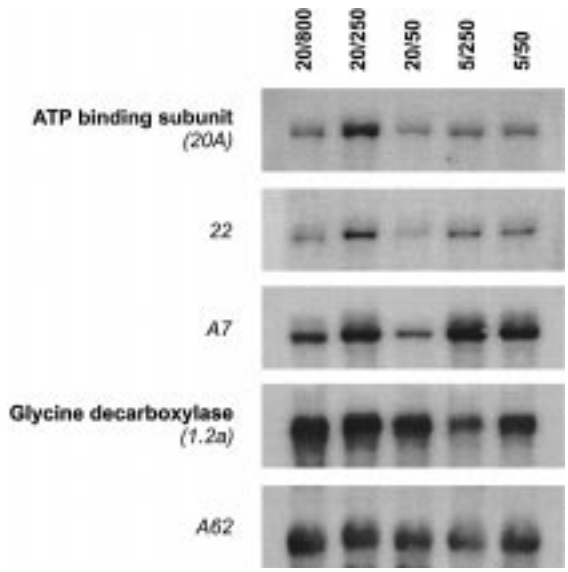

Figure 7. Expression analysis of unclassified cDNAs. Methods and abbreviations as in Figure 2.

5 °C. Finally, although rye grown at either 20/250 or 5/50 exhibited similar excitation pressures (Table 1), plants grown at 20/250 exhibited much higher transcript levels of the *Li2a* gene than plants grown at 5/50 (Figure 6A). Partial sequencing of this gene did not reveal any homology with known gene sequences in the data bank (Table 2).

Regulation of transcript levels by the interaction of growth temperature and irradiance

Figure 6B illustrates RNA hybridization blots of four transcripts that appear to be regulated by the combined effects of growth temperature and growth irradiance but not by excitation pressure. For example, the transcript levels of the *1.5B* gene were higher when plants are grown at 20/800 and 20/250 than in plants grown at 5 °C. However, in addition to the apparent growth temperature sensitivity of these transcripts, increasing growth irradiance at 5 °C induced higher levels of all transcripts. At a growth temperature of 20 °C, the highest transcript levels were observed in rye grown at 20/250 and the lowest levels were observed during growth at 20/50. Growth under high irradiance (20/800) appeared to induce lower but intermediate transcript levels compared to rye grown at 20/250 (Figure 6B). Because the level of expression observed for the *1.5B* transcript was significantly higher in rye grown at 20/250 than for plants grown at 5/50, the expression of this transcript cannot be regulated by excitation pressure. Similar trends were observed for the transcript levels of the *171*, *117* and the *A32* genes (Figure 6B). However, the temperature effect was less pronounced for the *117* and *A32* genes. Whether this group of genes have the same type of light regulation during growth at 5 °C will have to be confirmed with the inclusion of a 5/800 growth condition as an additional control. Sequence analyses indicated that the *A32* gene is a homologue of the barley *cp31* gene which codes for a chloroplast RNA-binding protein whereas the *1.5B* is a homologue of the wheat *Wcor518* gene coding for a proline-rich protein. The *171* and *117* genes did not reveal any homology with gene sequences present in the data bank. Churin *et al.* (1999) have demonstrated that the level of expression of *cp31* is positively affected by light. This is in accordance with our results since weak accumulation of the *A32* mRNA is observed at low light conditions at either 20 °C or 5 °C. The higher level of expression of these genes during 20 °C growth regimes and their down-regulation during growth at 20/800 could be consequences of metabolic adjustments. These include a higher growth rate at 20 °C and a feedback

down-regulation of genes involved in pathways that do not have to function at maximal rates.

Unclassified genes

The genes *20A*, *22* and *A7* remained unclassified based on their expression profiles (Figure 7). The analysis of the three 20 °C growth regimes indicate that these genes could be positively regulated by the 250 μmol m^{-2} s^{-1} light irradiance or negatively regulated by high PSII excitation pressure (20/800). However the similar expression levels of these genes at both 5 °C regimes does not permit to differentiate between these possibilities. On the other hand, the *1.2a* and the *A62* transcripts appear to be negatively regulated during growth at 5/250. Transcripts of these genes accumulated to higher but similar levels in all 20 °C growth regimes and in the 5/50 growth regime. Based on this observation these genes could not be classified as low-temperature-regulated genes. Therefore, these results suggest that these genes may be regulated by multiple factors that our experimental conditions were unable to identify. Sequence analysis did not permit to identify the nature of the *22* and *A7* genes whereas the *20A* gene is a homologue of the tomato ATP-binding subunit gene (Table 2). Moreover, the *1.2a* and *A62* genes show, respectively, homology with the P protein subunit of the multi-enzyme glycine decarboxylase complex (GDC) (Wolpert *et al.*, 1995) and with a rice EST of unknown function (Table 2). GDC is a key enzyme of the photorespiratory C-2 cycle of C3 plants involved in the cleavage of glycine during photorespiration.

Potential of using this experimental design for delineating gene regulation

The experimental system used in this study was designed to differentiate among three components underlying cold acclimation of plants: low but non-freezing temperatures, irradiance and excitation pressure. Growth of winter rye at five specific regimes allowed us to categorize 37 of the 42 genes examined into four broad groups: those regulated by excitation pressure; those regulated by low temperature; those regulated by irradiance; and those regulated by light and temperature in an independent but additive manner. The results from our study indicate that excitation pressure may be an important component regulating the expression of many genes. Because PSII excitation pressure can be modulated by many abiotic stresses, it may represent an important sensing mechanism for abiotic stresses in general and a signal for eliciting molecular and physiological responses.

Since five of the 42 genes examined could not be unambiguously categorized into any one of the four groups and additional controls are needed to confirm certain observed regulations, our experimental design has some limitations. Nevertheless, this study illustrates the importance of understanding the interactions of multiple abiotic stresses to which a plant may be exposed when examining plant gene expression. Our small-scale survey of the effects of light and temperature on gene expression underscores the necessity of proper controls in order to interpret properly the effects of growth conditions on the regulation of gene expression. This has important implications for functional genomic projects that initially rely on expression or regulation data to infer a function to a set of genes.

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