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Variation of Antifreeze Proteins during Cold Acclimation among Winter Cereals and Their Relationship with Freezing Resistance***

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ABSTRACT

Freezing-resistant plants can survive subzero temperatures by withstanding extracellular ice formation. During cold acclimation, their leaves accumulate antifreeze proteins (AFPs) that are secreted into the apoplast and have the ability to modify the normal growth of ice crystals. Three barley, two wheat and two rye cultivars were grown under two different temperature regimes (20/16℃ and 5/2℃, day/night). Apoplastic proteins from winter cereals were separated by SDS-PAGE and detected with antisera to AFPs from winter rye. Apoplastic proteins accumulated to much higher levels in cold-acclimated (CA) leaves compared with nonacclimated (NA) ones in winter cereals. After cold acclimation, the protein concentration of apoplastic extracts increased significantly from 0.088 mgmL^{-1} to 0.448 mgmL^{-1}, with about 5-fold increment. Also, the apoplastic protein content per gram leaf fresh weight in CA leaves ranged from 31 μg (gFW)$^{-1}$ to 120 μg (gFW)$^{-1}$ with an averaged value of 77 μg (gFW)$^{-1}$, and coefficients of variation of 54.9%. The CA leaves in Musketeer (a Canadian winter rye cultivar) showed the greatest AFPs and antifreeze activity followed by 'Geurumil' (a Korean winter wheat cultivar), and 'Dongbori 1' (Korean facultative barley cultivar). The proteins secreted into the wheat leaf apoplast at CA condition were more numerous than those observed in winter rye, where two β-1,3-glucanase-like proteins (GLPs), two chitinase-like proteins (CLPs) and two thaumatin-like proteins (TLPs) accumulated during cold acclimation. The proteins in barley leaf apoplast at CA conditions were a little different from those in wheat leaves. The AFPs were various among and within species. More freezing-resistant cultivars had more clear and numerous bands than less freezing-resistant ones. The high determination coefficient (R^2 = 91%) between freezing resistance and AFPs per gram leaf fresh weight indicated that the amount of AFPs was highly related to freezing resistance in winter cereal crops.

Key words : antifreeze proteins (AFPs), antifreeze activity, apoplast, cold acclimation, winter cereal, freezing resistance.

Freezing-resistant crop species including winter rye, wheat, and barley, can survive below zero temperatures after cold acclimation. During cold acclimation, winter rye leaves accumulate antifreeze proteins (AFPs) that are secreted into the apoplast and have the ability to modify the ice crystal formation (Griffith et al., 1992; Marentes et al., 1993; Antikainen & Griffith, 1997). The AFPs in freezing-tolerant plants may inhibit ice recrystallization during freezing and thawing cycles and prevent plants from mechanical disruptions caused by growing ice crystals (Knight & Duman, 1986). Thus, increases in the level of extractable AFPs can be one of the factors that enhance freezing resistance of the winter rye leaves (Marentes et al., 1993).

Six AFPs ranging from 16 to 35 kD in molecular mass have been isolated from winter rye leaves (Hon et al., 1994), and were recently found to be similar to members of three classes of pathogenesis-related (PR) proteins such as chitinases, β-1,3-glucanases and thaumatin-like proteins (Hon et al., 1995). One of the CLPs purified from cold-acclimated winter rye leaves had both antifreeze and chitinase activities. These results suggested that AFPs accumulated in winter rye leaves at low temperatures might play a role in nonspecific disease resistance as well as freezing resistance (Griffith et al., 1997).

Measurable antifreeze activity has been observed in 21 dicotyledons and 9 monocotyledons (Griffith et al., 1992; Urrutia et al., 1992; Duman & Olsen, 1993; Duman et al., 1993; Griffith & Ewart, 1995). As shown by immunolocation in tissue prints (Antikainen et al., 1996), the β-1, 3-glucanase-like proteins (GLPs), chitinase-like proteins (CLPs) and thaumatin-like proteins (TLPs) with antifreeze activity are localized in the apoplast of cold-acclimated winter tissues. The distribution of these proteins in the intercellular spaces within the epidermis and mesophyll is correlated with the location of both epiphytic and extracellular ice, suggesting that these proteins are involved in modifying the growth of ice. Moreover, the GLPs, CLPs and TLPs present in nonacclimated plants lacked antifreeze activity and were generally localized in different cell types, which suggests that the AFPs may be different isoforms of pathogenesis-related (PR) proteins (Antikainen & Griffith, 1997).

In order to understand the role of AFPs in winter cereals, we investigated the variations of AFPs in winter cereal crops, and the relationship between the accumulation levels of AFPs and freezing resistance.

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MATERIALS AND METHODS

Plant materials

About 20 seeds per cultivar for 3 barley cultivars (*Hordeum vulgare* L. cvs. Sacheon 6, Dongbori 1 and Suwon 18), 2 winter wheat cultivars (*Triticum aestivum* L. cvs. Geurumil and Olmil), and 2 rye cultivars (*Secale cereale* L. cvs. Musketeer and Gazele) were sown in coarse vermiculite in 15 cm pots. The pots were placed in a 20/16℃ (day/night) growth chamber with 16 h daylength and with photosynthetic photon flux density (PPFD) of 250 μ mol photons $m^{-2}s^{-1}$ for 1 wk for germination. The plants were grown in two different chambers; one was maintained at 20/16℃ for 3 wks (nonacclimated; NA), and the other was maintained at 5/2℃ for 7 wks (cold-acclimated; CA). The plants were watered weekly with modified Hoagland solution (Huner & Macdowall, 1976).

Extraction of apoplastic proteins

Apoplastic proteins were extracted from leaves of winter cereals as described by Hon et al. (1994). Plant leaves were harvested, cut into 3~4 cm sections and the leaf fresh weight per pot was measured. Apoplastic proteins were extracted in 20 mM ascorbic acid and 20 mM $CaCl_2$ (pH 3) for 30 min by vacuum infiltration followed by centrifugation at 800 g for 30 min. Protein concentrations were determined using the Bradford (1976) protein assay, as modified by Bio-Rad, with BSA as the standard protein.

Antifreeze activity assay

Antifreeze activity in extracts was assayed by monitoring the ability of the apoplastic proteins to modify the growth habit of ice crystals as described by Hon et al. (1994) and DeVries (1986). The freezing stage temperature was controlled by a nanoliter osmometer (Clifton Technical Physics, Hartford, NY, USA). The sample was flash frozen to −40℃ to form small ice crystals and then thawed until only one ice crystal remained in the well. The temperature was then slowly decreased and ice crystal morphology was determined qualitatively by observing crystal growth in solution (DeVries, 1986) and interpreted as described by Hon et al. (1994). So as to quantify the effects of the apoplastic proteins on ice crystal growth, a rating scale from 0 to 5 was applied. In short, ice crystals grown in pure water form circular plates (rating = 0; no activity). Ice crystals grown in a dilute antifreeze protein solution form hexagonal discs (rating = 1), crystals grown in moderate concentration of AFPs form hexagonal columns (rating = 3), and crystals grown in high concentrations of AFPs form complex hexagonal bipyramids (rating = 5; high activity).

SDS-PAGE and immunoblotting

Equal volume of apoplastic proteins extracted from equal fresh weight of leaves was separated in 15% SDS polyacrylamide gels according to Laemmli (1970). For immunoblotting, proteins were transferred onto 0.45 μm nitrocellulose membranes using the Mini Trans-Blot cell (Bio-Rad) according to manufacturer's instructions. The blots were blocked overnight in a buffer of 25 mM Tris-HCl (pH 7.6), 140 mM NaCl and 1% (w/v) skim milk, followed by 2 h of incubation with either the anti-GLP antiserum (dilution 1:3,000) and anti-TLP antiserum (1:15,000), or overnight with the anti-CLP antiserum (1:2,000) (Antikainen et al., 1996). The immunoreaction was detected by alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St Louis, MO, USA) with 5-bromo-4-chloro-3-indolylphosphate-toluidine salt (BCIP) and nitro blue tetrazolium (NBT) as substrates.

RESULTS AND DISCUSSION

Quantification of traits related to antifreeze proteins

The growth rates in spring types (Sacheon 6 and Gazele) were higher and also their developmental stages were faster than those in winter types (Table 1). Especially, even at low temperatures (5/2℃), the rates of leaf emergence in Sacheon 6 and Gazele were greater (about 1.7 leaves), and the rate of stem elongation was remarkably different from winter types (Suwon 18 and Musketeer). Slow growth for winter types may indicate general adaptation to unfavorable conditions that enable plants to conserve limited resources. However, it is also possible that some cases of slow growth are consequences of tolerance mechanisms which are specific to some environmental stresses (Hoffman & Parsons, 1991).

Apoplastic proteins accumulated to much higher levels in CA leaves than in NA leaves (Table 1). In NA leaves, the mean apoplastic protein concentration (APC; 0.088 $mgmL^{-1}$) 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 with coefficient of variation (CVs) of 25.0%. After cold acclimation, the protein concentration of apoplastic extracts ranged from 0.196 $mgmL^{-1}$ to 0.842 $mgmL^{-1}$ with a mean of 0.448 $mgmL^{-1}$. This represented an increase in the mean apoplastic protein concentration of 4.9-fold over NA leaves (Table 1). The ratio of apoplastic protein concentration in CA leaves to that in NA leaves was the greatest for Geurumil (8.8-fold) followed by Musketeer (6.7-fold) and Suwon 18 (5.0-fold). Also, variations among CA leaves of species were very high with CVs of 54.9%. The CA leaves in Geurumil exhibited the greatest antifreeze protein concentration (0.842 $mgmL^{-1}$) followed by Musketeer (0.679 $mgmL^{-1}$), Suwon 18 (0.514 $mgmL^{-1}$), and Dongbori 1 (0.425 $mgmL^{-1}$). The extractable apoplastic protein content per gram leaf fresh weight (AP) in CA leaves ranged

Table 1. Summary of some agronomic traits, antifreeze proteins, antifreeze activity observed in apoplastic extracts in 3 barley, 2 wheat and 2 rye cultivars grown at cold-acclimated (CA) and nonacclimated conditions (NA).

Cultivars	CA							NA		Ratio	Freezing@ resistance (0-9)	Growth habit
	PH (cm)	LN (no.)	SL (cm)	FW (g/pot)	AP (μg/gFW)	APC (mg/mL)	AF (0-5)	APC (mg/mL)	APCC (mg/mL)			
Sacheon 6	40	5.1	4.3	66	31	0.223	2	0.061	0.201	3.7	8	S
Dongbori 1	18	4.5	–	55	107	0.425	4	0.116	0.444	3.7	4	F
Suwon 18	22	3.5	–	32	89	0.514	4	0.102	0.461	5.0	4	W
Geurumil	26	4.5	–	41	115	0.842	5	0.096	0.457	8.8	2	W
Olmil	25	4.5	–	69	47	0.258	2	0.085	0.202	3.0	6	F
Musketeer	26	3.5	–	62	120	0.679	5	0.101	0.406	6.7	1	W
Gazele	42	5.2	7.5	65	31	0.196	2	0.058	0.273	3.4	9	ES
Mean	28.4	4.4		55.7	77.1	0.448	3.4	0.088	0.349	4.9	4.9	
SD	9.1	0.68		14.1	39.7	0.246	1.40	0.022	0.111	2.1	3.0	
CV (%)	32.0	15.5		25.3	51.5	54.9	41.2	25.0	31.8	42.9	61.2	

PH; plant height, LN; leaf number, SL; stem elongation length, FW; leaf fresh weight per pot, AP; apoplastic protein content per gram leaf fresh weight, APC; apoplastic protein concentration, AF; antifreeze activity, APCC; apoplastic protein concentration concentrated, Ratio; APC of CA plants / APC of NA plants, S; spring type, F; facultative type, W; winter type, ES; extremely spring type, SD; standard deviation, CV; coefficient of variation.@ Classified from 0 (high resistance) to 9 (high sensitivity) by combination of electrical conductivity test, survival rates with low temperature and field test.

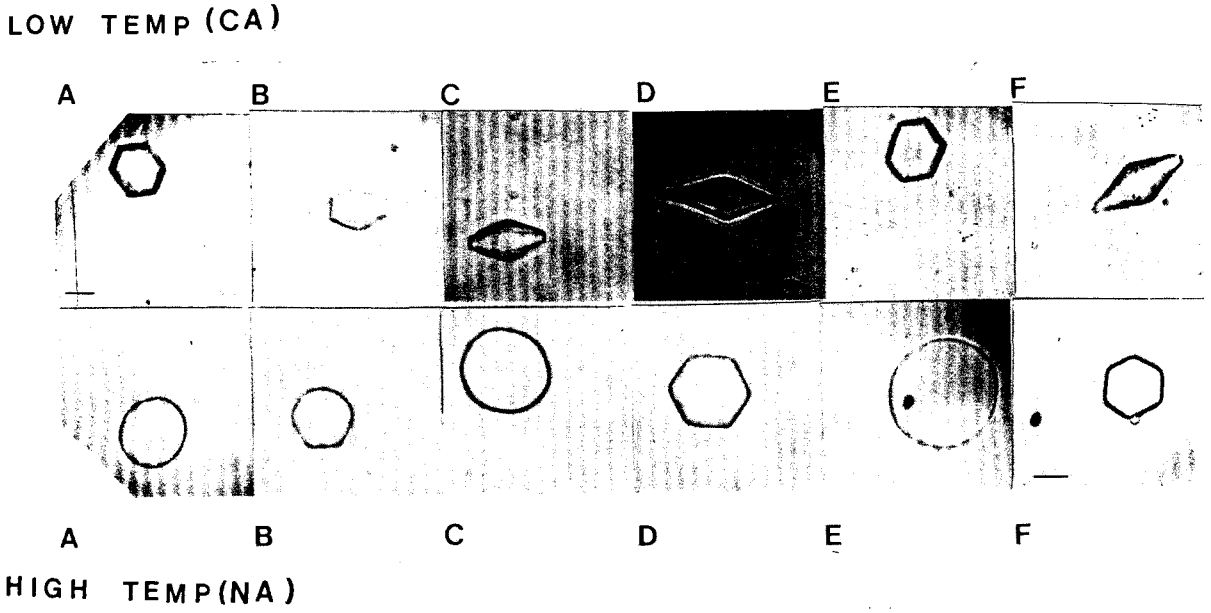

Fig. 1. Antifreeze activity of AFPs in CA and NA plants. The antifreeze activity was rated as follows: circular disc, rating = 0; hexagonal disc, rating = 1; hexagonal column, rating = 3, and hexagonal bipyramid, rating = 5. A : Sacheon 6 (rating = 2), B : Dongbori 1 (rating = 4), C : Olmil (rating = 4), D : Geurumil (rating = 5), E : Gazele (rating = 2), and F : Musketeer (rating = 5). Magnification bar = 17 μm.

from 31 $\mu g(gFW)^{-1}$ to 120 $\mu g(gFW)^{-1}$ with an averaged value of 77 $\mu g(gFW)^{-1}$. The CA leaves in rye Musketeer showed the greatest antifreeze proteins (AFPs) per gram leaf fresh weight of 120 $\mu g\ (gFW)^{-1}$ followed by Geurumil (115 $\mu g(gFW)^{-1}$), Dongbori 1 (107 $\mu g(gFW)^{-1}$), and Suwon 18 (89 $\mu g(gFW)^{-1}$). The AFPs in spring types, Sacheon 6 and Gazele showed the lowest values (31 $\mu g\ (gFW)^{-1}$).

Apoplastic extracts from leaves of all NA and CA plants were assayed for antifreeze activities (Fig. 1). The relative antifreeze activity could be quantified by comparing the inhibition of ice crystal growth among apoplastic

extracts (Table 1). Antifreeze activity was not detectable in any of the NA plants because only circular or disc-shaped ice crystals formed in the apoplastic extracts. In contrast, the antifreeze activity in apoplastic extracts from CA leaves of the different species ranged from 2 (low) in Sacheon 6, Olmil, and Gazele, to 5 (high) in Geurumil and Musketeer. Geurumil and Musketeer had higher AFP contents and antifreeze activity than other cultivars.

The freezing resistance that was classified from 0 (highly resistant) to 9 (highly sensitive) was evaluated by combination of electrical conductivity test, survival rates with low temperature (−8℃ and 6 h) and field test (Table 1). The rye cultivar, Musketeer has the greatest freezing resistance (1), followed by Geurumil, Dongbori 1 and Suwon 18, Olmil, Sacheon 6, and Gazele. Gazele headed out even under CA (5/2℃) condition. In general, freezing-resistant cultivars have more AFPs per gram leaf fresh weight, higher protein concentration, antifreeze activity and more expression levels of proteins in CA leaves compared with NA leaves.

Immunodetection of AFPs in NA and CA conditions

The apoplastic proteins in the CA and NA leaves of the different winter cereals were denatured and separated by 15% SDS-PAGE (Fig. 2A and 3A). For immunodetection of AFPs, antisera that were previously raised against three classes of AFPs in Musketeer (Antikainen et al., 1996) were used for blotting. The glucanase-like proteins (GLPs), thaumatin-like proteins (TLPs) and chitinase-like proteins (CLPs) were detected in CA and NA plants with antisera raised against AFPs from winter rye (Fig. 2 and 3). Apoplastic proteins corresponding to the GLPs, TLPs, and CLPs were expressed at very low levels in NA leaves (Fig. 3). Especially, CLPs were not observed except for Dongbori 1 and Suwon 18. There were variations of AFPs among and within species for CA leaves.

Antiserum raised against the 32 kD GLP from winter rye recognized two pairs of polypeptides (35 and 32 kD) in the apoplastic extracts of Geurumil after cold acclimation (Fig. 2B). The 32 and 35 kD GLPs were detectable in Musketeer and Geurumil but only 35 kD GLP was present in the barley and others. The 35 kD GLP were present at low levels in the apoplast of NA leaves of Dongbori 1, Suwon 18, Geurumil, and Gazele (Fig. 3B). In the apoplast of NA leaves of Sacheon 6 and Olmil, the GLPs were not detectable.

Antiserum raised against the 25 kD TLP from winter rye recognized three pairs of polypeptides (25, 22, and 16 kD) in the CA leaves of Dongbori 1, Suwon 18, and Geurumil, and the 25 and 16 kD TLPs were observed in rye Musketeer (Fig. 2C). However, the 16 kD TLP was not observed in Sacheon 6, Olmil, and Gazele. Three pairs of polypeptides were present in NA leaves of Dongbori 1 and Suwon 18, but absent in the NA leaves of other varieties (Fig. 3C).

Antiserum raised against the 35 kD CLP from winter rye recognized two pairs of polypeptides (35 and 28 kD

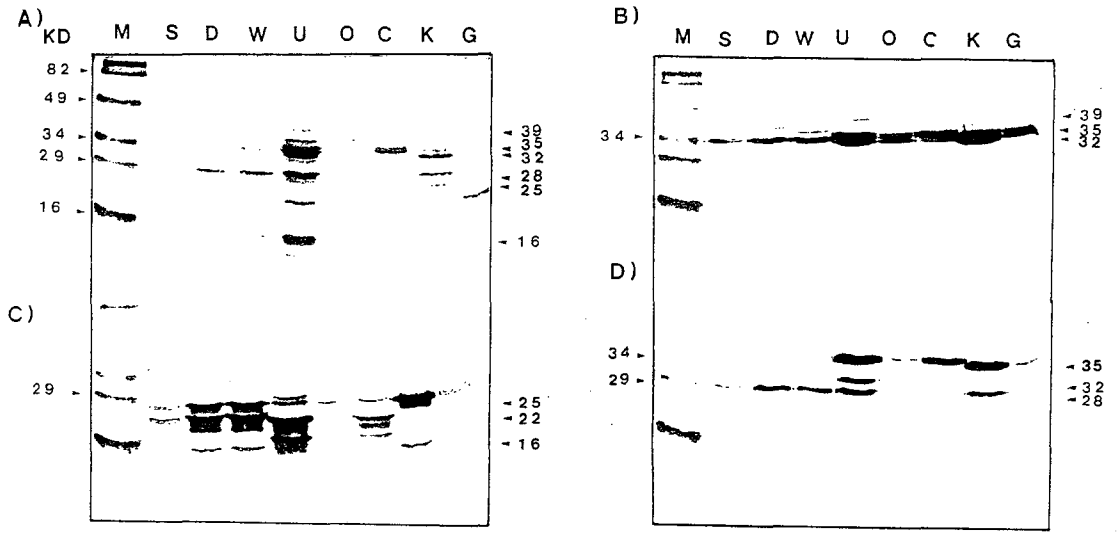

Fig. 2. Accumulation of apoplastic polypeptides and immunodetection of antifreeze proteins in Sacheon 6(S), Dongbori 1 (D), Suwon 18(W), Geurumil(U), Olmil(O), Chinese Spring(C), Musketeer(K), Gazele(G) grown at CA condition. A, Polypeptides were separated from an equal volume (20 uL) of each apoplastic 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. Prestained protein standards are shown in lanes labelled M in kD on the left-hand side of panel A.

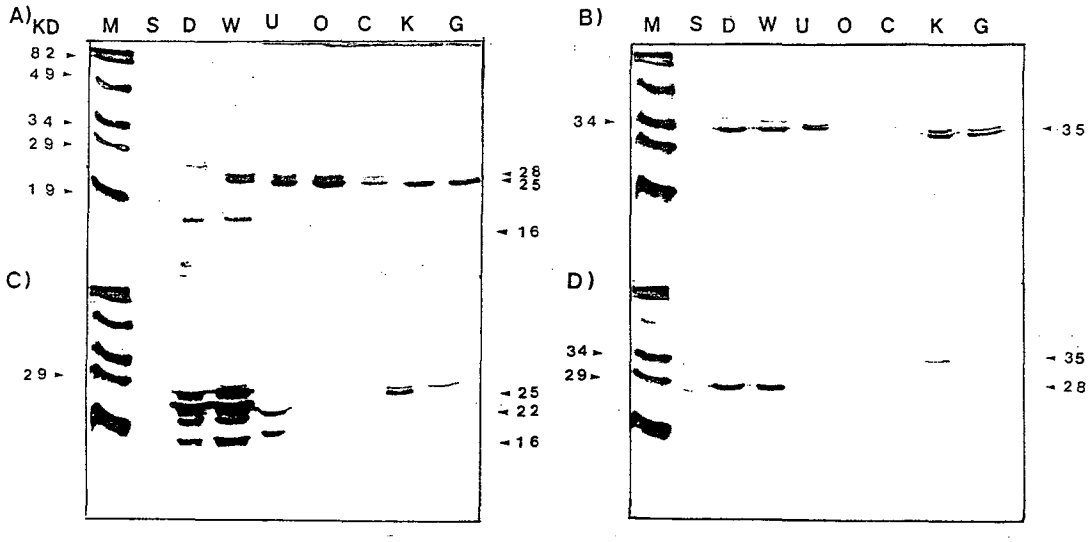

Fig. 3. Accumulation of apoplastic polypeptides and immunodetection of antifreeze proteins in Sacheon 6(S), Dongbori 1 (D), Suwon 18(W), Geurumil(U), Olmil(O), Chinese Spring(C), Musketeer(K), Gazele(G) grown at NA condition. A, Polypeptides were separated in a 15% SDS-polyacrylamide gel. B, Immunoblot of extracts probed with anti-GLP antiserum. C, Immunoblot of extracts probed with anti-TLP antiserum. D, Immunoblot of extracts probed with anti-CLP antiserum. Prestained protein standards are shown in labelled M.

CLPs) in CA leaves of Musketeer, and three pairs (35, 32, and 28 kD CLPs) in Geurumil (Fig. 2D). The 28 kD CLP was present in CA leaves of Dongbori 1 and Suwon 18 (Fig. 2D). The CLPs were not clearly detectable in spring or facultative, and less freezing-resistant cultivars such as Sacheon 6, Olmil, and Gazele.

The proteins secreted into the wheat leaf apoplast at CA condition, were more numerous than those observed in winter rye, where two GLPs (35 and 32 kD), two TLPs (25 and 16 kD), and two CLPs (35 and 28 kD) accumulated durig cold acclimation (Hon et al., 1995). However, the proteins in barley leaf apoplast at CA conditions, were a little different from those in wheat leaf apoplast. The AFPs secreted into the leaf apoplast at low temperature were various among and within winter cereals, showing that varieties with more freezing resistance have more clear and numerous bands than freezing-sensitive varieties. Within species, less freezing-resistant varieties showed less blots at even CA conditions. The expression of AFPs was a little different among different winter cereals and varieties of the same species. Freezing-resistant varieties might have higher expression levels for AFPs in the leaf apoplast than the freezing-sensitive varieties.

The six apoplastic AFPs that accumulated at low temperature were identified as two GLPs, two CLPs, and two TLPs in the winter rye (Hon et al., 1994), but different isoforms of chitinase and TLP were observed (Hon et al., 1995). Immunoblotting with anti-AFP antisera revealed that 35 and 32 kD GLPs, the 35 and 28 kD CLPs, and the 25 and 16 kD TLPs are localized in the apoplast of winter rye tissues, and related to antifreeze activity (Hon et al., 1994). They contribute to freezing tolerance of CA winter rye leaves (Marentes et al., 1993). On the other hand, the 88, 72, and 30 kD CLPs and the 27 kD TLP are thought to be localized intracellularly and their role is not known (Antikainen et al., 1996).

In CA wheat leaves, two GLPs (35 and 32 kD), three CLPs (35, 32, and 28 kD), and three TLPs (25, 22, and 16 kD) were immunologically detected. The accumulations of the 35 and 32 GLPs, 32 and 28 kD CLPs, and 25 kD TLP were various among lines (Griffith et al., 1997). The presence of multiple antifreeze polypeptides with closely related amino acid composition is common in other overwintering plant species that also produce AFPs. The heterogeneity in AFPs has been attributed to the expression of multigene families and to posttranslational modification of the proteins (Hon et al., 1994).

There was a positive correlation between the expression of the wheat cold-regulated gene Wcs 120 and freezing tolerance (Guy, 1990; Houde et al., 1992; Limin et al., 1997; Mohapatra et al., 1988). The increased accumulation of the Wcs 120 proteins in 5A with a concomitant increase in cold tolerance implies that a gene located on chromosome 5A controls expression of the Wcs 120 gene family (Limin et al, 1997). The results suggest that the chromosome 5A have a regulatory effect on the expression of the Wcs 120 gene family located on the group 6 chromosomes of homeologous parts. However, it is necessary to investigate the relationship between proteins accumulated by Wcs 120 gene families and AFPs within the apoplast in the future.

Table 2. Correlation coefficients among the characteristics related to freezing resistance in cold-acclimated (CA) and nonacclimated (NA) barley, wheat and rye cultivars.

Characteristic	CA				NA	
	PH	CAP	CAPC	CAF	NAPC	NAF
FR; Freezing resistance	0.741	−0.961**	−0.917**	−0.947**	−0.829*	−0.885**
PH; CA plant height		−0.760*	−0.529	−0.636	−0.965**	−0.748
CAP; CA apoplastic protein		−	0.897**	0.977**	0.882**	0.961**
CAPC; CA protein concentration			−	0.949**	0.641	0.845*
CAF; CA antifreeze activity				−	0.781*	0.956**
NAPC; NA protein concentration					−	0.876**
NAF; NA antifreeze activity						−

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

Correlations between antifreeze proteins and freezing resistance

Freezing resistance was numerically classified from 0 (highly resistant) to 9 (highly sensitive) by combination of electrical conductivity test, survival rates with low temperature (−8℃, 6 h) and field survival rates. The numerical classification was a little arbitrary in order to be fitted with Investigation Criteria for Agricultural Experiment & Research (Rural Development Administration; RDA, 1983). The degree of freezing resistance was all significantly correlated with CA apoplastic protein content per gram leaf fresh weight, apoplastic protein concentration, and antifreeze activity, NA apoplastic protein concentration and antifreeze activity, respectively (Table 2). CA apoplastic protein content per gram leaf fresh weight was highly positively correlated with protein concentration and antifreeze activity in CA plants, respectively (0.897** and 0.977**). The presence of antifreeze activity was correlated with the development of freezing resistance within the group of monocotyledons examined (Antikainen & Griffith, 1997).

The freezing resistance in winter cereals was fitted to a simple linear regression (Table 3). The degree of freezing resistance was constant throughout the CA apoplastic proteins (CAP), CA antifreeze activity (CAF), CA protein concentration (CAPC), NA antifreeze activity (NAF), and NA protein concentration (NAPC), ranging from 0.072 to 112.843 (Table 3). The linear regressions were highly significant. The degree of freezing resistance on apoplastic proteins per gram leaf fresh weight in winter cereals was 0.072 $\mu g(gFW)^{-1}$ (Fig. 4 and Table 3). The determination coefficient (R^2) for each of these traits was about 62 to 91%, showing that AFPs could be major factors for freezing resistance in winter cereals (Chun et al.,* 1998). Among the factors related to AFPs, the apoplastic protein content per gram leaf fresh weight had the greatest relationship with freezing resistance in winter cereals. Therefore, apoplastic protein content could be an useful criterion for selecting freezing-resistant lines in the

Table 3. Linear regression equations for freezing resistance (FR) in cold-acclimated (CA) and nonacclimated (NA) barley, wheat and rye cultivars.

Simple linear regression equation	R^2
FR = 10.396 − 0.072** CAP	0.908
FR = 11.756 − 2.012** CAF	0.877
FR = 9.813 − 11.058** CAPC	0.810
FR = 12.583 − 4.917** NAF	0.741
FR = 14.836 − 112.843* NAPC	0.624

CAP; CA apoplastic protein content per gram leaf fresh weight,
CAF; CA antifreeze activity,
CAPC; CA apoplastic protein concentration,
NAF; NA antifreeze activity,
NAPC; NA apoplastic protein concentration,
*, ** Significant at 5% and 1% levels, respectively.

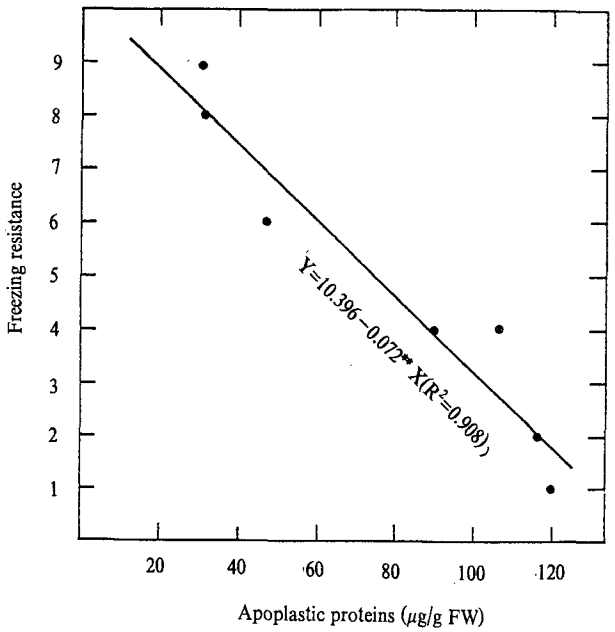

Fig. 4. Relationship between apoplastic protein content per gram leaf fresh weight and freezing resistance in winter cereal crops grown at CA condition.

breeding programs.

CONCLUSIONS

In winter cereal crops, antifreeze proteins and antifreeze activity were various among species after cold acclimation, and exhibited much higher levels and activities at CA condition compared with NA condition. The winter types, which were more freezing-resistant, had the characteristics of higher accumulations of AFPs and activities than spring types with less freezing resistance. The expression of AFPs was slightly different among different winter cereal crops and within cultivars of the same species. Freezing-resistant cultivars might have higher expression levels for AFPs in the leaf apoplast than the freezing-sensitive cultivars. The high determination coefficient (R^2 = 91%) between freezing resistance and AFPs per gram leaf fresh weight indicated that the amount of AFPs was highly related to freezing resistance in winter cereal crops. The apoplastic protein content could be an useful criterion for selecting freezing-resistant lines in the breeding programs.

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