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Genetic analysis of the accumulation of COR14 proteins in wild (*Hordeum spontaneum*) and cultivated (*Hordeum vulgare*) barley

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Abstract The *co*ld-*r*egulated (COR14) protein of 14 kDa is a polypeptide accumulated under low-temperature conditions in the chloroplasts of barley leaves. In *H. vulgare* the COR14 antibody cross-reacts with two proteins, with a slightly different relative molecular weight around the marker of 14.4 kDa, referred to as COR14a and COR14b (high and low relative molecular weight, respectively). In a collection of *H. spontaneum* genotypes a clear polymorphism was found for the corresponding COR proteins. While some accessions showed the same COR pattern as cultivated barley, in 38 out of 61 accessions examined the COR14 antibody cross-reacted with an additional cold-regulated protein with a relative molecular weight of about 24 kDa (COR24). The accumulation of COR24 was often associated with the absence of COR14b; the relationship between the COR14b/COR24 polymorphism and the adaptation of *H. spontaneum* to different environments is discussed. By studying COR14 accumulation in cultivated barley we have found that the threshold induction-temperature of COR14a is associated with the loci controlling winter hardiness. This association was demonstrated by using either a set of 30 cultivars of different origin, or two sets of frost-tolerant and frost-sensitive F1 doubled-haploid lines derived from the cross Dicktoo (winter type)× Morex (spring type). These results suggest that the threshold induction-temperature of COR14a can be a potential biochemical marker for the identification of superior frost-resistant barley genotypes.

Key words Cold-acclimation · Cold-regulated protein · Molecular marker · Frost resistance · *Hordeum* spp.

Introduction

The *co*ld-*r*egulated (COR14) protein of 14 kDa is a polypeptide accumulated under low-temperature conditions in the chloroplasts of barley leaves (Crosatti et al. 1995). The corresponding gene, *pt59* (Cattivelli and Bartels 1990), is expressed in leaves of barley, as well as of other related cereals, during cold-hardening. Therefore, the accumulation of COR14 has been related to the acquisition of frost-resistance. Several studies have shown that clones and antibodies corresponding to cold-regulated genes and proteins cross-react with homologous genes and proteins among different plant species, mainly winter cereals (Cattivelli and Bartels 1990; Houde et al. 1992; Crosatti et al. 1994, 1995), suggesting that the molecular response to low temperatures is a well-conserved mechanism. Nevertheless, very little is known about the genetic variability for hardening-related genes within a single plant species and the function of most COR proteins is still unknown.

An analysis of gene-expression patterns and/or of protein accumulation has sometimes shown a positive correlation between the amount of COR transcripts/proteins and the degree of cold-resistance (Mohapatra et al. 1989; Dunn et al. 1990; Houde et al. 1992). Our recent work has demonstrated that in cultivated barley the induction-temperature threshold for COR14 is different between a winter and a spring cultivar (Crosatti et al. 1995). In the present paper we have analyzed the threshold temperature for COR14 induction in a large number of genotypes in order to verify the existence of genetic linkage between the COR14 threshold-induction temperature and frost-resistance. In addition, by studying COR14 expression in cultivated and wild barley, a wide genetic variability for COR14 was detected within *Hordeum spontaneum* accessions.

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Materials and methods

Plant material and growing conditions

The following plant genotypes were used:

Sixty-one wild barley (*H. spontaneum* L.) accessions collected in Israel, Turkey and Iran (see Table 1A) as previously described by Nevo et al. (1979, 1986a, b, c). The wild barley populations here tested can be classified as follows: high-altitude populations, which experience cold winters (Mt. Hermon, 1400 m; Mt. Meron, 1150 m; Maalot, 500 m; Khoramabad, 1800 m; and Diyarbakyr, 1200 m); hot desert populations (Ein Zukim and Wadi Qilt); coastal plain populations (Ashqelon and Caesarea); and Jordan valley populations (Tabigha and Mehola).

Thirty barley (*Hordeum vulgare* L.) cultivars, mainly winter types, previously evaluated for frost-tolerance in winter field trials (Rizza et al. 1994) (see Table 1B).

Ten barley F_1-derived doubled-haploid lines bred from the cross Dicktoo (winter type)×Morex (spring type), kindly supplied by Dr. P. Hayes. These genotypes were rated as resistant (doubled-haploid lines nos. 20, 33, 50, 55, 80) or susceptible (doubled-haploid lines nos 18, 32, 44, 45, 92) on the basis of winter field-survival tests (Hayes et al. 1993).

Twelve wheat-barley ditelosomic addition lines (Islam et al. 1981 1983), kindly provided by Dr. A. K. M. S. Islam, and their two parents Betzes (barley) and Chinese Spring (wheat). The nomenclature of the barley chromosome is based upon the equivalent wheat homoeologous group (Ainsworth et al. 1986).

Seeds were sown in pots of 10-cm diameter, each containing 15 seeds in a medium containing sand and peat (1:1) and grown until first-leaf stage in a growth chamber with a daily regime of 10 h light (160 mol photons $m^{-2}s^{-1}$) at 20°C and 14 h darkness at 15°C. Plants at the first-leaf stage were cold-acclimated in a daily regime of 10 h light (160 mol photons $m^{-2}s^{-1}$) and 14 h darkness either at +3°C/+1.5°C for 7 days in order to detect COR14 polymorphisms and to map the corresponding gene (after 7 days of cold treatment many COR proteins are well accumulated), or at the constant temperature of +6, +8, +10, +12°C in order to reveal the COR14 threshold-induction temperature. Plants of the cultivar Onice, cold-acclimated for 7 days (+3°C/+1.5°C), were used as a positive control.

Protein extraction, electrophoresis and Western blotting

Barley leaves were frozen and ground to a fine powder in liquid nitrogen. The proteins were precipitated with 10% (w/v) trichloroacetic acid and 0.07% 2-mercaptoethanol in acetone, washed with 0.07% 2-mercaptoethanol in acetone and solubilized using 50 µl of SDS loading buffer (Tris-HCl pH 6.8 60 mM, SDS 2%, 2-mercaptoethanol 5%) for each 1 mg of powder (Laemmli 1970). The samples were boiled for 5 min, centrifuged for 10 min at 10000 *g* and 50 µl of supernatant were loaded onto a 12% SDS-Polyacrylamide gel overlaid with a 4% staking gel. Proteins were electroblotted onto a nitrocellulose membrane (BA83, Schleicher and Schuell) according to Szewczyk and Kozloff (1985) and probed with the COR14 polyclonal antibody (Crosatti et al. 1995). Western blotting was performed with an ECL kit (Amersham) according to the manufacturer's instruction; the antibody working dilution was 1:3500. Densitometric scanning of films after antibody exposure, was performed with a Beckman DU65 spectrophotometer equipped with gel-scanning-area software.

Chloroplast preparation

Leaves of wild barley plants, cold-acclimated for 7 days, were homogenized in 340 mM sorbitol, 0.4 mM KCl, 0.04 mM EDTA, 2 mM Hepes-KOH, pH 7.8 after Cerovic and Plesnicar (1984). The homogenate was layered on Percoll 40% (v/v) and centrifuged for 1 min at 1700 *g*. The intact chloroplasts were destroyed by adding SDS loading buffer (Laemmli 1970).

Thylakoids were isolated after Lapointe et al. (1991). One gram of leaves was homogenized in extraction buffer [0.4 M sorbitol, 50 mM Tricine (pH 7.8), 10 mM NaCl], filtered through two layers of Miracloth and centrifuged at 3000 *g* for 2 min. The pellet was washed once in buffer containing 0.1 M sorbitol, 50 mM Tricine (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$ 1 mM NH_4Cl and dissolved in SDS loading buffer

Freezing test

The extent of cold damage due to freezing was tested by measuring the rate increase in ion release according Van De Dijk et al. (1985) and Rizza et al. (1994). Plants of the cultivars Onice and Gitane were cold-acclimated for 21 days in a daily regime of 10 h light (160 mol photons $m^{-2}s^{-1}$) and 14 h darkness either at +3°C/+1.5°C or at a constant temperature of +8°C; unhardened plants were also included as a control. After cold-acclimation the plants were stored at −3°C (±0.5°C) for 16 h and subsequently subjected to a gradual 2°C h^{-1} lowering of the temperature down to −8, −10, −12°C (±0.5°C); plants were kept in these conditions for 18 h in the dark. After gradual thawing, a sample of 35 leaf segments of about 0.5 cm for each replication were placed in a vial containing 25 ml of de-ionized H_2O, degassed under vacuum for 20 min and stirred at 25°C for 2 h and 30 min. Ion release was measured by a digital conductivity meter and membrane damage was determined as the percentage of maximum possible injury induced by autoclaving of the samples. The experimental design was a split-split-plot with four replications. The cultivar was assigned to the main plot, temperature to the subplot and the hardening condition to the sub-subplot.

Results

COR14 polymorphisms in *H. spontaneum*

In *H. vulgare* the COR14 antibody cross-reacts with two proteins with slightly different relative molecular weights around the marker of 14.4 kDa (Fig. 1, lane 1); both of them are induced by low temperatures (and are therefore absent in the control sample, Fig. 1 lane 2). Hereafter we will refer to these two polypeptides as COR14a and COR14b (high and low relative molecular weight, respectively). In the *H. spontaneum* collection a clear polymorphism was found for the corresponding COR proteins. While some accessions showed the same COR pattern as cultivated barley (see genotype 7–7 in Fig. 1), in 38 out of the 61 *H. spontaneum* accessions examined the COR14 antibody cross-reacted with an additional cold-induced protein with a relative molecular weight of about 24 kDa (COR24). The accumulation of COR24 was most often associated with the absence of COR14b (e.g. genotypes 9–35 and I-18–25 in Fig. 1). Nevertheless, a few exceptions were detected in genotypes 9–26 and 22–24 where COR24 was expressed together with COR14a and COR14b (Fig. 1). COR14a was found in all samples examined. A large variation in the amount of COR24 accumulated by *H. spontaneum* plants under low temperatures has also been detected. Most of the accessions expressing COR24 accumulated this protein at a high level (e.g. genotype 9–35 in Fig. 1), but there are also a few accessions expressing COR24 at low level (e.g. genotypes 22–24 and 9–26 in Fig. 1).

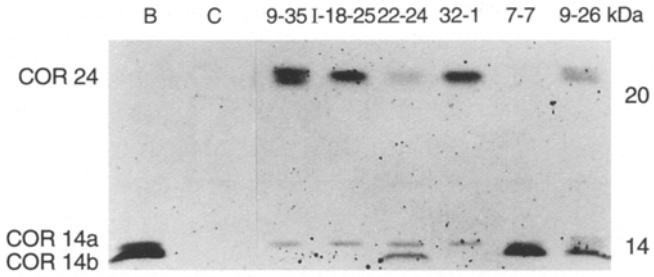

Fig. 1 COR14 polymorphism. Proteins were isolated from a collection of *H. spontaneum* accessions after 7 days of cold treatment and equal amounts were separated onto SDS-PAGE and hybridized with COR14 antibody. The different genotypes are described according to their population numbers (see Table 1). *B* cultivated barley hardened for 7 days; *C* sample from non-hardened *H. spontaneum* plants. COR14a, COR14b and COR24 are indicated. Each accession is described by two numbers, the first indicates the population (see Table 1A), the second the single genotype tested

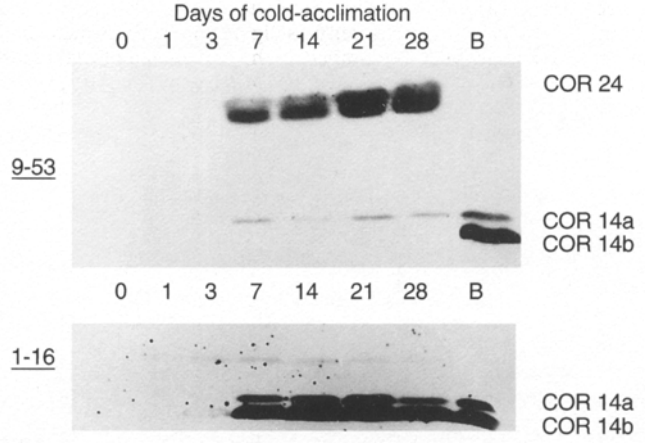

Fig. 2 Cold-triggered accumulation of COR14 and COR24. Equal amounts of proteins, extracted from *H. spontaneum* plants [accessions 9–53 (representing the genotypes with COR14a and COR24) and 1–16 (representing the genotypes with COR14a and COR14b)], were separated by SDS-PAGE and hybridized with COR14 antibody. *Numbers* indicate the days of cold-acclimation. *B* sample from cultivated barley hardened for 7 days

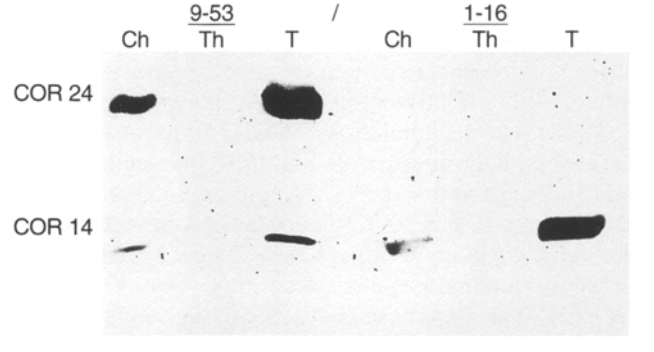

Fig. 3 Chloroplast localization of COR14 and COR24. Protein extracts isolated from intact chloroplasts and thylakoids of *H. spontaneum* plants, accessions 9–53 (Mt. Meron) and 1–16 (Mt. Hermon), were separated by SDS-PAGE and hybridized with COR14 antibody. *Th* thylakoids; *Ch* intact chloroplasts; *T* total protein extract. COR14 and COR24 are indicated

The COR14/24 polymorphism did not affect either the expression kinetics or the subcellular localization of the proteins. Indeed when the cold-triggered accumulation of COR14 and COR24 was studied by using genotypes 9–53 and 1–16, the same accumulation kinetics were detected for COR14 and COR24 (Fig. 2). It has already been reported that in *H. vulgare* COR14a and COR14b are localized in the stroma of the chloroplasts (Crosatti et al. 1995) and a specific experiment has shown that COR14 and COR24 are also accumulated in the stroma of the chloroplasts in *H. spontaneum* (Fig. 3). The common expression pattern and subcellular localization of COR14b and COR24, together with their high immunological relationship, suggest that these two proteins have probably the same, even if still unknown, function.

The nearly complementary frequencies of COR14b and COR24 appear in the last two columns of Table 1. Notably, COR14b is absent in all coastal plain and desert populations that are in frost-free localities. COR14b displays high frequencies in the Jordan valley, Mt. Hermon, Galilee (excepting Meron), Turkey and Iran. Thus COR14b and COR24 are primarily polymorphic in the cooler highlands, in Israel, Turkey and Iran. Exceptions are Maalot and Tabigha in Israel.

Chromosomal localization

Genetic mapping of the gene coding for COR14b was carried out with the wheat-barley ditelosomic addition-line series (Islam et al. 1981). The antibody recognized both the barley COR14b and the corresponding wheat protein. Nevertheless, due to the different electrophoretic mobilities of the two polypeptides (Crosatti et al. 1995) it was possible to distinguish each of them. All the addition lines available, plus the two parents Betzes (barley) and Chinese Spring (wheat), were cold-acclimated for 7 days in order to obtain full expression of the COR proteins. The addition line carrying the long arm of the barley chromosome 2 was the only one where the barley COR14b was present with the corresponding wheat protein (Fig. 4). Due to the identical electrophoretic mobility between COR14a and the corresponding protein of wheat, the gene coding for COR14a was not mapped by using the wheat-barley ditelosomic addition lines. Attempts to map the COR14 locus by using the corresponding cDNA clone failed to detect a convenient polymorphism.

Analysis of COR14 threshold induction-temperature in cultivated barley

By comparing a winter and a spring cultivar, previous work has shown, that the threshold-induction-temperature of COR14 is different in frost-resistant and frost-sensitive genotypes of *H. vulgare* (Crosatti et al. 1995). Here we have extended this analysis to a large number of cultivars with the aims of (1) verifying the association between the threshold temperature and the level of resistance, and (2)

Table 1 **A** *H. spontaneum* accessions used for COR14 investigation. A genetic description of these genotypes can be found in previous papers (Nevo et al. 1979, 1986a, b) according to the population numbers. The frequences of the polymorphic cold-regulated proteins calculated among accessions belonging to the same population, are indicated. **B** *H. vulgare* cultivars used for COR14 investigation. The cultivars are listed according to their level of frost resistance, Onice being the most tolerant and Pilastro and Gitane the most sensitive (Rizza et al. 1994). W=winter growth habit; S=spring growth habit

A – *H. spontaneum*

Population number	Site of collection	Number of genotypes	Freq. COR14b	Freq. COR24
	Israel populations			
1	Mt. Hermon	7	0.57	0.43
7	Tabigha	6	1.00	0.00
9	Mt. Meron	7	0.14	1.00
10	Maalot	5	1.00	0.00
22	Mehola	6	1.00	0.17
23	Wadi Qilt	5	0.00	1.00
26	Ceasarea	4	0.00	1.00
28	Ashqelon	5	0.00	1.00
32	Ein Zukim	6	0.00	1.00
	Turkey population			
T-9	Diyarbakir 38Km W	4	0.25	0.75
	Iran population			
I-18	Khoramabad 30Km E	6	0.33	0.67
Total		61	0.44	0.62

B – *H. vulgare*

Cultivars	Origin	W/S	Cultivars	Origin	W/S
Onice	Italy	W	Baraka	France	W
Fior 1189	Italy	W	Criter	France	W
Fiction	France	W	Igri	Germany	W
Alfeo	Italy	W	Fior 418	Italy	W
Trebbia	Italy	W	Barberousse	France	W
Fior 768	Italy	W	Panda	France	W
Plaisant	France	W	Robur	France	W
Etrusco	Italy	W	Red	Italy	W
Arda	Italy	W	Selvaggio	Italy	W
Frost	Sweden	W	Flash	France	W
Tipper	Great Britain	W	Elan	France	W
Fior 1006	Italy	W	Jaidor	France	W
Fior 1143	Italy	W	Timura	Germany	W
Express	France	W	Pilastro	Italy	W
Dahila	France	W	Gitane	France	S

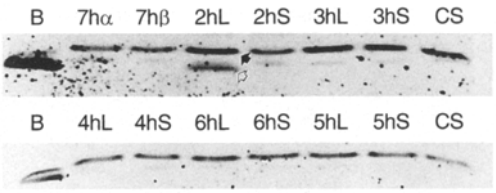

Fig. 4 Mapping of the gene coding for COR14 using the wheat-barley ditelosomic addition lines. Proteins were isolated from each ditelosomic addition line plus the two parents (Betzes *B* and Chinese Spring *CS*) after 7 days of cold treatment and equal amounts were separated onto SDS-PAGE and hybridized with COR14 antibody. The *open arrow* indicates the barley COR14 protein; the *black arrow* indicates the wheat homologous protein. The addition lines are described according to the wheat homologous nomenclature

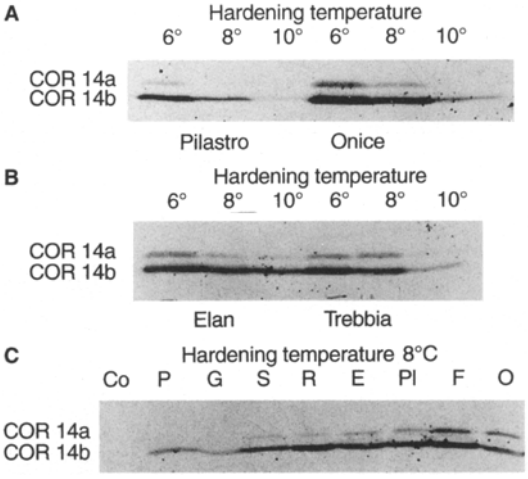

Fig. 5 Analysis of COR14a threshold induction-temperature. Equal amounts of proteins extracted from barley plants exposed for 7 days at the indicated temperatures were separated onto SDS-PAGE and hybridized with COR14 antibody. In *A* the COR14 accumulation is shown for the frost-sensitive cultivar Pilastro in comparison with the frost-tolerant cultivar Onice, while in *B* two winter cultivars with an intermediate level of frost-tolerance (Elan and Trebbia), are compared. In *C* two frost-sensitive cultivars (Pilastro *P* and Gitane *G*), four cultivars with an intermediate level of frost-tolerance (Selvaggio *S*, Red *R*, Etrusco *E* and Plaisant *Pl*), and two frost-tolerant cultivars (Fior 1189 *F* and Onice *O*) were acclimated for 7 days at +8°C and compared for COR14a expression. *Co* control sample from non-acclimated plants. COR14a and COR14b are indicated

testing the possible use of the COR14 antibody as a tool for selection. Rizza et al. (1994) have assessed the frost-resistance capacity of 30 barley cultivars, mainly winter type (Table 1B), under field conditions. The results identify Onice and Fior 1189 as the cultivars with the highest level of frost-resistance, while Pilastro and Gitane were those most severely damaged by cold. All these cultivars were cold-acclimated for 7 days at constant temperature (6°, 8° and 10°C) and subjected to Western analysis. Concerning COR14 accumulation, when the most frost-resistant cultivars were compared with the frost-sensitive ones (i.e. Onice vs Pilastro, Fig. 5A) a clear difference was detected: at the temperature of 8°C COR14a accumulates in the former (i.e.Onice in Fig. 5A) but not the latter cultivar (i. e. Pilastro in Fig. 5A). Nevertheless, two cultivars with an intermediate level of frost resistance showed the same threshold induction-temperature (i.e. Elan vs Trebbia, Fig. 5B). Based on these results we have analyzed the expression of COR14a in all the cultivars of Table 1B when exposed to 8°C for 7 days (Fig. 5C). The frost-sensitive cultivars Pilastro and Gitane did not accumulate COR14a, the cultivars with the highest frost-resistant capacity (Onice and Fior 1189) accumulated COR14a at high level, while all the other genotypes tested showed a low level

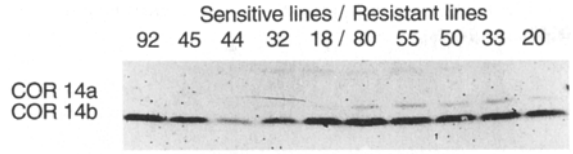

Fig 6 Analysis of COR14a threshold induction-temperature in resistant and susceptible F_1-derived doubled-haploid lines. Equal amounts of proteins extracted from plants exposed for 7 days at +8°C were separated onto SDS-PAGE and hybridized with COR14 antibody. Doubled-haploid lines Nos. 20, 33, 50, 55 and 80 were rated as frost-tolerant while doubled-haploid lines Nos. 18, 32, 44, 45 and 92 were rated as frost-susceptible (Hayes et al. 1993). COR14a and COR14b are indicated.

Table 2 Mean values (%) of membrane damage in two contrasting barley cultivars (Onice – winter and Gitane – spring) acclimated for 21 days under different hardening conditions and frozen at different temperatures. Unhardened plants were used as a control. $LSD_{(0.05)}$ Hardening condition=2.4; interaction: "Cultivar×Temperature× Hardening"=5.6

Hardening conditions	Gitane			Onice			Mean
	−8°C	−10°C	−12°C	−8°C	−10°C	−12°C	
+1 5/3°C	30	57	86	20	36	62	48
+8°C	73	97	98	52	87	87	84
Control	95	98	98	96	99	99	97

accumulation of COR14a (in Fig. 5C 4 cultivars out of the 26 genotypes with an intermediate level of frost resistance tested are reported).

In order to test whether the threshold induction-temperature of COR14a is genetically linked with the frost-resistant capacity, a further experiment has been performed with the double-haploid lines deriving from the cross Dicktoo× Morex (Hayes et al. 1993). When plants from five resistant and five susceptible doubled-haploid lines were acclimated at +8°C for 7 days and tested for the presence of COR14a, all the resistant genotypes showed protein accumulation, while only one of the susceptible genotypes (N 18) showed a faint band coresponding to COR14a (Fig. 6). These results support the suggestion that the gene controlling the accumulation of COR14a is genetically linked to, or pleiotropic with, the frost-resistance phenotype.

All the experiments concerning the analysis of the COR14a threshold induction-temperature have been carried out at +8°C. In order to test whether this temperature really increases plant frost tolerance, we have measured the level of membrane damage induced by an artificial freezing test in leaves of the cultivars Onice (a frost-resistant genotype that accumulates COR14a at +8°C) and Gitane (a frost-sensitive genotype that does not accumulate COR14a at + 8°C) after 21 days of acclimation at different temperatures. The results are reported in Table 2. Plants acclimated at + 8°C showed a level of damage close to that of unhardened plants suggesting that such a temperature, in both cultivars, induces only a minimal level of hardening.

Discussion

Analysis of COR14/24 polymorphism

The existence in *H. spontaneum* of a cold-regulated protein of 24 kDa immunologically related to COR14 has already been reported by Crosatti et al. (1995). In the present paper, by examining 61 wild barley accessions, we have found that COR14b and COR24 are both present in *H. spontaneum* with nearly complementary frequencies. Our previous work (Crosatti et al. 1995) has already shown that plants expressing COR24 have a single cold-induced mRNA of 0.9 kb, the same size as that found for COR14 in *H. vulgare* and corresponding to the size of the full-length clone *pt59*. These findings suggest that the accumulation of COR24 is probably due to a post-translation modification of COR14. The expression of COR24 is often associated with the absence of COR14b, though in two *H. spontaneum* accessions (9–26 and 22–24) COR14b and COR24 are accumulated together. These results may be due to heterozygosity so that two alleles were present in the same accession, although the analyses of the allozyme pattern within *H. spontaneum* accessions (Nevo et al. 1979) indicated that heterozygotes are present only rarely (in the order of 0.001).

H. spontaneum is polymorphic for COR24 and COR14b in highland regions exposed occasionally or routinely to frost (i.e. northern Israel, Turkey and Iran). By contrast COR24 is monomorphic and COR14b is absent in the warm desert and the mild, usually frost-free, coastal plain populations. Thus, the polymorphism of COR24 and COR14b may be climatically adapted. This conclusion needs larger sample sizes for validation.

The threshold induction-temperature of COR14a is associated with winter hardiness

Many biochemical characters have been proposed as markers for the selection of frost-resistant cereal plants: e.g. in wheat, proline and abscisic acid content have been related to the extent of freezing resistance (Dörffling et al. 1990). On the other hand, the exposure of barley plants to low temperatures resulted in an increase in proline equally in resistant and susceptible cultivars (Murelli et al. 1995). A clear correlation between the degree of freezing tolerance and the accumulation of a specific set of cold-regulated proteins has been found in wheat, and the corresponding antibody has been proposed as a marker to select for freezing tolerance in all cereal species (Houde et al. 1992). Nevertheless, the effectiveness of all those markers has usually been assessed by using few cultivars with contrasting frost-resistance capacity or by comparing plant species with different levels of cold adaptation. In our work, by using either a group of 30 cultivars or two pools of resistant and susceptible doubled-haploid genotypes (Hayes et al. 1993), we have shown that the accumulation of COR14a at +8°C is as effective as other traits (i.e. field survival or

LT50) in discriminating between resistant and susceptible genotypes of cultivated barley. By using the same F_1-derived doubled-haploid lines a single quantitative trait locus (QTL) controlling several traits associated with winter hardiness has been mapped onto chromosome 7 (5H, according to the equivalent wheat homoeologous group) (Hayes et al. 1993; Pan et al. 1994). Our results, obtained with two pools of contrasting doubled-haploid genotypes, suggest that the accumulation of COR14a is directly controlled by a locus localized within, or linked to, the chromosome region identified by the QTL controlling winter hardiness. A different threshold-induction temperature between spring and winter barley for the accumulation of the mRNA corresponding to *pt59* (the gene coding for COR14), was reported by Brauer et al. (1994).

By comparing the Western analyses reported in Figs. 5C and 6 it can be seen that the signals corresponding to COR14a accumulation in the doubled-haploid resistant genotypes (Fig. 6) are weaker than those reported for Fior 1189 and Onice (Fig. 5C). Indeed, testing the frost resistance capacity of the doubled-haploid genotypes in comparisons using Gitane and Onice (our standards), after 21 days of acclimation we have found that, while the sensitive genotypes performed very similarly to Gitane, the resistant genotypes were less frost resistant than Onice. Therefore, in barley the accumulation of COR14a at 8°C can be considered to be a precise marker linked to frost-resistance.

COR14a is highly immunologically related with COR14b, but further experiments are required for a fine molecular characterization of these two proteins. They may be encoded by two genes having different regulation mechanisms. The gene coding for COR14b, localized on chromosome 2, is induced equally in resistant and susceptible genotypes when the temperature is below 10°C (Fig. 5A), while the gene coding for COR14a is expressed at lower temperatures in a cultivar-dependent manner. Alternativly, COR14a can derive from a post-translation modification of COR14b. Regardless of the origin of COR14a its accumulation per se, does not produce fully hardened plants (see Table 2), although its threshold induction-temperature appears to be associated with the level of frost resistance. The precise mapping of the character "COR14a threshold induction-temperature" will help to better understand the relationship between the QTL(s) controlling winterhardiness and specific molecular variations induced by low temperatures.

Our results provide a good example of how a molecular marker, such as the COR14 antibody, can not only be a breeding tool for selecting superior genotypes with increased frost resistance, but may also serve as a useful marker for studying plant adaptation to different thermal environments.

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