Genetic Analysis of Apoplastic Proteins in Barley Crosses

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ABSTRACT

Antifreeze proteins (AFPs) accumulate in the leaves of barley during cold acclimation, where they may inhibit ice recrystallization and produce freezing resistance of the plant. Four parental diallel crosses of the barley varieties were used to determine the heritability of AFPs and the relationship between the accumulation level of AFPs and freezing resistance. The concentration of apoplastic proteins in the cold-acclimated leaves was increased in the mean by four-fold over as compared with that of nonacclimated. The diallel cross analyses revealed that the gene of Sacheon 6 was dominant and those of Reno and Dongbori 1 were recessive. The AFPs had high narrow-sense heritabilities. The general combining ability effects of Reno and Dongbori 1 were much higher than the other parents. The bands of 32-kD for GLP, 35- & 28-kD for CLP and 25-, 22- & 16-kD for TLP were observed in the apoplastic extracts from cold-acclimated plants, but there were no clear differences between the parents and F1 hybrids. The concentrations of AFPs were significantly correlated with the degree of freezing resistance, indicating that the concentration of AFPs in the plant is the very important factor for freezing resistance.

Abbreviations : AFP - antifreeze protein; GNA - nonacclimated in growth chamber; GCA - cold-acclimated in growth chamber; GLP - glucanase-like protein; CLP - chitinase-like protein, TLP - thaumatin-like protein

Key words : Genetic analysis, Antifreeze proteins, Cold acclimation, Freezing resistance, Barley

INTRODUCTION

Over-wintering plants such as winter wheat, barley, rye and oats are encountered with various stress conditions and sometimes severe situations in winter season. Since temperature is a major uncontrollable climatic factor, freezing resistance to low temperature and inheritable characteristics for temperature stress resistance are very important factors for developing new variety in winter cereals (Atici & Nalbantoglu, 2003). Freezing resistance is controlled with related genes, environmental factors and their interactions. The objective of this study is to determine the heritability of AFPs in winter barley.

During cold acclimation, winter barley accumulates antifreeze proteins (AFPs) that are secreted into the apoplast and have the ability to modify the growth of intercellular ice (Griffith *et al.*, 1992, Marentez *et al.*,

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1993). The expression of AFPs was affected by environmental factors such as drought and external ABA application (Chun & Griffith, 1998). The function of AFPs in freezing-resistant plants may inhibit ice recrystallization during prolonged exposure to low temperature (Knight & Duman, 1986; Griffith & Anitikainen, 1997). Consequently, on accumulating AFPs in acclimated plant leaves, the freezing resistance increased (Hon *et al*, 1994). Six polypeptides have been isolated from cold-acclimated winter rye leaves. These AFPs were ranged from 16 to 35-kD in molecular mass and were shown to have antifreeze activity (Hon *et al.*, 1994 and 1995). The polypeptides are similar to plant pathogenesis-related proteins (Hon *et al.*, 1995), β,-1,3-endoglucanases, endochitinases and thaumatin-like proteins (Chun & Griffith, 1998). In fact, one of chitinase-like proteins (CLPs) was purified to homogeneity from the cold-acclimated winter rye and was exhibited both antifreeze and chitinase activities (Hon *et al.*, 1995), suggesting that the AFPs may play a role in nonspecific disease resistance as well as in freezing tolerance (Griffith *et al.*, 1997). The AFPs in wheat chromosome substitution lines were controlled with several major genes on chromosome 5A and 5D (Chun *et al.*, 1998), which appear to carry major genes affecting freezing resistance (Sutka *et al.*, 1997).

In this study, we determined the heritability of AFPs by using partial diallel crosses in barley varieties. In addition, we examined the relationship between the accumulation level of AFPs and freezing resistance for the effective selection of lines in the barley.

MATERIALS AND METHODS

All experiments were done at the and physiology laboratory of Sunchon National University from 1999 to 2001. Four cultivars of barley with different degree of freezing resistance (*Hordeum vulgare* L., cvs Sacheon 6, Oweolbori, Dongbori 1 and Reno) were grown and used to make 6 cross combinations in a partial diallel design. Twenty seeds of each F1 hybrid and parent were surface-sterilized in a 0.3% hypochlorite solution for 5 min, rinsed with distilled water several times, planted in 15-cm pots of coarse vermiculite, and germinated at 20 ℃/16℃ (day/night) with a 16-h daylength and a PPFD of 300 $\mu molm^{-2}s^{-1}$ for one week. Nonacclimated (NA) plants were grown under the same conditions for an additional 2 weeks. Cold-acclimated (CA) plants were transferred to acclimate at 5℃/2℃ (day/night) with an 8-h daylength and a PPFD of 300 $\mu molm^{-2}s^{-1}$ for an additional 7 weeks. NA plants are similar in physiological age to CA plants (Griffith & McIntyre, 1993). Plants were watered with modified Hoagland solution (Huner & Macdowall, 1976).

Apoplastic proteins were extracted from the leaves of NA and CA plants in growth chamber plants as described by Hon et al. (1994). Plant leaves were harvested, cut into 3 ~ 4 cm sections and the leaf fresh weight per pot were 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 800g for 30 min. Protein concentrations were determined by the method of Bradford (1976) with some modifications, using BSA as standard.

The apoplastic proteins extracted from the leaves were separated in 15% SDS polyacrylamide gels according to the method of Laemmli (1970). For immunoblotting, proteins were transferred onto nitrocellulose membranes using the Mini Trans-Blot Cell (Bio-Rad) according to manufacturer's instructions. The blots were blocked overnight with 1% skim mik in 25 mM Tris/140 mM NaCl buffer, pH 7.6, then membranes were incubated for 2 hours 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-indolyphosphate- toluidine salt (BCIP) and nitro blue tetrazolium (NBT).

The freezing resistance was tested by reduction of 2, 3, 5-tripenyl tetrazolium chloride (TTC) to a water-insoluble red formazan as previously described (Chun *et al.*, 2000). The data were expressed as the mean of 4 replications per treatment.

RESULTS AND DISCUSSION

Population mean of AFPs in parents and their F1 hybrids

The mean concentrations of apoplastic proteins in 4-parent partial diallel crosses were shown in Table 1. The apoplastic protein concentrations in GNA leaves ranged from 38.1 to 91.9 $\mu g\ ml^{-1}$ with a mean of 60.7 $\mu g\ ml^{-1}$. After cold acclimation at 5℃ for 7 weeks, the concentrations ranged from 165.5 to 364.9$\mu g\ ml^{-1}$ with a mean of 245.6$\mu g\ ml^{-1}$, which showed an increase in the mean by four-fold over. When the amount of apoplastic proteins was calculated as leaf fresh weight (FW) basis, the extractable protein content also increased by two-fold over in GCA leaves (26.4 $\mu g(gFW)^{-1}$) as compared with GNA leaves (12.7 $\mu g(gFW)^{-1}$).

Reno showed the highest value of the apoplastic proteins among parents, followed by Dongbori 1£æOweolbori£æSacheon 6, which showed the similar tendency to the degree of freezing resistance (Chun & Griffith, 1998). The combination of Dongbori 1 and Reno was the greatest for expressions of apoplastic proteins among F_1 hybrids. It was known that expression of the AFP gene was rapidly induced by low temperature (Atici & Nalbantoglu, 2003) and apoplastic proteins secrete into the leaf apoplast where ice forms,

Table 1. Mean values for antifreeze protein (AFP)concentrations and contents in 4-parent F_1 diallel crosses grown in growth chamber

C Cross	AFPs concentration($\mu g/ml$)		AFPs content(μg/g FW)	
	GNA	GCA	GNA	GCA
S6×S6	43.2	184.2	8.4	17.1
S6×OW	38.1	165.5	8.1	17.0
S6×D1	47.0	196.1	9.0	22.4
S6×RE	60.5	235.5	11.6	23.3
OW×OW	47.7	217.2	9.2	21.8
OW×D1	55.7	220.5	15.0	25.3
OW×RE	67.3	275.5	16.2	27.7
D1×D1	70.0	279.2	13.7	31.1
D1×RE	85.4	316.9	16.6	34.4
RE ×RE	91.9	364.9	18.9	43.8
Mean	60.7	245.6	12.7	26.4
SD	18.0	62.8	3.9	8.3
CV(%)	29.7	25.6	30.7	31.4
Ratio	4.05		2.08	

S6; Sacheon 6, OW; Oweolbori, D1; Dongbori 1, RE; Reno,

SD; standard deviation, CV; coefficient of variation.

Table 2. Mean squares for Wr-Vr of antifreeze protein concentrations in 4-parent F1 diallel crosses grown in growth chamber

Source	DF	Mean square	
		GNA	GCA
Replications	3	1354.0	48496.0
Arrays	3	549.6*	7959.3 ns
Error	9	90.6	2644.0

* Significant at 5% level. ns ; not significant. Wr; covariance of each array, Vr; variance of each array.

which increased freezing resistance of the plant (Hon *et al.*, 1994).

Genetic analysis of AFPs

The variance of each array (Vr) and covariance of each array with the non-recurrent parents (Wr) were calculated for each replication from the diallel table (Table 2). Tests of significance of the difference between arrays in (Wr-Vr) were performed by an analysis of variance (Hayman, 1954). If the array variance in analysis of variance of (Wr-Vr) over replications was not significant, an adequate basis was thought to exist to warrant the graphical analysis. The Wr/Vr graph (Figure 1) also provides test of significance for the presence of dominance ($b \neq 0$), the average dominance (sign of the Wr intercept), and the existence of non-allelic interaction ($b \neq 1$). The Wr-Vr for apoplastic proteins except that of GNA were not significant among the treatments, which fitted to additive-dominance model applicable in case of no interaction among non-allelic genes. The linear regression coefficient of Wr on Vr for apoplastic proteins was 1.01 (Figure 1). They were not different from 1.0 by t-test (β=1) and linear regression lines passed above original point (0, 0), showing that apoplastic proteins were partially dominant with non-allelic genes in F_1 hybrids. The parents having dominant and allelic genes with small variance and covariance are

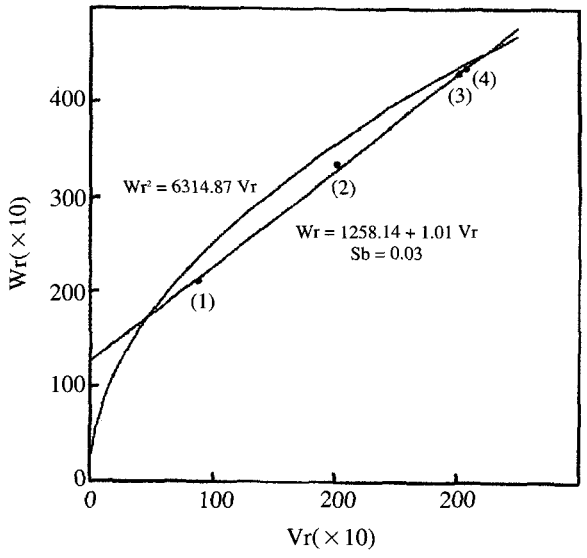

Fig. 1. Regression of Wr on Vr for AFPs in 4-parent F_1 diallel cross of cold-acclimted plants in growth chamber . The parents are; (1) Sacheon 6, (2) Oweolbori, (3) Dongbori 1, (4) Reno.

placed on near original point of linear regression lines. However, the parents having recessive and allelic genes with large variance and covariance are placed on the end of the original point. The nearer to the point of the origin on the Wr/Vr graph a cultivar is located, the more it has dominant alleles (Hayman, 1956). The sign of Wr intercept on the Wr/Vr graphs indicate the degree of dominance. Over-dominance, complete or partial dominance correspond to less than zero, equal to zero or larger than zero, respectively. The values of the Wr intercept in F_1s were larger than zero (1258.14), which indicates partial dominance for apoplastic proteins. Sacheon 6 that showed the lowest accumulation level of apoplastic proteins was dominant, whereas Reno and Dongbori 1 were recessive for the plants grown in chambers (Figure 1). The estimated genetic components of variation for the homogeneous 4-parent F_1 in growth chambers were shown in Table 3 (Hayman, 1954). The additive (D) and dominant (H_1 and H_2) components were always significant since they were greatly exceeded twice their standard errors, indicating the

Table 3. Genetic components of variation and their standard errors for antifreeze protein concentrations in 4-parent F_1 diallel crosses grown in growth chamber and field

Component	Estimate	
	GNA	GCA
D	502.7 ± 12.2	6310.8 ± 47.4
H_1	83.4 ± 35.3	1161.9 ± 137.7
H_2	68.9 ± 32.6	1059.9 ± 127.1
h^2	38.9 ± 22.1	1561.4 ± 86.2
F	- 160.5 ± 31.2	- 1515.9 ± 121.7
E	0.6 ± 5.4	4.1 ± 2.1
$(H_1/D)^{1/2}$	0.41	0.43
$H_2/4H_1$	0.21	0.23
K_d/K_R	0.44	0.50
h^2/H_2 (K)	0.56	1.47
Heritability (B)	1.00	1.00
Heritability (N)	0.95	0.94

existence of both additive and dominant variation.

In the presence of unequal gene frequencies, the sign and magnitude of F can be used to determine the relative frequencies of dominant to recessive alleles in the parental population, and to determine the variation in the dominance level over loci (Mather & Jinks, 1971). Negative values of F implied an excess of recessive alleles. The average degree of dominance $[(H_1/D)^{1/2}]$ in intra-locus gene interaction for apoplastic proteins were much smaller than 1.00 with $D > H_1$, indicating the partial dominance with great additive gene action. The relationship between H_1 and H_2 provides an estimate for overall allele frequencies at loci exhibiting dominance. The ratio of $H_2/4H_1$ $(= u \times v)$ has a maximum value of 0.25 when the frequencies are equal between dominant and recessive alleles ($u = v = 0.5$). The ratio ranged 0.21 ~ 0.25 with nearly equal frequency of positive and negative alleles for apoplastic proteins. The h^2/H_2 (K) ranged 0.56 ~1.47 and it showed

Table 4. Mean squares of general combining ability (GA) and specific combining ability (SA) for antifreeze proteins in 4-parent F_1 diallel crosses grown in growth chamber

Source	DF	Mean square	
		GNA	GCA
GA	3	926.3**	11063.8**
SA	6	22.4**	387.4**
Error	27	0.64	4.08
GA/SA		41.35	28.56

** Significant at 1% level.

Table 5. The general combining ability (GA) effects for antifreeze protein concentrations in 4-parent F_1 diallel crosses grown in growth chamber and field

Parents	GA effect	
	GNA	GCA
Sacheon 6	-11.91	-43.72
Oweolbori	-7.81	-21.96
Dongbori 1	4.13	10.68
Reno	15.59	55.00
S.E.(Gi)	0.28	0.71
S.E.(Gi-Gj)	0.46	1.17

that the character was controlled by at least 2 major genes. Heritability analysis revealed that apoplastic proteins had high narrow-sense heritabilities and it was considered that this character could be selected for freezing resistance in

the segregating generation of barley breeding program.

Combining ability effects for AFPs

The combining ability mean squares for apoplastic proteins in 4-parent diallel crosses were shown in Table 4. Mean squares of both general combining ability (GA) and specific combining ability (SA) were highly significant for all treatments. Moreover, GA mean

Table 6. Simple correlation coefficients among traits related to freezing resistance in 4-parent F1 diallel crosses

Variable	X_1	X_2	X_3	X_4	X_5
X_1: AFPs conc.(GNA)	1				
X_2: AFPs conc.(GCA)	0.988**	1			
X_3: AFPs content(GNA)	0.922**	0.911**	1		
X_4: AFPs content(GCA)	0.964**	0.976**	0.902**	1	
X_5 TTC(L-13)	0.975**	0.988**	0.915**	0.967**	1

L-13; absorbance ratios of TTC reduction treated with -13℃ in 55-day old plants in field.

** Significant at 1% level.

squares for all treatments were much larger than those of SA (28.6 ~ 41.4 times), indicating that variance components due to additive effects might be larger than those due to non-additive effects for the apoplastic proteins, although both additive and non-additive effects were important. The GA effect of the parents for apoplastic proteins was shown in Table 5. The GA effect of Reno was much higher than the other parent cultivars (15.6 ~ 55.0). The large and positive GA value for apoplastic proteins indicated that Reno was a desirable parent with high accumulation of apoplastic proteins. Sacheon 6 and Oweolbori showed negative GA effects, whereas Dongbori 1 showed positive GA effects in all treatments.

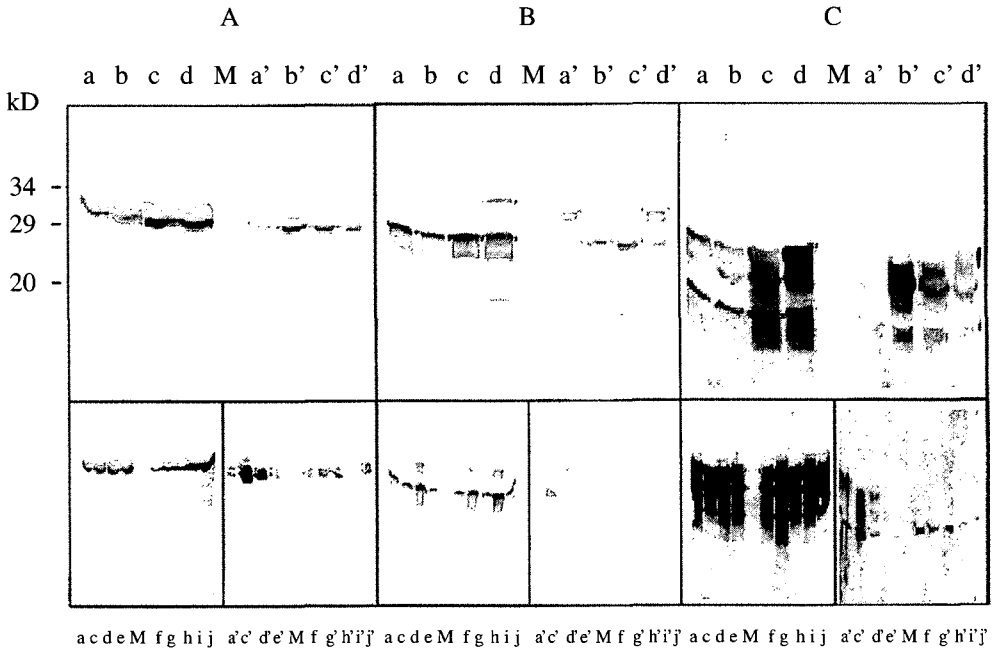

Fig. 2. Immunodetection of antifreeze proteins in apoplastic extracts from 4-parent F_1 diallel crosses. A; Immunodetection of antifreeze protein probed with anti-GLP antiserum. B; Immunodetection of antifreeze protein probed with anti-CLP antiserum. C; Immunodetection of antifreeze protein probed with anti-TLP antiserum. a; Sachon 6(L), b; Oweolbori(L), c; Dongbori 1(L), d; Reno(L), a'; Sachon 6(H), b'; Oweolbori(H), c'; Dongbori 1(H), d'; Reno(H), e; (S6×OW)-L, f; (S6×D1)-L, g; (S6×RE)-L, h; (OW×D1)-L, i; (OW×RE)-L, j; (D1×RE)-L, e'; (S6×OW)-H, f'; (S6×D1)-H, g'; (S6×RE)-H, h'; (OW×D1)-H, i'; (OW×RE)-H, j'; (D1×RO)-H, M; Prestained protein standard. H for nonacclimated, L for cold-acclimated.

Immunodetection of AFPs in the parents and F_1 hybrids

For immunodetection of apoplastic proteins, antisera that were previously raised against three classes of AFPs in Musketeer (Antikainen et al., 1996) were used for blotting. Antiserum raised against the 32-kD GLP from winter rye recognized one pair of polypeptide in the apoplastic extracts of GCA and GNA parent leaves except Sacheon 6 (Fig. 2A).

The results for F_1 hybrids were not different from those of their parents. Antiserum raised against the 35-kD CLP from winter rye recognized two polypeptides (35- and 28-kD) in GCA and GNA parent leaves, and two polypeptide bands were clearly detectable in the GCA leaves of Reno (Fig. 2B). The results for F_1 hybrids were similar to those of their parents, but there were no detectable bands in GNA leaves of all cultivars. Antiserum raised against the 25-kD TLP from winter rye recognized three polypeptides (25-, 22- and 16-kD) in the GCA leaves of parents (Fig. 2C). Three bands of TLP were detected in GNA leaves of Oweolbori, Dongbori 1 and Reno, but not in the Sacheon 6. The F_1 hybrids had three clear bands in GCA leaves, but not in all GNA leaves.

Correlation between freezing resistance and AFPs

The simple correlation coefficients related between freezing resistance and apoplastic proteins in parents and their F_1 hybrids were shown in Table 6. The degree of freezing resistance, estimated with absorbance ratio of TTC reduction treated at -13℃ for 55-day old plants in field, was significantly correlated with AFP concentration and content per gram leaf fresh weight in all treatments (Table 6). The concentrations and contents of AFPs among the treatments were positively correlated. Thereupon, it was considered that AFP concentration or content could be a useful criterion for selecting freezing-resistant lines in the breeding programs.

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