Variation in Antifreeze Proteins during Cold Acclimation among Winter Cereals, and Their Relationship with Freezing Resistance

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Objectives : Overwintering cereal plants can survive freezing temperature even below -20°C. During cold acclimation, their leaves accumulate antifreeze proteins (AFPs) that are secreted into the apoplast and have the ability to modify the growth of ice (Griffith et al. 1992, Marentes et al. 1993). The goal of this study was to investigate the variation of AFPs in winter cereals, and the relationship between the accumulation of AFPs and freezing resistance in order to better understand the role of antifreeze proteins in overwintering plants.

Materials and Methods : The plants after germination were either maintained at 20/16°C for 21d (NA), or transferred to 5/2°C for 49 d (CA). Apoplastic proteins were extracted from leaves (Hon et al. 1994). Protein concentrations were determined using the Bradford protein assay(1976). Antifreeze activity in extracts was assayed by monitoring the ability of the apoplastic proteins to modify the growth habit of ice crystals in a manner and interpretation (Hon et al. 1994). Equal amounts of extracted apoplastic proteins were separated in 15% SDS polyacrylamide gels (Laemmli 1970). For immunoblotting, proteins were transferred onto 0.45 µm nitrocellulose membranes. The blots were blocked overnight in a buffer of 25 *m*M Tris-HCl, 140 *m*M NaCl and 1% skim milk powder, followed by 2h of incubation with either the anti-GLP antiserum, anti-TLP antiserum, or overnight with the anti-CLP antiserum.

Results and Discussion : Apoplastic proteins accumulated to much higher levels in CA leaves compared with NA leaves in winter cereals. In NA leaves, the apoplastic protein concentration (0.088 mg/mL) and levels of extractable apoplastic protein per gram leaf fresh weight (not presented here) were very low. However, the varietal difference in protein concentration was very high (CV: 25.0%). After cold acclimation, the protein concentration of apoplastic extracts ranged from 0.196 mg mL^{-1} to 0.842 $mgmL^{-1}$ with a mean of 0.448 $mgmL^{-1}$, which represented an increase in the mean apoplastic protein concentration of 4.9-fold over NA leaves. The extractable apoplastic protein content per gram leaf fresh weight in CA leaves ranged 31 $\mu gg^{-1}FW$ to 120 $\mu gg^{-1}FW$ with an averaged value of 77 $\mu gg^{-1}FW$. There was not observable antifreeze activity in apoplastic extracts of NA leaves from the different species (1~2). In contrast, the antifreeze activity in apoplastic extracts from CA leaves of the different species ranged from 2 (low) in Sacheon 6, Olmeal and Gazele to 5 (high) in Glumeal and Musketeer.

Apoplastic proteins corresponding to the GLPs, TLPs and CLPs were not clearly observed in NA leaves. Antiserum raised against the 32-kD GLP from winter rye recognized two pairs of polypeptides (35 and 32kD) in the apoplastic extracts of only Glumeal after cold acclimation. The 35-kD GLP was present in the other varieties. Antiserum raised against the 25-kD TLP from winter rye recognized three pairs of polypeptides (25, 22 and 16 kD) in the CA Dongbori 1, Suwon 18 and Glumeal, and the 25- and 16-kD TLPs were observed in Musketeer. Antiserum raised against the 35-kD CLP from winter rye recognized two pairs of polypeptides in CA leaves Musketeer, but three pairs in Glumeal. The 28-kD CLP was present in CA leaves of Dongbori 1 and Suwon 18. The freezing resistance(FR) was all significantly correlated with CA extractable protein content, protein concentration and antifreeze activity, NA protein concentration and antifreeze activity, respectively. The determination coefficient(R^2) for each of these traits was about 62 to 91%, showing that AFPs is very important factor for freezing resistance in winter cereals.

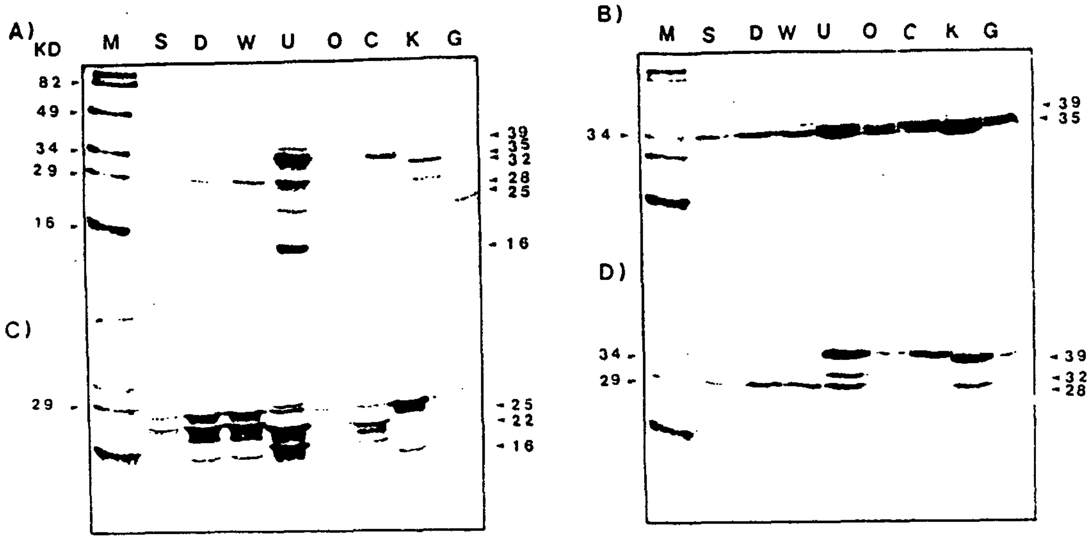

Fig. 1. Accumulation of apoplastic polypeptides and immunodetection of antifreeze proteins in Sacheon 6(S), Dongbori 1(D), Suwon 18(W), Glumeal(U), Olmeal(O), Chinese Spring(C), Musketeer(K), Gazele(G) grown at CA conditions. A, Polypeptides were separated from an equal volume (20 uL) of each apoplastic extract per gram leaf fresh weight in a 15% SDS-polyacrylamide gel stained with Coomassie brilliant blue. B, Immunoblot of apoplastic extracts probed with anti-GLP antiserum. C, Immunoblot of apoplastic extracts probed with anti-TLP antiserum. D, Immunoblot of apoplastic extracts probed with anti-CLP antiserum.

Table 1. Summary of some agronomic traits, antifreeze activity, antifreeze proteins observed in apoplastic extracts in 3 barley, 2 wheat and 2 rye varieties grown at cold-acclimated condition.

Variety	Cold-acclimated condition(CA)						
	PH(cm)	LN(no.)	SL(cm)	FW(g/pot)	EP(ug/g)FW	LPC(mg/mL)	AF(1-5)
Sacheon 6	40	5.1	4.3	66	31	0.223	2
Dongbori 1	18	4.5		55	107	0.425	4
Suwon 18	22	3.5		32	89	0.514	4
Glumeal	26	4.5		41	115	0.842	5
Olmeal	25	4.5		69	47	0.258	2
Musketeer	26	3.5		62	120	0.679	5
Gazele	42	5.2	7.5	65	31	0.196	2
Mean	28.4	4.4		55.7	77.1	0.448	3.4
SD	9.1	0.68		14.1	39.7	0.246	1.40
CV(%)	32.0	15.5		25.3	51.5	54.9	41.2

PH; Plant height, LN; leaf number, SL; stem elongation length, FW; fresh leaf weight per pot, EP; extractable protein per FW, LPC; protein concentration of CA plants, AF; antifreeze activity, SD; standard deviation

Table 2. Summary of some agronomic traits, antifreeze activity, antifreeze proteins observed in apoplastic extracts in 3 barley, 2 wheat and 2 rye varieties grown at non- acclimated conditions.

Variety	Nonacclimated condition(NA)						Ratio (LPC/ HPC)	Freezing resistance (0-9)	Growth habit
	PH(cm)	LN(no.)	FW(g/ pot)	HPC(mg /mL)	PCC (mg/mL)	AF (1-5)			
Sacheon 6	65	4.5	70	0.061	0.201	1	3.7	8	S
Dongbori 1	34	3.5	37	0.116	0.444	2	3.7	4	F
Suwon 18	35	3.0	44	0.102	0.461	2	5.0	4	W
Glumeal	35	3.2	63	0.096	0.457	2	8.8	2	W
Olmeal	36	4.0	60	0.085	0.202	1	3.0	6	F
Musketeer	38	3.8	66	0.101	0.406	2	6.7	1	W
Gazele	44	4.5	71	0.058	0.273	1	3.4	9	ES
Mean	36.7	3.8	58.7	0.088	0.349	1.6	4.9	4.9	
SD	3.5	0.59	13.2	0.022	0.111	0.53	2.1	3.0	
CV(%)	9.5	15.5	22.5	25.0	31.8	33.1	42.9	61.2	

PH; Plant height, LN; leaf number, FW; fresh leaf weight per pot, HPC; protein concentration of NA plants, PCC; protein concentration concentrated, AF; antifreeze activity, S; spring type, F; facultative type, W; winter type, ES; Extremely spring type, SD; standard deviation

Table 4. Linear regression equations for freezing resistance (FR) in cold-acclimated(CA) and nonacclimated(NA) barley, wheat and rye varieties.

Simple linear regression equation	R^2
FR = 10.396 - 0.072** CEP	0.908
FR = 11.756 - 2.012** CAF	0.877
FR = 9.813 - 11.058** LPC	0.810
FR = 12.583 - 4.917** NAF	0.741
FR = 14.836 - 112.843* HPC	0.624

CEP; CA extractable protein, CAF; CA antifreeze activity, LPC; CA protein concentration, NAF; NA antifreeze activity, HPC; NA protein concentration, *, ** Significant 5% and 1% levels, respectively.

Table 3. Correlation coefficients(r) among the characteristics of freezing resistance in cold-acclimated(CA) and nonacclimated(NA) barley, wheat and rye varieties.

Characteristic	CA					NA
	CPH	CEP	LPC	CAF	HPC	NAF
FR: Freezing resistance	0.741	-0.961**	-0.917**	-0.947**	-0.829*	-0.886**
CPH: CA plant height		-0.760*	-0.529	-0.636	-0.965**	-0.748
CEP: CA extractable protein		-	0.897**	0.977**	0.882**	0.961**
LPC: CA protein concentration			-	0.949**	0.641	0.845*
CAF: CA antifreeze activity				-	0.781*	0.956**
HPC: NA protein concentration					-	0.876**
NAF: NA antifreeze activity						-

*, ** Significant 5% and 1% levels, respectively.