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Cold-modulated small proteins abundance in winter triticale (x *Triticosecale*, Wittm.) seedlings tolerant to the pink snow mould (*Microdochium nivale*, Samuels & Hallett) infection

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Two winter triticale (x *Triticosecale* Wittmack) model cultivars: Hewo (tolerant to pink snow mould) and Magnat (sensitive) were used to test the effect of cold-hardening (4 weeks at 4°C) on soluble ≤50 kDa protein profiles of the seedling leaves. The presence and abundance of individual proteins were analysed *via* two-dimensional gel electrophoresis (2-DE) and Surface-Enhanced Laser Desorption/Ionization Time-of-Flight (SELDI-TOF). Up to now, no proteomics analysis of triticale response to hardening has been performed. Thus, the present paper is the first in the series describing the obtained results. In our experiments, the exposure to the low temperature-induced only quantitative changes in the leaves of both cultivars, causing either an increase or decrease of 4–50 kDa protein abundance. Among proteins which were cold-accumulated in cv. Hewo's leaves, we identified two thioredoxin peroxidases (chloroplastic thiol-specific antioxidant proteins) as well as mitochondrial-β-ATP synthase subunit and ADP-binding resistance protein. On the contrary, in hardened seedlings of this genotype, we observed the decreased level of chloroplastic RuBisCO small subunit PW9 and epidermal peroxidase 10. Simultaneous SELDI-TOF analysis revealed several low mass proteins better represented in cold-hardened plants of tolerant genotype in comparison to the sensitive one and the impact of both genotype and temperature on their level. Based on those results, we suggest that indicated proteins might be potential candidates for molecular markers of cold-induced snow mould resistance of winter triticale and their role is worth to be investigated in the further inoculation experiments.

Key words: Cereals, cold-hardening, pathogen tolerance, thiol-specific peroxidases, two-dimensional gel electrophoresis, Surface-Enhanced Laser Desorption/Ionization Time-of-Flight

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Abbreviations: AFPs, Anti-Freeze Proteins; IEF, Isoelectrofocusing; LTPs, Lipid-Transfer Proteins; MALDI-TOF, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight; PR, Pathogenesis-Related proteins; Prx, peroxiredoxin; ROS, reactive oxygen species; SAR, Systemic Acquired Resistance; SELDI-TOF, Surface-Enhanced Laser Desorption/Ionization Time-of-Flight; 2-DE, two-dimensional gel electrophoresis

INTRODUCTION

According to the regular reports, pink snow mould resulting from *Microdochium nivale* (Samuels & Hallett) infection is one of the most serious diseases of winter cereals and grasses in temperate and cold climatic areas (Tronsmo *et al.*, 2001; Prończuk *et al.*, 2003, Ren *et al.*, 2015, Stricker *et al.*, 2017). *M. nivale* infects also winter triticale (*x Triticosecale* Wittmack), the man-made hybrid cereal, lowering the quality and quantity of its yield. Variable levels of triticale pink snow mould infection were reported from field experiments and from tests under control conditions (Cichy & Maćkowiak, 1993; Hudec & Bokor, 2002; Sliesaravičius *et al.*, 2006; Golębiowska & Wędzony, 2009; Zhukovsky & Ilyuk, 2010; Szechyńska *et al.*, 2011; 2013; Arseniuk & Góral, 2015).

In response to fungal attack and other stresses, higher plants can produce special proteins belonging to Pathogenesis-Related proteins (PR-proteins), which are classified into PR1-17 families (Okushima *et al.,* 2000). They are expressed mainly in leaves, but also in other plant organs (Van Loon, 1997; Van Loon, 2006). Among others, glucanases (PR2) and chitinases (PR3, 4, 8 and 11) are suggested to decompose fungal cell wall, while PR1, peroxidases (PR11) and thaumatins function as antibiotics, membrane permeabilizers of the invaders and inhibitors of fungal proteases. Their activity can inhibit pathogenesis/infection progress (Van Loon & Van Strien, 1999). PR-proteins can be a part of the first defensive barrier since they are produced in cell wall appositions at the early stage of pathogenesis before concise host-pathogen contact. They are also suggested to play a role in Systemic Acquired Resistance SAR (Tuzun & Somanchi, 2006). A potential role in defensive response to *M. nivale* infection was proposed for catalase and peroxidase (Golębiowska *et al.,* 2011), chitinase (Żur *et al.,* 2013) and thiol-specific antioxidant protein (Golębiowska-Pikania & Golemiec, 2015) in winter triticale; chitinase, endochitinase, 1,3-β glucanase in winter rye (Hiilovaara-Teijo *et al.*, 1999; Yeh *et al.*, 2000); chitinase, endochitinase, 1,3-β glucanase, PR1-a protein and peroxidase in winter wheat (Ergon *et al.*, 1998) and thaumatin-like proteins in winter wheat (Kuwabara *et al.*, 2002). The predicted molecular mass of those proteins ranges from 13 to 50 kDa. Despite the above studies, the 2-DE analysis of cereal PR protein profiles has not been performed so far.

As many authors proved, cold-hardening is the most important factor activating cereal defense responses to *M. nivale* infection (Laroche 1997; Ergon *et al.*, 1998; Browne *et al.*, 2008; Golębiowska & Wędzony, 2009;

Dubas *et al.*, 2011; Szechyńska *et al.*, 2011, 2013, 2015). The maximal resistance was detected exclusively in cold-hardened plants but genotypes differ in their ability to obtain cold-induced resistance (Hömmo, 1994; Ergon *et al.*, 1998; Gołębiowska & Wędzony, 2009; Gołębiowska & Golemiec, 2015). The relationship between frost resistance and snow mould resistance remains indistinct (Ergon *et al.*, 1998; Ergon & Tronsmo, 2006). Cold-hardening enhances snow mould resistance since stronger and more rapid transcription of genes encoding different PR-proteins were found in hardened compared to unhardened winter wheat plants after *M. nivale* inoculation (Ergon *et al.*, 1998; Hiilovaara-Teijo *et al.*, 1999; Gaudet *et al.*, 2000; Kuwabara *et al.*, 2002; Gaudet *et al.*, 2003a,b). However, the complete influence of cold on PR activity is prominent especially in inoculated plants (Ergon *et al.*, 1998; Gaudet *et al.*, 2000). PR-proteins accumulating in the apoplast of winter rye during cold acclimation were shown to exhibit glucanase and chitinase activity in addition to antifreeze activity (Hiilovaara-Teijo *et al.*, 1999; Yeh *et al.*, 2000) unlike PR proteins produced at warmer temperatures. Additionally, winter rye AFPs (Anti-Freeze Proteins) were apparently isoforms of PR-proteins specifically induced at low temperature (Yeh *et al.*, 2000). Moreover, in winter wheat experiments, a thaumatin-like protein was induced by cold (Kuwabara *et al.*, 2002). Gene transcription of defensin, purothionine, and LTPs (Lipid-Transfer Proteins) was maximal after 2-3 weeks of cold-hardening and remained constant for 7 days of dehardening (Gaudet *et al.*, 2003a). Expression of LTPs mentioned above was observed in non-hardened plants neither in laboratory nor under the field conditions.

Our previous investigations documented genotype-dependent, increased resistance of triticale after 4 weeks of cold-hardening at 4°C (Gołębiowska & Wędzony, 2009) and antioxidative enzyme involvement in the mechanisms of cold-induced snow mould resistance (Gołębiowska *et al.*, 2011; Gołębiowska & Golemiec, 2015; Gawrońska & Gołębiowska, 2016). However, no proteomic analysis of the reaction of triticale to cold-hardening has been done so far. Thus, extensive studies at the molecular level were performed and the present paper is the first in the series describing the obtained results. For that purpose, two winter triticale cultivars selected in the previous research (Gołębiowska & Wędzony, 2009): snow mould-tolerant cv. Hewo and sensitive cv. Magnat were used as the plant model to test the effect of cold-hardening on soluble protein patterns in seedling leaves. In this paper, we proposed the hypothesis that the protein expression profiles of those two genotypes differ after cold treatment and that some of the changes caused by hardening might be significant under snow cover and pathogen infection. The presence and amount of individual proteins were analyzed *via* 2-DE in fully-hardened plants in comparison to the control non-hardened seedlings. Parallel analyses were also performed *via* Surface-Enhanced Laser Desorption/Ionization Time-of-Flight (SELDI-TOF).

MATERIALS AND METHODS

Plant material. Two winter hexaploid triticale (*x Triticosecale* Wittmack, 2n=6x=42) cultivars, significantly different in respect to their cold-induced resistance to *M. nivale* infection in the field and under controlled conditions (Gołębiowska & Wędzony, 2009) were used in the study: tolerant cv. Hewo (Strzelce Plant Breeding – IHAR Group Ltd., Poland) and sensitive cv. Magnat (Danko Plant Breeders Ltd., Poland). Plants were surface-sterilized and grown as described previously (Gołębiowska & Wędzony, 2009) in the climatic chamber at 8h/16h (day/night) photoperiod, at 16°C/12°C, RH=60–67%, for 7 days. On the 7th day, plants were supplemented once with Hoagland & Arnon's (1938) sterile medium, 0.05 m^3 per pot.

Starting from the 8th day after potting, half of the plants were subjected to the prehardening for 14 days at 12°C/12°C, with 8h/16h (day/night) photoperiod. Then, they were hardened at 4°C/4°C for 28 days in the same light regime. The remaining control, non-hardened plants were grown constantly at 16°C/12°C, 8h/16h (day/night) photoperiod, until achieving the same stage of seedling development according to the Zadok's scale (Zadok *et al.*, 1974) as the fully cold-hardened plants, i.e. for the next 14 days (21 days from potting in total).

Plant sampling. The protein analysis was conducted on fully-expanded leaves (2nd in appearance) of 5 different seedlings of each genotype from the control non-hardened and the cold-hardened plants. Freshly cut samples were immediately frozen in liquid nitrogen and stored at -80°C until protein extraction. Analyses were performed in 3 biological replicates consisting of 5 leaves each, in a single growth experiment.

Protein isolation and purification. Leaf samples (ca 1 g fresh weight) were ground to a fine powder in liquid nitrogen using mortar and pestle. Then proteins were extracted according to Giavialisco and others (Giavialisco *et al.,* 2003, modified) protocol, in 5:1, v/w portions of extraction buffer consisted of 8 M Urea, 2 M Thiourea, 2%, w/v C7BzO (Sigma), 2%, v/v IPG Buffer (Amersham), 50 mM DTT and 1%, v/v proteinase inhibitors cocktail (Sigma, P 9599). After samples centrifugation at 4°C and 18 000 rpm for 10 min, protein concentration in the obtained supernatant was determined by the Bradford method (Bradford, 1976). Protein quantification was performed in triplicates against a standard curve of bovine serum albumin (BSA). Aliquots of proteins (500 μg) were stored at –80°C until further assay. Before analysis, each portion was purified *via* unspecific precipitation with Proteo Precipitation Kit (Sigma) according to the given protocol. Obtained pellet was resuspended in 0.5% IPG Buffer NL 3-10 (Amersham).

Two-dimensional gel electrophoresis. Protein samples were analyzed in triplicates. The aliquots were thawed, mixed with 3.2 μl of ampholytes (pH 3–10), adjusted to the final volume of 315 μl with the Isoelectrofocusing (IEF) sample solution and then loaded onto immobilized pH gradient (IPG) strips (pH 4–7, 18 cm, non-linear gradient, Amersham Biosciences) in an isoelectric focusing unit (Ettan™ IPGphor II™, Amersham Biosciences). IEF was performed according to Berkelman & Stendstedt (2004) protocol at 20°C for 20 h. After the first dimension IPG strips were equilibrated for 15 min in 5 ml of SDS equilibration buffer (1.5 M Tris-HCl pH 6.8, 6 M Urea, 30% (v/v) glycerol, 5% (w/v) SDS) with 2% (w/v) DTT followed by 15 min with the same buffer but containing 2.5% (w/v) iodoacetamide (IAA) instead of DTT. The strips were then transferred onto 12.5% SDS-polyacrylamide gels and overlayed with 0.5% (w/v) agarose in SDS running buffer with some addition of bromophenol blue as tracking dye. The second dimension electrophoresis was conducted according to Berkelman & Stendstedt (2004) at 10°C at 10 mA/gel until the dye front reached the bottom of the gel (approx. 16 h) by using Ettan Dalt Six electrophoresis unit (Amersham Biosciences). Simultaneously, molecular mass marker 10–200 kDa (PageRuler™ Pro-

tein Lader, Fermentas) was run. Gels were stained overnight in Colloidal Coomassie Brilliant Blue (CBB G-250) (Sigma) according to given protocol and digitalized using Image Scanner (Amersham Biosciences).

Analysis of protein expression. Image analyses (normalization, spot matching, expression analyses and statistics) were performed with PDQuest 8.0 software (Bio-Rad). First, images of gels were inverted, centralized and cropped using the same anchor spot, then the correlation coefficient between replicates was checked. The Master Gel was selected automatically and used for all bioinformatics analysis. The spot relative intensities were normalized to the total density in the gel images. Based on the MW of PR proteins reported in the literature, soluble proteins in the range of 13 kDa to 50 kDa were analysed using the software. One-way ANOVA statistical analysis was performed with a 95% significance level to determine which protein species were differentially abundant between the samples collected from control and cold-hardened plants of both genotypes. On the basis of the above calculations, spots showing a statistically significant ($p<0.05$) increase or decrease in abundance (at least 2-fold) were selected and manually picked for digestion and identification.

Mass spectrometric identification of the protein species. Proteins were in-gel digested with trypsin according to the protocol described by Shevchenko and others (Shevchenko *et al.,* 1996). The extracts of the obtained tryptic peptides were then spotted onto an AnchorChip target plate (MTP AnchorChip 384 T F, Bruker) and left to dry at ambient temperature. The anchors were subsequently covered with a solution of α-cyano-4-hydroxycinnamic acid (0.7 mg CHCA in 85% ACN, 15% H_2O, 0.1% TFA and 1 mM $NH_4H_2PO_4$) and again left to dry. All spectra were collected using ultrafleXtreme MALDI-TOF/TOF mass spectrometer and Compass 1.3 software for instrument control and data processing (both from Bruker Daltonik, Bremen, Germany). MS spectra were acquired in positive reflectron mode and externally calibrated using Peptide Calibration Standard II (Bruker). Fragment spectra were obtained by post-source decay (PSD) and internally calibrated on immonium ions. Protein identification was based on the peptide mass fingerprint confirmed by fragment spectra (PMF + MS/MS). MS and PSD spectra were peak-picked in flexAnalysis 3.3, sent to BioTools 3.2 (both Bruker software packages) and submitted to database search with the use of Mascot 2.4 (Matrix Science, London, England, http://www.matrixscience.com) in-house server. Five custom databases were created for this purpose based on the protein databases for *Triticosecale* (120 entries), *Secale* (945 entries), *Triticum* (50 862 entries), *Aegilops* (39 589 entries) and *Hordeum* (38 447 entries), taken from NCBI Taxonomy Browser. MS and MS/MS mass tolerance was 50 ppm and 0.5 Da, respectively. The identification results obtained were examined in terms of the score level (greater than 64) and the number of matched peptides (more than 2).

Surface-Enhanced Laser Desorption/Ionization (SELDI) and data analysis. The protocol for protein pattern analysis was performed according to Tang and others (Tang *et al.,* 2004, modified), using different protein chips (microcolumns): NP20 (neutral), HP50 (hydrophobic), CM10 (exchanging weak cations) and Q10 (exchanging strong anions). Samples (1–10 µl) of crude leaf extracts prepared in 10 mM Tris/HCl pH=7.0 buffer were analysed in 5 replicates for both control non-hardened and cold-hardened plants in the Protein Biological System II mass spectrometer reader (Ciphergen Biosystems, Fremont, CA). Peak detection accurate to the second decimal place was performed using ProteinChip Software 3.1 (Ciphergen Biosystems, Fremont, CA). The molecular mass below 2000 Da was eliminated from analysis because this area contains adducts and artifacts of the Energy Absorbing Molecule (EAM) and possibly other chemical contaminants. The spectra of samples generated under the same condition were grouped together and baseline subtracted then normalized to the total ion current of m/z starting from 1500 Da. Peak information was exported into MS Excel and peak intensity was calculated for each spectrum. Differences among the means were statistically examined with a Student's *t*-test using STATISTICA® version 13.0 software at $p<0.05$.

RESULTS

In the 2-DE electrophoregrams of winter triticale seedling leaves, the mean total number of 470 soluble proteins ranged from 13 kDa to 200 kDa was found. In the present analysis, 2-DE profiles of 360 soluble proteins sized from 13 kDa to 50 kDa were compared between biological replicates and between different genotypes/treatments. Within this protein group, quantitative differences in protein abundance were observed for proteins of different MW (14 kDa to 50 kDa) and pI (4.6 to 7.0) (Table 1). No qualitative changes were noted between the experimental objects.

In cold-hardened seedling leaves of the snow mould tolerant cv. Hewo, increased mean content of twelve proteins and decreased mean content of ten proteins were observed in comparison to the non-hardened control plants of this genotype (Table 1). Among cold-accumulated proteins, two chloroplastic enzymes 2-Cys peroxiredoxin BAS1 and Thioredoxin peroxidase, as well as ADP-binding resistance protein, were identified (Table 2, Fig. 1A). Proteins with the lowered concentration after exposure to the low temperature were identified as chloroplastic Ribulose bisphosphate carboxylase small chain

Table 1. The number of spots showing quantitative changes in abundance in leaves of model winter triticale seedlings.

Comparison	Differential spots*	≥2-fold up-regulated spots* (%)	≥2-fold down-regulated spots* (%)	MW [kDa]	pI
A: Hewo cold-hardened *vs.* control	22	12 (55%)	10 (45%)	15–50	4.6–6.8
B: Magnat cold-hardened *vs.* control	34	29 (85%)	5 (15%)	16–50	4.6–6.5
C: Control: Hewo *vs.* Magnat	10	7 (70%)	3 (30%)	16–50	4.9–6.7
D: Cold-hardened: Hewo *vs.* Magnat	12	6 (50%)	6 (50%)	14–49	4.9–7.0
Total	78	54 (69%)	24 (31%)	14–50	4.6–7.0

*Change in abundance was calculated by dividing the mean %vol of a spot in leaves of plants of the first object to mean %vol of that spot in leaves of plants of the second one.

Table 2. The list of identified protein species in leaves of cold-hardened and non-hardened seedlings of *M. nivale* tolerant and sensitive winter triticale cultivars.
NCBI database was used for identification. The results are based on peptide mass fingerprint confirmed by fragment spectra (PMF + MS/MS). CHL – chloroplast; CT – cytoplasm; EX - extracellular region/secreted; MT – mitochondrion.

Biological process*	Spot No. (SSP)	Spot volume**	NCBI accession No.	Identified protein	Protein symbol on gel	Subcellular localization	Organism	Me/Mt [kDa][a]	pIa	Score/ C [%][b]	A) Hewo: hardened *vs.* control***	B) Magnat: hardened *vs.* control***	C) control: Hewo *vs.* Magnat***	D) hardened: Hewo *vs.* Magnat***
Photosynthesis	4301	3327344.8	P26667 EC=4.1.1.39	Ribulose bisphosphate carboxylase small chain PW9	RCA	CHL	*Triticum aestivum*	16/19	4.9	230/90	174x ↓	13x ↓	150x ↑	4x ↓
	4313	58871.0	Q5I3E8	Peroxidase 10	POX10	EX	*Triticum monococcum*	23/37	5.1	475/57	10x ↓	31x ↑	9x ↑	33x ↓
Oxidation-reduction	6309	581440.9	P80602 EC=1.11.1.15	2-Cys peroxiredoxin BAS1	TSA1/1	CHL	*Triticum aestivum*	25/23	6.1	135/85	57x ↑	30x ↓	14x ↑	44x ↑
	6510	2001378.6	O81480	Thioredoxin peroxidase	TSA1/2	CHL	*Secale cereale*	49/28	6.1	470/33	3x ↑	5x ↓	5x ↑	17x ↑
Energy pathway	5406	480697.8	Q6YDA3	ADP-binding resistance protein	RP	CT	*Arachis stenosperma*	38/18	5.5	324/35	2x ↑	19x ↓	not changed	4x ↑
	5510	186484.9	EC=3.6.3.14, Q41534	β-ATP synthase subunit	ATP2	MT	*Triticum aestivum*	49/59	5.8	213/60	13x ↓	9x ↓	3x ↑	4x ↑

[a]Me – experimental mass weight; Mt – theoretical mass weight; theoretical values were retrieved from the protein database. [b]The score and protein sequence coverage (C) [Best Score in Biotools software]. *The protein function was assigned using a gene ontology browser QuickGO (www.ebi.ac.uk/QuickGO) and UniProt database (www.uniprot.org). **The average spot volume on the reference 2-DE gel of *M. nivale* tolerant winter triticale cv. Hewo after cold-hardening. ***Fold-change in abundance was calculated by dividing the mean % vol of a spot in leaves of the fist object to mean % vol of that spot in leaves of the second one.

PW9, extracellular/secreted Peroxidase 10 and mitochondrial β-ATP synthase subunit (Table 2, Fig. 1A).

After hardening of the sensitive cv. Magnat seedlings, the increased accumulation of twenty-nine proteins and decreased accumulation of five proteins were noted in comparison to the control plants of this genotype (Table 1). Among identified proteins, only peroxidase 10 showed increased level after cold-hardening, while five remaining: chloroplastic Ribulose bisphosphate carboxylase small chain PW9, 2-Cys peroxiredoxin BAS1 and Thioredoxin peroxidase, as well as mitochondrial β-ATP synthase subunit and ADP-binding resistance protein, revealed decreased concentration in comparison to the control plants (Table 2, Fig. 1B).

Differences between cv. Hewo and cv. Magnat control seedlings involved seven proteins with the increased concentration as well as other three proteins with the decreased abundance (Table 1). Leaves of the control cv. Hewo seedlings showed a higher concentration of peroxidase 10, Ribulose bisphosphate carboxylase small chain PW9, 2-Cys peroxiredoxin BAS1, Thioredoxin peroxidase as well as β-ATP synthase subunit when compared to cv. Magnat non-hardened plants (Table 2, Fig. 1C).

In cold-hardened plants, differences between the model genotypes included six proteins with the increased and six proteins with the decreased expression level (Table 1). Among them, four proteins with the higher abundance in cv. Hewo leaves were identified as

2-Cys peroxiredoxin BAS1, Thioredoxin peroxidase, ADP-binding resistance protein and β-ATP synthase subunit (Table 1, Fig. 1D). Proteins with the decreased level after cold-hardening in tolerant genotype in comparison to sensitive one were identified as Ribulose bisphosphate carboxylase small chain PW9 and Peroxidase 10 (Table 2, Fig. 1D).

SELDI-TOF analysis of the same plant samples revealed the presence of twenty-one low molecular weight proteins (4.3–10.7 kDa) with the majority of them visible on CM10 columns (Table 3). After cold-hardening, only quantitative changes in the concentration of those molecules were noted in both triticale cultivars (Table 3). Cold-hardened seedling leaves of tolerant cv. Hewo showed the increased mean concentration of seven proteins (ST2-3, ST6-7, ST13-14 and ST21) and the decreased mean concentration of four proteins (ST1, ST12, ST15 and ST20), when compared to the non-hardened control plants of this genotype (Table 3A). In sensitive cv. Magnat plants those changes involved twelve (ST1-3, ST5-6, ST11, ST13-17 and ST21) and three (ST8, ST12 and ST20) proteins, accordingly (Table 3B). In control plants of cv. Hewo the concentration of ten proteins (ST1, ST3-4, ST6, ST8 and ST13-17) was increased whereas the concentration of three proteins (ST10-12) was decreased in relation to the control plants of cv. Magnat (Table 3C). After cold hardening, four proteins (ST3, ST6 and ST13-14) had higher and seven (ST5, ST11-12, ST15-17 and ST21) had a lower level in cv. Hewo leaves in comparison to cv. Magnat cold-hardened plants (Table 3D). After exposure to the low temperature, the maximal experimental differences between cv. Hewo and cv. Magnat leaf profiles were found for ST6 protein (6.8 kDa, 203%) as well as ST15 protein (8.9 kDa, 32%). The concentration of

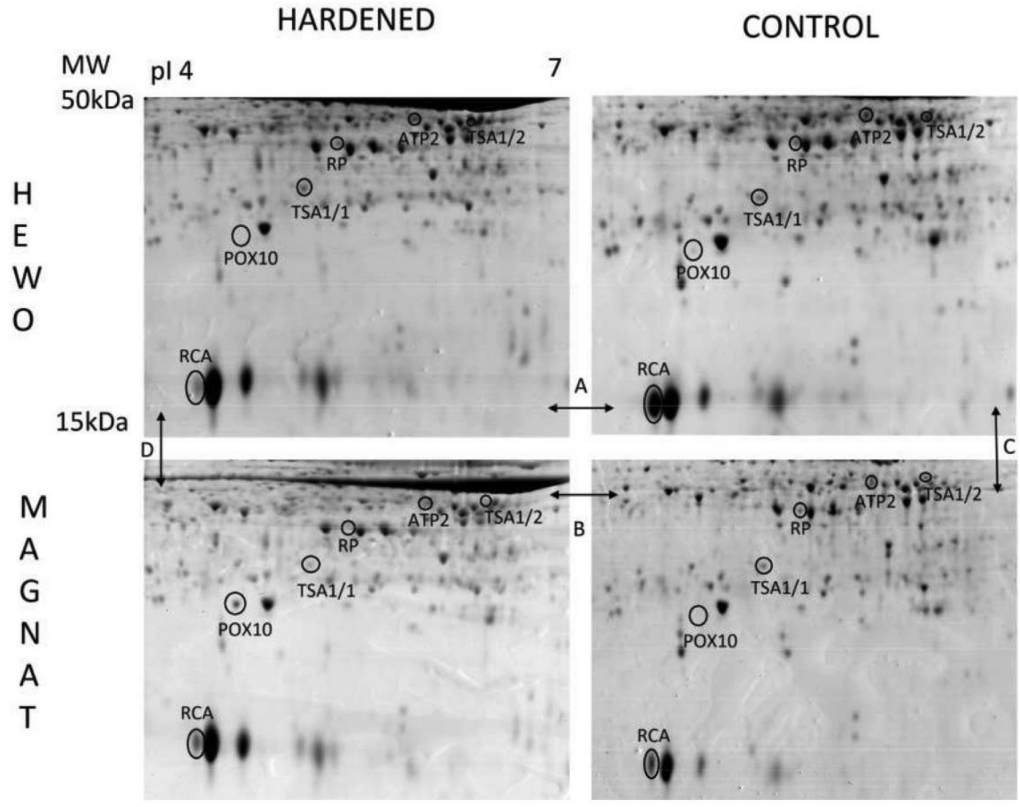

Figure 1. The comparison of soluble 14–50 kDa leaf protein 2-DE profiles between winter triticale (*xTriticosecale* Wittmack) seedlings of:
(**A**) snow mould tolerant cv. Hewo, cold-hardened *vs*. non-hardened control; (**B**) snow mould sensitive cv. Magnat, cold-hardened *vs*. non-hardened control; (**C**) cv. Hewo *vs*. cv. Magnat non-hardened control; (**D**) cv. Hewo *vs*. cv. Magnat cold-hardened plants. Circles indicate the position of identified protein species differentially abundant in compared objects. Spot details are described in Table 2. Abbreviations: ATP2 – β-ATP synthase subunit, mitochondrial; POX10 – peroxidase 10, extracellular/secreted; RCA – Ribulose bisphosphate carboxylase small chain PW9, chloroplastic; RP – 38 kDa ADP-binding resistance protein; TSA1/1 – 2-Cys peroxiredoxin BAS1, chloroplastic; TSA1/2 – Thioredoxin peroxidase, chloroplastic.

ST4, ST9, ST10, ST18-19 was not changed by hardening in leaves of both genotypes, additionally, ST9, ST18-19 proteins had equal level in all experimental objects (Table 3).

DISCUSSION

In our experiments, the exposure to the low temperature (4 weeks at 4°C) caused only quantitative changes in the protein patterns of seedling leaves of the two model winter triticale cultivars. Those changes included either increase or decrease of 4.3–10.7 kDa and 14-50 kDa soluble protein concentration, detected *via* SELDI-TOF and 2-DE analysis, accordingly (Tables 1 and 3).

The group of proteins with the cold-caused abundance drop was better represented in 2-DE profiles of snow mould tolerant cv. Hewo seedling leaves, while in sensitive cv. Magnat plants many proteins revealed increased abundance after hardening (Table 1). A similar effect was observed by Golebiowska-Pikania *et al.* (2017) in winter barley seedlings: the maximal number of up-regulated proteins in hardened (20 days at 4/2°C) plants was observed for the most freezing-sensitive DH line while the minimal for the freezing-tolerant one. This may suggest the strong low-temperature stress occurring in sensitive genotypes. Additionally, in present experiments, after exposure to the low temperature, changes of Peroxidase 10, 2-Cys peroxiredoxin BAS1, Thioredoxin peroxidase and ADP-binding resistance level in tolerant cv. Hewo plants had the opposite direction in comparison to the sensitive cv. Magnat (Table 2).

Even without hardening, Ribulose bisphosphate carboxylase small chain PW9, Peroxidase 10, 2-Cys peroxiredoxin BAS1, Thioredoxin peroxidase, and β-ATP synthase subunit had different (higher) abundance in cv. Hewo leaves in comparison to cv. Magnat ones (Table 2). Among identified proteins, only 38 kDa protein with homology to ADP-binding 18 kDa resistance protein showed no difference in abundance between model cultivars without hardening (Table 2). On the contrary, after cold treatment, its content increased in cv. Hewo seedlings leaves and it had a higher level in relation to cv. Magnat plants growing under the same conditions. This protein with an unknown function was described by Bertioli and others (Bertioli *et al.,* 2003) in peanut (*Arachis stenosperma*) young leaves. In our experiment its increase was cold-specific.

The other protein cold-accumulated in plants of tolerant winter triticale genotype, 2-Cys peroxiredoxin BAS1 is homologous to a chloroplastic thiol-specific antioxidant 23 kDa protein found in wheat leaves by Tsunoyama and others (Tsunoyama *et al.,* 1996). The Cys-64-SH group of this enzyme was indicated as the primary site of oxidation by hydrogen peroxide, and the oxidized Cys-64 rapidly reacts with Cys-185-SH of the other subunit to form an intermolecular disulfide, which might subsequently be reduced by thioredoxin (Tsunoyama *et al.*, 1996). In our work, we also identified the second thioredoxin peroxidase, with increased accumulation in cv.

Table 3. The comparison of SELDI-TOF protein profiles of the winter triticale seedling leaves.
↑ – increased accumulation; ↓ – decreased accumulation of individual protein; n/c – not changed.

Protein		Abundance in plant leaves				
Symbol		Properties	A) Hewo: hardened *vs.* control	B) Magnat: hardened *vs.* control	C) control: Hewo *vs.* Magnat	D) hardened: Hewo *vs.* Magnat
	MW [Da]	Affinity				
ST 1	4.3	Q10/CM10	↓ 75%	↑ 135%	↑ 164%	n/c
ST 2	4.4	CM10	↑ 156%	↑ 163%	n/c	n/c
ST 3	6.4	CM10	↑ 599%	↑ 546%	↑ 461%	↑ 130%
ST 4	6.6	CM10	n/c	n/c	↑ 142%	n/c
ST 5	6.7	CM10	n/c	↑ 232%	n/c	↓ 131%
ST 6	6.8	CM10	↑ 854%	↑ 383%	↑ 450%	↑ 203%
ST 7	6.9	Q10/CM10	↑ 145%	n/c	n/c	n/c
ST 8	7.0	CM10	n/c	↓ 70%	↑ 138%	n/c
ST 9	7.5	CM10	n/c	n/c	n/c	n/c
ST 10	7.6	CM10	n/c	n/c	↓ 180%	n/c
ST 11	8.4	Q10	n/c	↑ 120%	↓ 125%	↓ 148%
ST 12	8.6	Q10	↓ 62%	↓ 66%	↓ 135%	↓ 178%
ST 13	8.7	CM10	↑ 295%	↑ 144%	↑ 203%	↑ 145%
ST 14	8.8	CM10	↑ 194%	↑ 206%	↑ 159%	↑ 144%
ST 15	8.9	CM10	↓ 49%	↑ 457%	↑ 168%	↓ 312%
ST 16	9.0	CM10	n/c	↑ 242%	↑ 160%	↓ 139%
ST 17	9.1	CM10	n/c	↑ 266%	↑ 154%	↓ 177%
ST 18	9.4	CM10	n/c	n/c	n/c	n/c
ST 19	9.5	CM10	n/c	n/c	n/c	n/c
ST 20	10.2	Q10	↓ 53%	↓ 52%	n/c	n/c
ST 21	10.7	Q10	↑ 180%	↑ 290%	n/c	↓ 162%

Hewo hardened seedling leaves, with homology to 28 kDa rye protein (Berberich *et al.*, 1998). Cold-enhanced *Tsa* gene expression of the foliar thiol-specific antioxidant protein in *M. nivale* tolerant winter triticale seedlings after 4 weeks of chamber culture at 4°C was also previously documented in our qPCR analysis (Gołębiowska-Pikania & Golemiec, 2015). In seedling leaves of cv. Hewo growing in the same hardening conditions we showed lower cold-mediated accumulation of hydrogen peroxide as well as a higher increase in the total unspecific peroxidase activity in comparison to cv. Magnat plants (Gołębiowska *et al.*, 2011). Other authors reported a protective role of 2-Cys peroxiredoxins in photosynthesis (Baier & Dietz, 1999; Horling *et al.*, 2002) and in oxidative stress-dependent switching from the peroxidase to molecular chaperone function (Jang *et al.*, 2004), including the plant cellular defensive signaling mechanisms against oxidative stress (Jang *et al.*, 2006). 2-Cys PRXs can interact with at least 18 other proteins and regulate them by their redox state as described in Rhee & Woo review (Rhee & Woo, 2011). On the basis of the above results, it can be assumed that in the tolerant cv. Hewo seedlings the cold-caused increased production of hydrogen peroxide is balanced by the thioredoxin peroxidases activity which we confirmed by spectrophotometric, qPCR and proteomic analyses.

In cv. Hewo leaves, the β-ATP synthase subunit had also higher level in cold-hardened plants, and it is homologous to wheat mitochondrial 59 kDa chain with the molecular function of creating ATP from ADP in the presence of a proton gradient across the membrane (Abulafia *et al.*, 1996). Such a result may suggest increased energy production at low temperature in snow mould tolerant genotypes.

On the contrary, in hardened cv. Hewo plants we showed a decreased level of RuBisCO small subunit PW9 and peroxidase 10 in comparison to cv. Magnat ones (Table 1). The first protein was discovered in wheat chloroplast by Broglie and others (Broglie *et al.,* 1993) as a 19 kDa transit protein, belonging to RuBisCO small chain family. It was reported that protein expression of this subunit decreases in leaves of winter rape moved to cold-hardening conditions but increases in young leaves expanding at the low temperature (Singh & Johnson-Flanagan, 1994). Similarly to our results, other authors also observed in 2-DE profiles a decreased accumulation of several photosynthesis-related proteins in winter wheat subjected to 63 day-long hardening at 4°C (Rinalducci *et al.*, 2011). The decrease in susceptibility to photoinhibition exhibited following cold-hardening of winter and spring cultivars of wheat was not due to an increased capacity of repair of photoinhibitory damage at 5°C but reflected intrinsic properties of the cold-hardened photosynthetic apparatus (Hurry & Huner, 1992). The down-accumulated peroxidase 10 identified by us was homologous to 37 kDa class III peroxidase extracted from the powdery mildew-attacked epidermis of einkorn wheat (*Triticum monococcum*), involved in response to oxidative stress (Liu *et al.*, 2005).

Similarly to the results described above, only quantitative changes caused by the low temperature on protein concentration were noted in SELDI-TOF analysis (Table 3). Like in 2-DE profiles, more proteins with the increased concentration were present in leaves of cold-hardened plants of cv. Magnat in comparison to non-hardened control of this genotype than in the similar comparison performed for cv. Hewo seedlings, while the number of proteins with the decreased level was almost equal (Table 3A, B). In leaves of both genotypes, the concentration of ST2-3, ST6, ST13-14 and ST21 increased and the concentration of ST12 and ST20 decreased after cold-hardening (Table 3A,B). Among those proteins, ST3 (6.4 kDa), ST6 (6.8 kDa), ST13 (8.7 kDa) and ST14 (8.8 kDa) had a higher concentration and ST12 had a lower concentration in cv. Hewo both in cold-hardened and non-hardened plants in relation to similarly treated cv. Magnat ones (Table 3C, D). Additionally, ST5, ST11, ST15-17 and ST21 revealed lower level in cold-hardened cv. Hewo plants in comparison to cold-hardened plants of cv. Magnat (Table 3D). Even without hardening - in control plants of cv. Hewo proteins: ST1, ST4, ST8 and ST15-17 had higher level and ST10-11 had lower level in relation to control cv. Magnat plants (Table 3C). It can be assumed, that several low mass proteins were better represented in cold-hardened cv. Hewo plants in comparison to cv. Magnat ones and the impact of both genotype and temperature on their concentration was detected in our experiment.

Based on the above results we conclude that genotype and cold treatment significantly influenced the quantity of the small proteins in winter triticale seedling leaves. The pattern of these proteins could be correlated with the level of the resistance to pink snow mould infection. Four cold-accumulated proteins: ADP-binding 38 kDa resistant protein, β-ATP synthase subunit, as well as 25 kDa and 49 kDa thioredoxin peroxidases, may possibly play a role in the plant preparation before fungal pathogen attack in tolerant winter triticale cultivar by maintaining the red/ox balance and energy pathway in seedling leaves. Present studies may indicate molecules for the further analysis of *M. nivale* pathogenesis process in winter cereals.

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