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WCS120 protein family and proteins soluble upon boiling in cold-acclimated winter wheat

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Summary

The amount of proteins soluble upon boiling (especially WCS120 proteins) and the ability to develop frost tolerance (FT) after cold acclimation was studied in two frost-tolerant winter wheat cultivars, Mironovskaya 808 and Bezostaya 1. Protein gel blot analysis, mass spectrometry (MS) and image analysis of two-dimensional gel electrophoresis (2-DE) gels were used to identify and/or quantify the differences in protein patterns before (non-acclimated, NA) and after 3 weeks of cold acclimation (CA) of the wheats, when FT increased from −4 °C (lethal temperature (LT_{50}), for both cultivars) to −18.6 °C in Bezostaya 1 and −20.8 °C in Mironovskaya 808. Only WCS120 protein was visible in NA leaves while all five WCS120 proteins were induced in the CA leaves. Mironovskaya 808 had higher accumulation of three members of WCS120 proteins (WCS120, WCS66 and WCS40) than Bezostaya 1. MS analysis of total sample of proteins soluble upon boiling showed seven COR proteins in the CA samples and only three COR proteins in the NA samples of cultivar Mironovskaya 808 (MIR). In conclusion, the level of the accumulation of WCS120, WCS66 and WCS40

Abbreviations: BEZ, cultivar Bezostaya 1; CA, cold-acclimated (cultivation at 2 °C); COR, cold-regulated; FT, frost tolerance; LEA, late embryonic abundant; LT_{50}, lethal temperature; LC, liquid chromatography; MIR, cultivar Mironovskaya 808; MS, mass spectrometry; MS/MS, tandem mass spectrometry; *m/z*, mass-to-charge ratio; NA, non-acclimated (cultivation at 17 °C); Q, quadrupoles; QM, queries matched; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TOF, time-of-flight; 2-DE, two-dimensional gel electrophoresis

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distinguished our two frost-tolerant winter wheat cultivars. Moreover, the differences of CA and NA samples of the MIR were shown by liquid chromatography (LC)–tandem mass spectrometry (MS/MS).

Introduction

The ability of plants to survive freezing temperatures, or frost tolerance (FT), depends on their genotype and is affected by environment. Higher levels of FT are developed after the exposure of plants to low non-freezing temperatures in the process called cold acclimation (Fowler et al., 1996; Thomashow, 1999).

Cold acclimation involves several biochemical and physiological changes. The research on cold acclimation and FT has been focused on the characterization of genes that are up- or down-regulated during cold acclimation and are important for the capacity of each genotype to develop FT (Houde et al., 1992a; Galiba et al., 1995; Fowler and Thomashow, 2002; Ohno et al., 2003; Kobayashi et al., 2004). A broad spectrum of cold-regulated (COR) genes is represented by the superfamily of *Cor* genes. Proteins encoded by these genes are mostly hydrophilic and soluble upon boiling. They are composed largely of a few amino acids with repeated amino acid sequence motifs, and most have amphipathic α-helixes (Thomashow, 1999). Wheat *Cor* genes coding late embryonic abundant (LEA) proteins (COR/LEA) have been described by many authors, for example: *wcs120* gene family (Houde et al., 1992b; Sarhan et al., 1997), *Wcor410* (Danyluk et al., 1994), *wcs19* (Chauvin et al., 1993; NDong et al., 2002).

The *wcs120* gene family encodes a group of highly abundant wheat proteins ranging in size from 12 to 200 kDa, namely WCS200 (MW = 200 kDa, p*I* = 6.5), WCS180 (180 kDa, 6.5), WCS66 (66 kDa, 7.28), WCS120 (50 kDa, 7.77), WCS40 (40 kDa, 7.30), WCS726 (21 kDa, 7.04) and WCS80 (12 kDa, 8.05) (Houde et al., 1992a; Ouellet et al., 1993; Sarhan et al., 1997). The expression of the first five major members of the *wcs120* gene family is inducible only by cold treatment, while *wcs726* and *wcs80* genes are also inducible by drought (Sarhan et al., 1997). The WCS120 proteins are rich in glycine and threonine, highly hydrophilic and soluble upon boiling. The WCS120 protein family shares sequence homology with the D11 dehydrin family (Sarhan et al., 1997).

Transcription and/or translation levels of *wcs120* gene alone or together with other *Cor* genes in the plant tissues have been used as an illustration of induction of *Cor* genes under cold treatment (Houde et al., 1992b; Danyluk et al., 2003; Shen et al., 2003; Kobayashi et al., 2004). Protein levels increased rapidly in both spring and winter cultivars following exposure to the cold but the accumulation of WCS120 protein was higher in winter than in spring cereals (Houde et al., 1992b; Fowler et al., 1996). Houde et al. (1992b) suggest that one of WCS120 proteins, the WCS120 protein, could be used as the molecular marker for FT in Gramineae. To date, the different levels of WCS120 proteins have not been detected between frost tolerant winter wheat cultivars like Mironovskaya 808 and Bezostaya 1. Of winter wheats, Mironovskaya 808 represents a high FT cultivar and Bezostaya 1 represents a middle FT cultivar (Gusta et al., 2001; Prášilová and Prášil, 2001).

The aim of the present study was to compare two winter wheat cultivars, Mironovskaya 808 and Bezostaya 1 with different levels of FT through analysis of their protein patterns. In this work, two-dimensional gel electrophoresis (2-DE), image analysis, and WCS120 antibody were used to study the level of accumulation of the members of WCS120 protein family in both winter cultivars. In addition, mass spectrometry (MS) analysis of proteins soluble after boiling was used to identify proteins in the non-acclimated (NA) and cold-acclimated (CA) winter wheat cultivar Mironovskaya 808 (MIR).

Materials and methods

Plants

Two winter wheat (*Triticum aestivum* L.) cultivars, Mironovskaya 808 (MIR) and Bezostaya 1 (BEZ) were investigated with regard to their differing abilities to develop FT. The seeds of these cultivars were kindly provided by the breeding company Selgen a.s. Prague.

Growth and cold acclimation

After a seed germination at 18 °C for 4 days, the seedlings were grown in soil at a constant tem-

perature of 17 °C under the conditions of a 12 h-photoperiod and an irradiance of 400 μmol m^{-2} s^{-1} supplied by a combination of vapor lamps and high intensity discharge lamps (LU/400/T/40, Tungsram, Hungary) in a growth cabinet (Tyler, Hungary) (Prášil et al., 2004). After 2 weeks of growth at 17 °C, the plants at three-leaf stage were exposed to a cold acclimation at 2 °C with the same photoperiod, for 3 weeks.

Frost tolerance

The level of FT was determined by direct freezing of plants in freezing boxes (MHM/52, MIRKOZ, Hungary). Plants taken from the soil were divided into bundles of 10 units and exposed to −4 °C for 20 h, followed by five different freezing temperatures in separate freezing boxes for 24 h. Temperatures in the boxes differed by 2 °C and were chosen according to the predicted FT of the plants. The rate of cooling and thawing was 2 °C h^{-1}. After thawing, the plants were cut at 2.5 cm from the crown, the roots were submerged in a dish filled with fresh water and the plants were placed in a greenhouse at 20 °C. After 5–6 days, the number of living and regenerating plants was determined for each freezing treatment. The lethal temperature (LT_{50}) (i.e. the temperature at which 50% of the plants were killed) was calculated according to the model of Janáček and Prášil (1991).

Protein extraction

Proteins soluble upon boiling were extracted with Tris buffer (0.1 M Tris–HCl, pH 8.0 containing "cømplete EDTA-free Protease Inhibitor Cocktail Tablets" (Roche)) from frozen plant leaves as described by Houde et al. (1995), with some modifications. The supernatant after 20,000*g*-centrifugation was boiled for 15 min and then centrifuged again for 20 min, at the same speed, to eliminate insoluble proteins. COR/LEA proteins are stable upon boiling, and the boiling step therefore was used to enrich these proteins in the sample. The soluble proteins were precipitated with five volumes of acetone and collected by centrifugation at 20,000*g* for 30 min. The pellet was dissolved in sample buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis (Laemmli, 1970) or in rehydration buffer for isoelectric focusing (Bio-Rad). The protein concentration was measured according to Bradford (1976).

Protein analysis

Proteins separated by 2-DE (O'Farrell, 1975) were visualized by Coomassie blue or silver staining. Isoelectric focusing was run on ReadyStrip IPG strips (pH 3–10) in PROTEAN IEF cell (Bio-Rad) according to the manufacturer's Instruction Manual until 35,000 Vh were reached. The rehydration buffer contained 9.8 M urea and 4% CHAPS. The focused proteins were then separated in the second dimension by 10% SDS-PAGE (Laemmli, 1970).

WCS120 proteins were identified on the basis of molecular weight (MW) and isoelectric point (p*I*) (Houde et al., 1995). "2-D SDS-PAGE Standards" (Bio-Rad) was used for the calibration of MW and p*I*.

The separated proteins were also identified by the protein gel blot analysis using the WCS120 polyclonal antibody (Houde et al., 1992a). The proteins were electrophoretically transferred to nitrocellulose (0.45 μm, Pharmacia Biotech). After blocking with BSA (3%) in TBS, the membrane was incubated with a 1:20,000 dilution of WCS120 antibody. After washing with TBS containing 0.05% Tween-20 and 0.2% Triton X-100 (Sigma), the secondary antibody (GAR-AP conjugate, Bio-Rad) was applied at 1:3000 dilution. The complex of proteins and antibodies was visualized by BCIP/NBT staining (Bio-Rad). SDS-PAGE Standards, broad range, (Bio-Rad) were used for the estimation of molecular weight (MW).

Image analysis of 2-DE gels

Image analysis was used to quantify the density of the protein spots on the 2-DE gels. All images were captured by ColorPage-SP2 (Genius)and transferred to the image analysis system LUCIA G v.4.80 (Laboratory Imaging Prague, Czech Republic), equipped with Dual Pentium MMX and Matrox Magic frame grabber. The background of gel was decreased by the function Intensity Transformation on 95–97% of maximal brightness (255). The spots were defined as the areas of minimally 10% lower brightness than the brightness of the background. The value of Integral Density was measured on these automatically defined spots. The calibration of density was adjusted as 0 = maximum of the brightness and 10 = black.

Mass-spectrometry/nano-liquid chromatography (LC)–tandem mass spectrometry (MS/MS)

MS was used to identify proteins in the total protein samples after acetone precipitation and

centrifugation (as described in Protein extraction) and to determine proteins from the bands chosen from Coomasie stained 2-DE gels.

The protein pellets were dissolved in 50 μL of 0.1 M NH_4HCO_3/10% acetonitrile. About 3 μg of trypsin (sequencing grade, Roche) were added and the samples were incubated at 37 °C overnight. The bands from stained SDS-gels were cut out, washed and treated with trypsin, according to Shevchenko et al. (1996). After the digestion, the peptides were extracted by shaking for 3 h with 100 μL of 5% formic acid. All tryptic peptide samples were dried and re-dissolved in 50 μL of 0.1% trifluoroacetic acid/5% acetonitrile.

LC–MS/MS was used to analyze the tryptic peptides. The analyses were performed on a quadrupoles (Q)-time-of-flight (TOF) Ultima Global (Micromass, Manchester, UK) at Risoe National Laboratory (Roskilde, Denmark). A nano-LC system (Famos, Switchos, Ultimate, LC-Packings, Amsterdam, The Netherlands) equipped with RP-columns was used and interfaced directly to the Q-TOF Ultima Global instrument using an electrospray source. The analytical nano-column (75 μm ID × 15 cm) was (self)-packed with Zorbax 300SB-C18, 3.5 μm (Agilent Technologies, Palo Alto, CA, USA) and a trap column (300 μm × 5 mm) packed with 5 μm C18, 100 Å, PepMap™ (LC Packings) was used. After binding of the peptides to the trap column, the column was washed with 0.1% trifluoroacetic acid, 2% acetonitrile and switched on-line with the analytical nano-column. The peptides were eluted and separated by a 92 min gradient of 5–50% acetonitrile in 1% acetic acid, 1% formic acid at a flow rate of 150 nL min^{-1}. The mass spectrometer was run in data-dependent mode (four most abundant ions in each cycle, peak threshold 40 counts s^{-1}, 0.5 s MS mass-to-charge ratio (m/z) 500–2000 and maximum 4 s MS/MS m/z 50–2000, continuum mode, 180 s dynamic exclusion).

Data reduction was performed using MassLynx4.0/ProteinLynx software (peptide filter QA threshold: 100; background subtract: polynomial order 2, below curve 40%; smooth: 4/1, Savitzky Golay, center: 4 channels, 80% centroid), and the resulting MS/MS data sets were used to search the NCBInr database using the Mascot search engine (Matrix Science Ltd., London, UK). The sub-database of green plants was searched with a tolerance of 200 ppm (parent ion) and 200 mmu (MS2) and oxidation of methionine as variable modification.

Statistical analyses were carried out with a multiple range test (LSD at the 5% level) of averages calculated from three repetitions (Unistat version 5.1, Unistat Ltd., London, UK).

Results

Two winter wheat cultivars Mironovskaya 808 (MIR) and BEZ showed similar low FT (LT_{50}0 about −4 °C) when cultivated at non-acclimated conditions 17 °C (NA). After 3 weeks of cold acclimation (CA), winter wheat MIR reached $LT_{50} = -20.8$ °C and BEZ $LT_{50} = -18.6$ °C (Table 1).

With respect to increased FT, water content in leaves decreased after cold acclimation (Table 1).

Quantitative analyses of proteins present in the soluble fraction after boiling were related to fresh weight and dry weight (Table 1). Concentration of proteins was identical in NA leaves of all analyzed cultivars. The level of proteins expressed per fresh weight significantly increased in winter cultivars after 3 weeks of cold acclimation, but the two cultivars had similar levels of proteins soluble upon boiling. In parallel, the level of proteins expressed per dry weight significantly decreased in winter cultivars after 3 weeks of cold acclimation, but also without any significant differences between both CA cultivars.

Qualitative and quantitative differences in pattern of soluble proteins stable upon boiling between cold- and non-acclimated leaf tissues of both wheat cultivars were observed on protein gel blots of 1-DE gels (Fig. 1) and 2-DE gels (Fig. 2). Protein

Table 1. Lethal temperature (LT_{50}), water content (WC, 100 × (g FW−g DW)/g FW), concentration of proteins soluble upon boiling expressed for fresh weight (CP−mg protein/g FW) in leaves of non-acclimated and cold-acclimated wheat cultivars Bezostaya 1 (BEZ) and Mironovskaya 808 (MIR)

cv.	Non-acclimated			Cold-acclimated		
	LT_{50} (°C)	WC (%)	CP (mg)	LT_{50} (°C)	WC (%)	CP (mg)
BEZ	−4.2a	88a	4.3a	−18.6a	74a	5.3a
MIR	−4.4a	88a	4.2a	−20.8b	74a	5.3a

Values denoted with the same letter are not significantly different between MIR and BEZ within the same treatment (NA or CA).

gel blot analysis showed different levels of WCS120 proteins in the NA and CA leaves. NA leaves had all WCS120 proteins invisible except a very low level of WCS120 protein. In the CA leaves of both winter cultivars all five main WCS120 proteins were visible but they had no significantly different levels on 1-DE protein gel blots.

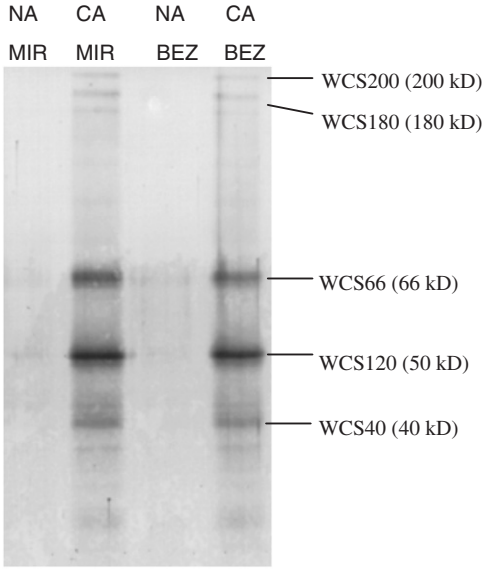

Figure 1. Protein gel blot analysis using the WCS120 antibody on proteins soluble upon boiling from cold acclimated (CA) and non-acclimated (NA) cultivars Mironovskaya 808 (MIR) and Bezostaya 1 (BEZ).

Five intensively silver-stained spots appeared after cold acclimation on 2-DE gels: MW 200 kDa—p*I* 6.5, MW 180 kDa—p*I* 6.5, MW 66 kDa—p*I* 7.28, MW 50 kDa—p*I* 7.77, MW 40 kDa—p*I* 7.30. All of them have been identified as WCS120 proteins, according to their MW and p*I* value on 2-DE gels (Fig. 2) and using polyclonal WCS120 antibody on W-blotted membranes (not shown). Furthermore, two of them were also confirmed by MS (WCS66 and WCS120, see Table 2). Three different methods confirmed the expression of cold acclimation-induced WCS120 proteins.

Level of WCS120 protein expression in analyzed cultivars was so distinct on 2-DE gels that image analysis was used to quantify particular WCS120 proteins. According to the sum of all spot densities, the lowest level of WCS120 proteins accumulation was in BEZ after cold acclimation. Individual WCS120 protein density demonstrated the highest accumulation of WCS66 protein that was almost one time higher in MIR than in BEZ. The average values of the density of proteins WCS180 and WCS200 showed no significant differences between both winter cultivars (Table 3). Integral density of image analyses revealed the presence of very small amounts of WCS120 proteins in both winter cultivars even in NA samples (Table 3).

LC–MS/MS was used to further characterize NA and CA proteins soluble upon boiling in winter wheat MIR. In this way, 33 proteins were identified in CA sample, while 42 proteins were identified in NA

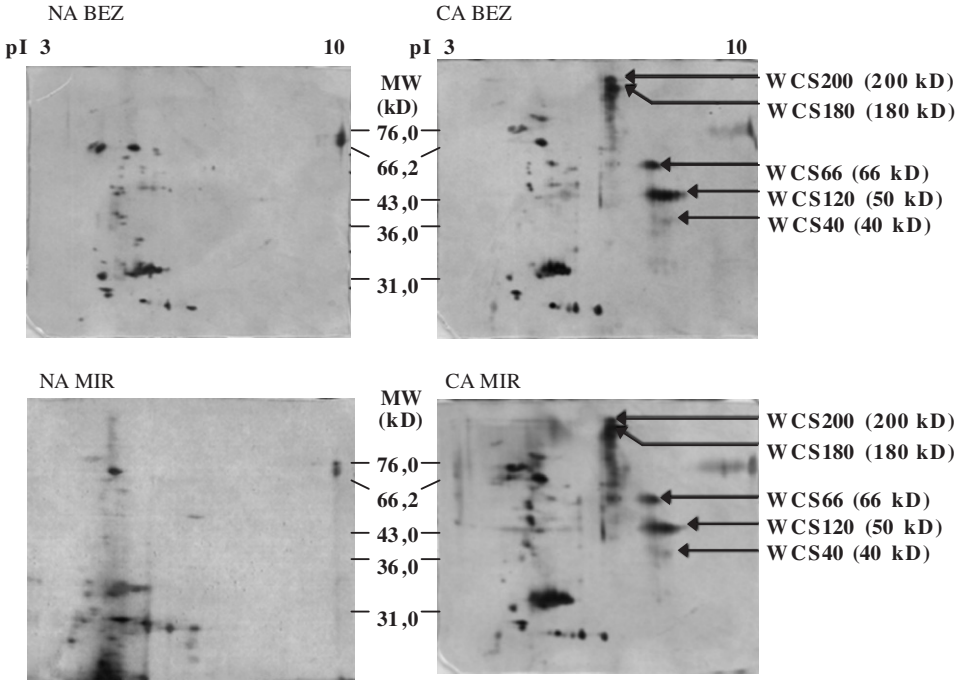

Figure 2. Comparison of 2-DE gels of proteins (soluble upon boiling) extracted from leaf tissue of non-acclimated (NA) and cold-acclimated (CA) winter wheat Bezostaya 1 (BEZ) and Mironovskaya 808 (MIR). 2-DE gels were silver-stained.

Table 2. Mass spectrometry (LC-MS/MS) identification of two WCS120 proteins from 2-DE gels of cold acclimated winter wheat Bezostaya 1 (shown in Fig. 2 as WCS120 and WCS66)

Protein	GIs	Mass	Score	Queries matched
Cold shock protein CS66/WCS66	gi|1169107	46,769	47	2
COR39 protein – wheat/WCS120	gi|7489666	38,796	80	3

Individual scores >34 indicate identity or extensive homology ($p<0.05$).

Table 3. Density of WCS120 protein spots obtained from image analysis of 2-DE gels of non-acclimated and cold-acclimated cultivars Bezostaya 1 and Mironovskaya 808

Protein	Integral density			
	Non-acclimated		Cold-acclimated	
	BEZ	MIR	BEZ	MIR
WCS 120	5.0a	7.8a	1220.0a	1734.5b
WCS 66	ND	ND	384.0a	692.7b
WCS 40	ND	ND	79.8a	102.1a
WCS 200	ND	ND	244.0a	288.7a
WCS 180	ND	ND	476.7a	412.5a
Suma	5.0a	7.8a	2404.5a	3231.5b

The given values represent the integral density of spots after subtraction of background density of the 2-DE gels. ND – non-detected.
Values denoted with the same letter are not significantly different between MIR and BEZ within the same protein and treatment.

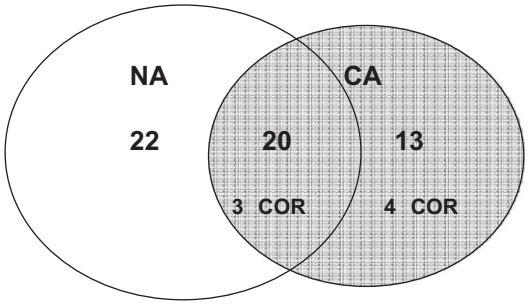

Figure 3. Results of protein identification by LC–MS/MS on total protein sample of non-acclimated (NA) and cold-acclimated (CA) leaves of cultivar Mironovskaya 808. The numbers represent total identified proteins including COR proteins, which are separately presented by numbers of identified COR proteins.

sample (see Fig. 3 and Table 4). The majority of the identified proteins fell into three categories: COR proteins, proteins of photosynthetic apparatus and carbon fixation (photosystem II oxygen-evolving complex protein 1, RuBisCO), and proteins related to translation (RNA-binding proteins, ribosomal proteins, ribonucleoproteins (GRP1, cp31AHv, Ps16, product of gene *blt801*). Proteins of the second and the third category were present in both samples. Only three COR proteins appeared in both samples (COR14a, WCOR719, cold-responsive LEA/RAB-related COR protein), while another four COR proteins were specific for CA samples (WCS19, WCOR726, WCOR615 and fragment of WCS200). Moreover, these three COR proteins found in the NA plants had a much lower number of matched peptides and also lower individual scores (2/128, 1/57, 1/55) than in the CA plants (5/251, 3/228, 5/289).

In addition to the above-mentioned protein groups, other proteins were detected in samples of MIR. Some were found only in CA (like cystein proteinase inhibitor) or in NA samples (like 14-3-3 proteins: GF14 mu, GRF15, 14-3-3 protein).

Discussion

The tolerance of plants to cold and frost is a quantitative inherited trait that involves a wide array of changes, including appearance and accumulation of COR proteins (COR/LEA). The *Cor/Lea* gene superfamily is a well-known group of cold-inducible genes with a positive correlation between levels of expression and the level of freezing tolerance in cereals (Sarhan et al., 1997; Vagujfalvi et al., 2000; Ohno et al., 2001; Kobayashi et al., 2004, 2005). Therefore, the members of this superfamily are possible markers for cold tolerance. However, they were usually used to distinguish spring from winter cultivars.

Our research was focused on the expression of these genes at the protein level in two winter wheat cultivars (Mironovskaya 808 and Bezostaya 1) with high but different levels of FT. Here, we compared NA with 3-week CA plants because this period of cold acclimation at regulated conditions has been shown to be necessary to reach the high FT in wheat (Fowler et al., 1996; Prášil et al., 2004). After the cold acclimation, Mironovskaya 808 was more frost tolerant than Bezostaya 1, which is consistent with

Table 4. Identification of proteins by LC-MS/MS in a total protein sample from non-acclimated (NA) and cold-acclimated (CA) plants of winter wheat Mironovskaya 808

Protein	Accession no.	Mass	Score (NA)	QM (NA)	Score (CA)	QM (CA)
Nucleic acid-binding protein – maize	S23780	33097	356	5	140	3
Oxygen-evolving enhancer protein 2	Q00434	27253	353	7	226	3
cp31AHv protein – barley	T05725	31903	326	7	250	7
Nucleic acid-binding protein – barley	T05727	30662	279	4	256	4
Photosystem II oxygen-evolving complex protein 1 – rice (strain Nihonbare)	A38889	26489	265	5	345	4
Ps16 protein – wheat	T06232	31829	230	7	257	5
Ribulose 1,5-bisphosphate carboxylase activase isoform 1 [*Hordeum vulgare* subsp. *Vulgare*]	AAA63163	47113	227	4	230	3
Oxygen-evolving enhancer protein 1	O49079	34848	135	4	152	3
Cold-responsive protein COR14a – wheat	**AAF17098**	**13537**	**128**	**2**	**251**	**5**
Ribosomal protein L12.1 precursor, chloroplast – rye	S30199	18105	108	1	110	1
Glycine-rich RNA-binding protein, low-temperature-responsive – barley (encoded by gene blt801)	S71453	15916	102	2	103	3
Plastocyanin, chloroplast precursor	P20423	15567	61	2	95	2
Thioredoxin M-type, chloroplast precursor (TRX-M)	Q41864	18061	61	1	36	1
RNA binding protein – barley	S53050	16789	58	2	66	2
Glycine-rich RNA-binding protein GRP1 – wheat	S71779	16245	58	2	66	2
WCOR719	**AAC49404**	**15755**	**57**	**1**	**228**	**3**
Cold-responsive LEA/RAB-related COR protein [*Triticum aestivum*]	**AAF68628**	**17150**	**55**	**1**	**289**	**5**
RuBisCO subunit binding-protein beta subunit (60 kDa chaperonin beta subunit) (CPN-60 beta)	Q43831	53379	53	1	74	2
Probable superoxide dismutase (EC 1.15.1.1) (Cu–Zn) precursor, chloroplast – wheat	T06229	20310	51	2	220	4
Ribulose-1,5-bisphosphate carboxylase/ oxygenase [*Lycopodiella cernua*]	CAC22264	ND	35	1	40	1
Ribulose 1,5-bisphosphate carboxylase/ oxygenase large chain [*Triticum aestivum*]	NP_114267	52817	126	3	ND	ND
Phosphoglycerate kinase, chloroplast precursor	P12782	49809	98	2	ND	ND
Ribulosebiphosphate carboxylase [*Peridictyon sanctum*]	CAA90006	52729	89	3	ND	ND
Oxygen-evolving enhancer protein 3-2	Q41806	22829	76	2	ND	ND
Calmodulin [*Lycopersicon esculentum*]	CAA75056	13307	61	1	ND	ND
Calmodulin	P04464	ND	61	1	ND	ND
Ribulose-biphosphate carboxylase large subunit [*Blandfordia punicea*]	CAA98038	51980	61	2	ND	ND
Ribulose 1,5-bisphosphate carboxylase large subunit [*Stenomesson variegatum*]	AAF97673	48276	58	2	ND	ND
Ribulose 1,5-bisphosphate carboxylase large subunit [*Cartonema philydroides*]	AAD02089	ND	53	1	ND	ND
Ribulose-1,5-bisphosphate carboxylase large subunit [*Peliosanthes campanulata*]	BAA83125	51841	53	2	ND	ND
Ribulose-1,5-bisphosphate carboxylase/ oxygenase [*Tremulina tremula*]	AAD50115	49394	53	2	ND	ND
GF14 mu [*Arabidopsis thaliana*]	AAB49334	29475	50	2	ND	ND
Acyl carrier protein I, chloroplast precursor	P02902	15964	48	1	ND	ND
rpS28 [*Hordeum vulgare* subsp. *vulgare*]	CAA04565	7481	44	1	ND	ND

Table 4. (*continued*)

Protein	Accession no.	Mass	Score (NA)	QM (NA)	Score (CA)	QM (CA)
14-3-3 protein [*Vigna angularis*]	BAB47118	28043	43	3	ND	ND
31 kDa ribonucleoprotein, chloroplast (RNA-binding protein cp31), putative [*Arabidopsis thaliana*]	NP_199836	ND	42	2	ND	ND
14-3-3 protein (grf15), putative [*Arabidopsis thaliana*]	NP_565347	ND	42	1	ND	ND
RuBisCO large subunit [*Hypecoum imberbe*]	AAB88833	ND	39	1	ND	ND
Putative methyl-binding domain protein MBD105 [*Zea mays*]	AAK40307	43684	37	1	ND	ND
Putative cytochrome P-450 like protein [*Oryza sativa* (japonica cultivar-group)]	NP_919986	ND	36	3	ND	ND
Ribulose-1,5-bisphosphate carboxylase, large subunit [*Mitchella repens*]	CAA93017	51608	35	2	ND	ND
Ribulose-1,5-bisphosphate carboxylase, large subunit [*Plocama pendula*]	CAA93028	52159	35	2	ND	ND
Oxygen evolving enhancer protein 1 precursor [*Bruguiera gymnorrhiza*]	BAA96365	35112	ND	ND	247	3
Cold acclimation protein WCOR615 – wheat	**T06812**	**17773**	**ND**	**ND**	**190**	**4**
Leaf-specific protein Wcs19 – wheat	**Q06540**	**19690**	**ND**	**ND**	**175**	**3**
ESTs AU070372(S13446),AU075541(S0353)	BAA82377	23165	ND	ND	129	2
Ribulose bisphosphate carboxylase activase B [*Triticum aestivum*]	AAF71272	47784	ND	ND	127	2
ES2A protein – barley	Q43478	18371	ND	ND	96	2
Cysteine proteinase inhibitor [*Triticum aestivum*]	BAB18768	13802	ND	ND	51	1
Dehydrin-/LEA group 2-like protein [*Lophopyrum elongatum*]	**AAC05923**	**12755**	**ND**	**ND**	**47**	**1**
Probable dehydrin WCOR726 – wheat	**T06804**	**12664**			**47**	**1**
200 kDa cold-induced protein {12 kDa fragment} [*Triticum aestivum* = wheat, Peptide Partial, 25 aa]/fragment of WCS200	**AAB31284**	**2729**	**ND**	**ND**	**44**	**1**
Plastocyanin, chloroplast precursor	P08248	15699	ND	ND	43	1
Ribulose-1,5-bisphosphate-carboxylase [*Euphorbia characias*]	CAA49390	6304	ND	ND	40	1
Ribulose-1,5-bisphosphate carboxylase oxygenase large subunit [*Heteranthera limosa*]	AAD09845	49484	ND	ND	40	2
Ribulose 5-bisphosphate carboxylase, large subunit [*Gaillonia yemenensis*]	CAC19566	51401	ND	ND	40	2

COR proteins are given in bold. Individual scores >34 indicate identity or extensive homology ($p<0.05$).
QM—queries matched, ND—non-detected.

published data (Sutka, 1981; Gusta et al., 2001; Prášilová and Prášil, 2001).

The decrease of water content during a cold acclimation is related to an accumulation of many compounds typical for a cold treatment (Levitt, 1980). The higher concentration of proteins soluble upon boiling (CP) in the CA leaves compared to NA leaves was found in our experiments when it was expressed per fresh weight (FW). However, the opposite result in CP was obtained after a recalculation per dry weight (DW) (Table 1). It was shown that major changes in water content were not due to a loss of water, but due to a high accumulation of the dry matter (mainly non-protein components such as sugars) in the mature leaf during cold acclimation (Prášil et al., 2001). Therefore, the expression of the concentration of soluble proteins per fresh weight may represent the more accurate method for determination of the protein ratio between NA and CA plants.

The differences in the protein patterns between boiled samples of CA and NA cultivars were recognizable on protein gel blots and 2-DE gels (Figs. 1 and 2). All five major WCS120 proteins classified by Sarhan et al. (1997) were visible and identified by both methods in CA plants, contrary to NA plants (cultivated at 17 °C), where only a low level of WCS120 protein was detectable in winter cultivars (Table 3). Kobayashi et al. (2004) did not detect any level of *wcs120* mRNA at 25 °C in NA winter (Mironovskaya 808) cultivar, while Fowler et al. (1996) observed very low levels of *wcs120* mRNA at 17 °C in NA wheats, i.e. at the same temperature which we used for cultivation of plants before acclimation. This could be explained by a threshold temperature below 25 °C but higher than 17 °C for the induction of *wcs120* genes. Different threshold temperatures were demonstrated by Crosatti et al. (1995, 1996) for *Cor14a* and *Cor14b* in barley and by Vagujfalvi et al. (2000, 2003) for a homologous gene of *Cor14b* in wheat. Questions of whether different WCS120 proteins have different threshold temperatures in various cultivars have not yet been addressed.

Several studies, e.g. Houde et al. (1992b), Fowler et al. (1996), Ohno et al. (2001), NDong et al. (2002), Danyluk et al. (2003), Takumi et al. (2003) and Kobayashi et al. (2004), showed higher level of expression of *Cor* genes in winter wheat (frost tolerant) than spring wheat (frost sensitive). The research based on protein gel blot analysis of some wheat COR proteins, e.g. WCS120 proteins (Fowler et al., 1996), WCS19 (NDong et al., 2002) or WCOR14, 15 and WDHN13 (Kobayashi et al., 2004) distinguished spring and winter wheat cultivars by protein accumulation. Recently, Gulick et al. (2005) found, using cDNA microarray, 65 genes regulated differently between the cold-acclimated spring cultivar Genlea and winter cultivar Norstar. However, research in this field has not focused on or shown evidence of differences in protein accumulation between winter wheat cultivars with different FT. In our studies, we found differences in the quantity of WCS120 proteins on the 2-DE gels (Table 3) between two cold-acclimated winter cultivars. Moreover, some of the WCS120 proteins showed strong correlations between their density on the 2-DE gels (Table 3) and the cultivar's FT (Table 1). The sum of the WCS120 proteins was also significantly higher in MIR than in BEZ. The most pronounced difference in protein accumulation was observed in the accumulation of WCS40 protein. Unfortunately, the spot of WCS40 had the lowest density in all 2-DE gels of CA plants, which limits the use of this protein for determination of differences in FT of winter wheat cultivars. Further, we found no differences between CA winter wheats in the accumulation of WCS180 and WCS200 proteins. On the other hand, the FT of CA wheat cultivars MIR and BEZ could be very clearly distinguished based on the accumulation of WCS120 and WCS66 proteins.

The use of LC–MS/MS made it possible to identify a number of proteins in the total sample of proteins soluble upon boiling (Fig. 3 and Table 4) and to obtain more detailed insight into the changes of protein patterns after cold acclimation of cultivar MIR.

We found a higher number of identified COR proteins in the CA samples (seven proteins) than in the NA samples (three proteins) (Fig. 3). The number of peptides of a specific protein and also the score could depend on the amount of the protein in the sample (Table 4). Consequently, the observed peptide numbers and scores could indicate a lower abundance of these three COR proteins in the NA samples (Table 4). Members of WCS120 proteins (WCOR726, WCS200) appeared only in CA samples. WCS19 and COR14a (WCOR14) belong to three LEA chloroplastic proteins and their accumulation is stimulated by light and cold temperature (Chauvin et al., 1993; NDong et al., 2002). WCOR719 is an actin-binding protein (Danyluk et al., 1996). Cold-responsive LEA/RAB-related COR protein is responsive to the abscisic acid pathway (Tsuda et al., 2000). WCOR615 shares sequence homology with cold-responsive LEA/RAB-related COR protein and therefore probably belongs to the abscisic acid-inducible proteins.

The presence of 22 proteins identified only in NA plants (like 14-3-3 homologues) and 13 proteins identified only in CA plants (like cystein proteinase inhibitor) indicate that NA and CA samples could be characterized also by other proteins than COR proteins.

The MS of total protein sample showed a high number of chloroplastic proteins (e.g. COR14a). This may be due to a higher abundance of these proteins compared to non-chloroplastic proteins in the leaves. However, most of the observed proteins or transcripts of genes encoding these proteins were found by others in plants after cold treatment. For example, Gulick et al. (2005), found a moderately increased level of transcript of RuBisCO large subunit, a moderately decreased level of transcript of RuBisCO small subunit and a moderately decreased level of transcript of plastid ribosomal protein CL9 in CA (36 days) winter wheat Norstar compared to NA control. Unfortunately, LC–MS/MS of total protein sample under our conditions showed qualitative, not quantitative, differences in proteins and therefore it is almost

impossible to compare our results directly with quantitative transcriptomic studies. Only large differences in the protein content (e.g. COR proteins) between CA and NA tissues could be determined based on our results (Table 4). The proteins identified as proteins of photosynthetic apparatus and carbon fixation, and proteins related to a translation were found in all samples. However, some of these proteins identified as the homologous proteins (like some of RuBisCO proteins) were found only in the CA or only in the NA plants. This result indicates that different protein isoforms are produced under the different conditions. Therefore, the protein isoforms found only in the CA samples could be associated with the response of plants to cold. Our results show that LC–MS/MS analysis is a useful tool for characterizing differences in protein level in plants with different level of FT.

In conclusion, it was possible to distinguish two winter wheat cultivars, differing in the degree of FT, by the accumulation of soluble proteins extracted from CA leaves, namely WCS120 and WCS66. A low level of WCS120 protein was possible to detect at 17 °C indicating that the threshold temperature for induction of this protein is high for the winter wheat cultivars. Further, the fact that cold-acclimated and non-acclimated samples of the MIR were different by LC–MS/MS also illustrated the possibility to distinguish the plants with different FT by this method.

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