The effect of cold hardening on leaf protein profile in barley DH lines varying in frost tolerance

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Introduction:

Barley (*Hordeum vulgare* L.) is one of the most important cereal grains, due to its wide utilization in the malting and brewing industry, for animal feed and human consumption. However, in Polish climatic conditions, its poor winter hardiness results in significant yield losses from cold injury almost every year. Therefore, the aim of presented research was the identification of factors that determine freeze tolerance and could improve selection efficiency of genotypes of potential value for future breeding programmes.

Since freeze tolerance is a polygenic trait resulting from multiple genes interactions, the protein expression profiling *via* 2-D PAGE seems to be the most effective method of the analysis. Another beneficial tool used in the study was doubled haploid (DH) technology, which enabled to achieve total homozygosity in one generation and increased the genetic diversity within DH progeny by the expression of recessive allele based traits.

Materials and methods:

Plant material

The plant material consisted of 10 doubled haploid (DH) lines of winter barley (*Hordeum vulgare* L.), produced from Polish breeding materials (F_1 generation) by *in vitro* anther culture method according to the combined methods of Jacquard et al. (2003) and Cistué et al. (2003) with several minor modifications. Studied genotypes were evaluated for their freezing tolerance according to Rapacz et al. (2011) and characterized as significantly different in respect of this trait. Among them, DH61, DH65, DH435 and DH602 lines were considered as partly frost resistant whereas DH158, DH561 and DH575 were the most susceptible. The remaining 3 lines (DH363, DH534, DH584) showed intermediate frost resistance.

The grains were planted in 30 cm x 38 cm x 9 cm plastic boxes filled with a mixture of soil, sand and peat substrate (1:1:1, v:v:v) in a randomized complete block design, in three replicates. Plants grew in a greenhouse conditions at 25°C/17°C day/night, 12/12h light/darkness up to the 5-leaf stage of development. Then they were subjected to a hardening period in the climatic chamber at 4°C/2°C day/night, 9/15h light/darkness for 3 weeks.

Samples collection

For proteomic analysis, seedlings leaves of 0.5g total fresh weight were collected after 3 weeks of cold-hardening. The control samples were harvested from non-hardened plants at the same phase of development. Leaves (always first in seedlings development) from three different plants were considered as one biological replicate. Collected samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Protein expression profiling

The changes in the abundance in protein species were analysed in triplicates using gel-based proteomics. The proteins were isolated according to the phenol-based procedure (Hajduch et al. 2005) and examined by 2-D electrophoresis and PDQuest ver. 8.0 Basic (Bio-Rad) software (**Fig. 1**). Chosen, the most interesting proteins highly differentiated across examined DH lines were identified by MALDI TOF/TOF MS/MS analysis.

Results:

Since cold-hardening is the factor inducing plant freeze tolerance, the variation among seedlings exposed to the low temperature was considered as the most important. Both, quantitative (807 spots) and qualitative (152 spots) differences in leaf protein expression have been detected in examined cold-treated genotypes (**Tab. 1**). Up to now, among them five proteins were successfully identified (**Tab. 2**). These identified proteins were classified as being mainly related to photosynthesis (RuBisCO large subunit, RuBisCO activase A and photosystem II oxygen-evolving enhancer protein 1) and its higher concentration was detected in the seedlings of partly frost resistant barley DH lines when compared to the susceptible ones.

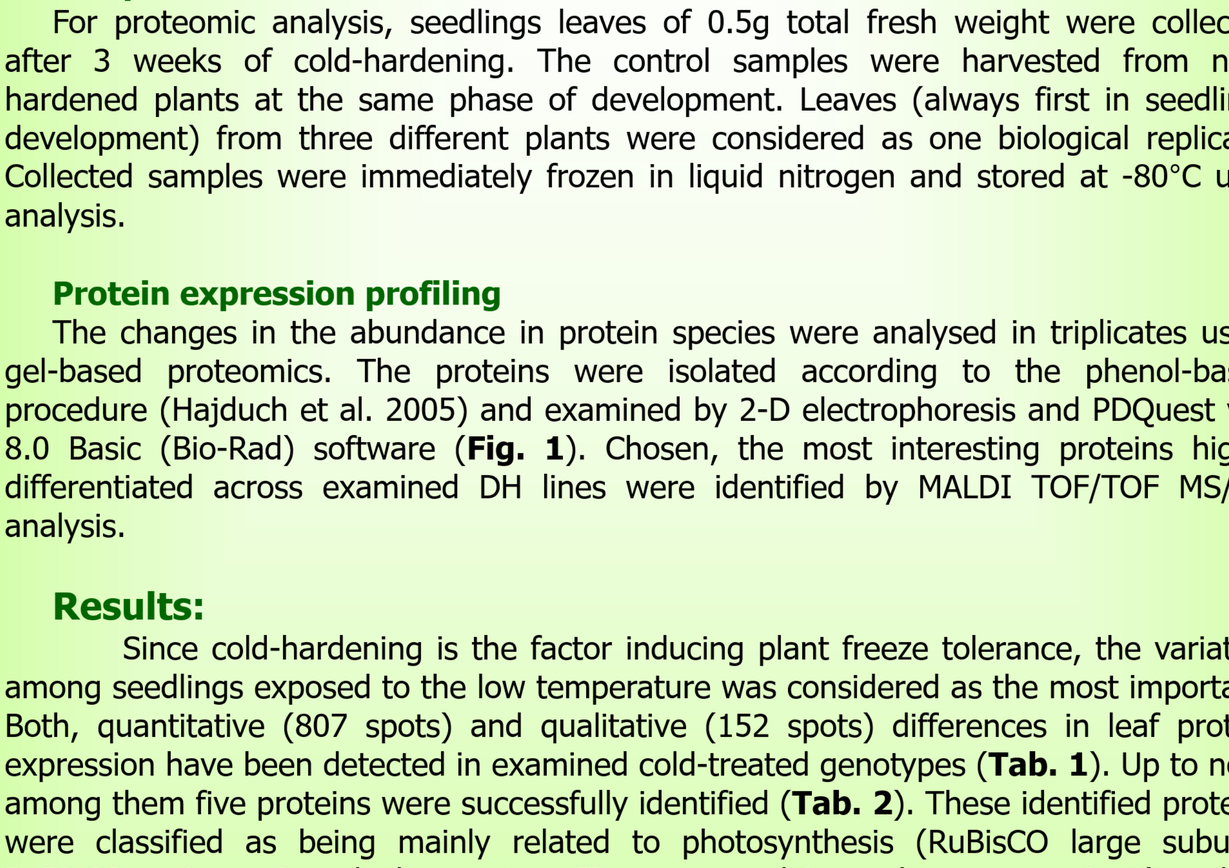

Figure 1. The Master Gel containing marked proteins with altered expression in frost resistant (DH65) in comparison to susceptible (DH158) cold-hardened winter barley seedlings.

Table 1. The most important quantitative (A) and qualitative (B) differences in leaf protein abundance between resistant and susceptible winter barley DH lines exposed to the cold-hardening.

A

Plant object	N.o. of differential spots	Min. 2-fold-up	Min. 2-fold-down
DH65 vrs DH61	17	15	2
DH435 vrs DH61	14	8	6
DH602 vrs DH61	2	2	0
DH534 vrs DH61	10	7	3
DH363 vrs DH61	12	5	7
DH584 vrs DH61	8	1	7
DH575 vrs DH61	8	3	5
DH158 vrs DH61	13	4	9
DH561 vrs DH61	12	8	4
DH435 vrs DH65	36	1	35
DH602 vrs DH65	13	1	12
DH534 vrs DH65	55	46	8
DH363 vrs DH65	55	35	18
DH584 vrs DH65	18	2	16
DH575 vrs DH65	19	3	16
DH158 vrs DH65	47	4	43
DH561 vrs DH65	46	4	32
DH602 vrs DH435	8	6	2
DH534 vrs DH435	10	7	3
DH363 vrs DH435	16	11	5
DH584 vrs DH435	12	7	5
DH575 vrs DH435	8	7	1
DH158 vrs DH435	19	9	10
DH561 vrs DH435	16	13	3
DH534 vrs DH602	6	2	4
DH363 vrs DH602	14	2	8
DH584 vrs DH602	6	3	3
DH575 vrs DH602	5	1	4
DH158 vrs DH602	18	9	9
DH561 vrs DH602	46	4	42
DH363 vrs DH534	13	5	8
DH584 vrs DH534	5	2	3
DH575 vrs DH534	12	9	11
DH158 vrs DH534	0	0	0
DH561 vrs DH534	20	9	11
DH584 vrs DH363	13	11	2
DH575 vrs DH363	21	12	9
DH158 vrs DH363	27	13	12
DH561 vrs DH363	19	16	3
DH575 vrs DH584	5	3	2
DH158 vrs DH584	17	13	4
DH561 vrs DH584	9	6	3
DH158 vrs DH575	26	13	13
DH561 vrs DH575	17	10	7
DH561 vrs DH158	34	18	16
Total	**807**	**404**	**392**

B

Plant objects	N.o. of differential spots	Min. 2-fold-up	Min. 2-fold-down
DH65 vsr DH61	3		3
DH435 vrs DH61	1		1
DH602 vrs DH61	12		6
DH534 vrs DH61	4		4
DH363 vrs DH61	6		6
DH584 vrs DH61	5		5
DH575 vrs DH61	8		8
DH561 vrs DH61	2		2
DH158 vrs DH61	5	2	
DH584 vrs DH65	1		1
DH65 vrs DH435	1		1
DH602 vrs DH435	7		
DH363 vrs DH435	2		2
DH584 vrs DH435	3	3	
DH158 vrs DH435	4		4
DH534 vrs DH602	2	1	1
DH363 vrs DH602	14	2	12
DH363 vrs DH534	7	7	
DH561 vrs DH534	1		1
DH575 vrs DH363	19		19
DH158 vrs DH363	2		2
DH561 vrs DH363	6		6
DH575 vrs DH584	1	1	
DH158 vrs DH584	34	34	
DH561 vrs DH584	2	2	
Total	**152**	**52**	**84**

Table 2. Mascot search results from BioTools analysis within *Hordeum vulgare* sequences of cold-accumulated proteins better represented in partly resistant DH lines when compared to the most susceptible ones.

No	Compared genotypes	Accesion number	Protein name	Protein sequence coverage	Calculated pI	Nominal mass (Mr) [Da]	Fold-up
1	DH65 vrs DH158	CAY37672.1	**Transketolaze**	10%	5.91	80410	2
2	DH65 vrs DH158 and DH561 as well as DH61 vrs DH158	P05698.2 (RBL_HORVU)	**RuBisCO large chain**	28%	6.22	53672	3, 12 and 2
3	DH65 vrs DH158 and DH561 as well as DH61 vrs DH158	Q40073.1 (RCAA_HORVU)	**RuBisCO activase A chloroplastic**	29%	8.04	51383	7, 6 and 5
		AAA62703.1 (RCAB_HORVU)	**RuBisCO activase B chloroplastic**	12%	7.59	47426	
4	DH65 vrs DH158	AAN59956.1 (Q8H1L9_HORVU)	**Actin**	11%	5.23	41929	4
5	DH65 vrs DH158	BAJ85472.1	**Photosystem II oxygen-evolving enhancer protein 1**	35%	5.75	34678	3

Conclusions:

The altered expression and up-regulated activities of photosynthesis-related proteins may indicate the potential role of this process in freeze tolerance acquisition.

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