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The cold-regulated genes are involved in the physiological response of barley to cold environment

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SUMMARY

Barley is grown from northern countries to the limits of the desert. Its adaptation to such different environments underlines the existence of evolutionary-shaped mechanisms that allow barley to cope with physical stresses like cold or drought. Recent studies allowed the identification of the molecular bases of frost resistance and the isolation of many cold regulated genes (*cor* genes). These results depict a complex situation where several signal transduction pathways co-operate for the expression of different classes of *cor* genes. The accumulation of the corresponding *cor* proteins has been associated with development of frost resistance, although a clear function for many of these proteins is still lacking.

Key words: barley, cold acclimation, cold-regulated gene.

YFIRLIT

Kuldastýrðir erfðavísar hafa áhrif á lífeðlisfræðileg viðbrögð byggs við kulda

Bygg er ræktað frá Norðurlöndum suður að jaðri eyðimarkanna. Aðlögun þess að svo breytilegum aðstæðum undirstrikar tilveru þróunarbúnaðar sem gerir því kleift að aðlagast mismunandi álagi, s.s. kulda eða þurrki. Nýlegar rannsóknir hafa sýnt fram á sameindarfræðilegan grunn frostþols og leitt til einangrunar margra kuldastýrðra erfðavísa (*cor*-genes). Þessar niðurstöður sýna flókið ferli þar sem nokkrir skynboðsferlar vinna saman til tjáningar mismunandi hópa *cor*-erfðavísa. Uppsöfnun tilheyrandi *cor*-próteina hefur verið tengd aukningu á frostþoli, enda þótt skilning vanti enn á hlutverki margra þessara próteina.

INTRODUCTION

Barley is grown in northern countries close to the polar circle or up to 4500 m above the sea level in the Himalayan mountains. Such a widespread distribution, despite the differences in the climatic conditions, already suggests that the barley gene pool should contain wide environmental adaptability and stress resistance. The genetic adaptation to cold climate can be achieved either by evolving a powerful frost tolerance ability or by limiting the life cycle to the short summer season (escape strategy). It is a known fact that the winter barley varieties are less hardy than winter wheat, rye and triticale, nevertheless barley is grown up to the Polar Circle because early maturing spring cultivars are able to complete their life cycle in the short summer season. Plant growth habit and heading date can therefore be considered as the basic traits involved in barley adaptation to environments since they allow it to synchronise the life cycle with seasonal changes. Nevertheless, since winter barley has a higher yielding potential than spring ones, there is a great interest to improve its frost resistance capacity.

Freezing tolerance, a fundamental component of winter-hardiness, is based on an inducible process known as hardening or cold acclimation that occurs when plants are exposed to low but non-freezing temperatures. Several genes regulated by low temperatures and sometimes also by drought, have been isolated from barley. The expression of some cold-regulated (*cor*) genes was found to be correlated with the frost tolerance capacity, suggesting that molecular probes could be used for selecting superior genotypes with increased frost resistance.

GENETIC LOCI CONTROLLING FROST RESISTANCE

Frost tolerance has generally been considered a polygenic trait, although specific loci are known to play more important role than other. Traditional genetic approaches have been used to describe characters such as heading-date or growth habit and their involvement in the plant adaptation to cold stress. More recently the application of the molecular marker technology for the analysis of quantitative traits has led to the identification of a relatively small number of quantitative trait loci (QTL) having a major effect on the ability of the plant to survive under stress conditions.

The growth habit trait, even if not directly involved in frost resistance, has been related to the plant winter survival ability. It is known that winter cultivars are generally hardier than their spring counterparts. In barley, winter habit depends on the presence of the dominant allele at locus *Sh* and of the recessive alleles at loci *sh2* and *sh3*. All the other allele combinations among these three genes are found in spring genotypes. The loci *Sh*, *Sh2* and *Sh3* are located on chromosomes 4H, 5H and 1H, respectively. The homozygote genotype *shsh* is epistatic with respect to the recessive allele *sh2* and *sh3*, and evinces a facultative behaviour towards the spring habit when sown in spring. The *Sh3* allele is epistatic with respect to alleles *Sh* and *sh2*. Without vernalization and in long-day conditions, all the *Sh3Sh3* cultivars are essentially spring types. *Sh2*, which is epistatic vis à vis alleles *Sh* and *Sh3*, has a series of multiple alleles which induce several spring-to-winter variants (Cattivelli *et al.*, 1994). Fowler *et al.* (1996) have reported that the loci controling vernalization requirements in wheat and in rye are responsible for the duration of the expression of cold-regulated genes, demonstrating a relationship between growth habit, frost resistance and expression of the genes involved in cold acclimation (see below). The genetic analyses of the frost resistance in barley has demonstrated that a QTL for winter survival on barley chromosome 7 (5H) is associated with the *Sh2* locus (Hayes *et al.*, 1993) and with QTLs for heading-data and vernalization response under long day conditions (Pan *et al.*, 1994). These results probably demonstrate genetic linkage rather than pleiotropic effects, indeed recombinants between vernalization requirement and win-

ter survival traits has been described (Doll *et al.*, 1989).

RFLP analyses of the homeologous 5A chromosome of wheat have proved that vernalization requirement and frost resistance are controlled by two different, but tightly linked loci (*Vrn-A1* and *Fr1* respectively) (Galiba *et al.*, 1995). In wheat the availability of chromosome substitution lines allowed the identification of chromosome carrying loci with relevant role in frost resistance. Thus, when the 5A chromosome of the frost-sensitive variety Chinese Spring was replaced by the corresponding chromosome of the frost resistant Cheyenne variety, the frost tolerance of Chinese Spring was greatly increased (Sutka, 1981; Veisz and Sutka, 1989). Conversily, when the 5A chromosome of Chinese Spring was substituted with the corresponding chromosome originated from a highly frost sensitive *Triticum spelta* accession, the frost resistance of the recipient Chinese Spring decreased (Galiba *et al.*, 1995). Because of its large effect on frost resistance, molecular-assisted selection for the *Vrn-A1-Fr1* 5A chromosome interval has been proposed as a tool to improve cold hardiness of cultivars (Storlie *et al.*, 1998). *Vrn-A1* locus has been found to form a homeologous series with *Vrn-B1* (formerly *Vrn2*) on chromosome 5B and *Vrn-D1* (formerly *Vrn3*) and on chromosome 5D (Snape *et al.*, 1997). Comparison of a common set of RFLP markers suggested that *Vrn-A1* locus is homologous to *Vrn-H1* (formerly *Sh2*) located in barley chromosome arm 5HL (Laurie *et al.*, 1995) and to *Vrn-R1* (formerly *Sp1*) located on rye chromosome arm 5RL (Plaschke *et al.*, 1993).

GENE EXPRESSION AND FROST RESISTANCE

The ability of barley to withstand cold stress situations is mediated by specific sets of genes which modify the cell metabolism enabling cells to cope better with low temperatures. This stress can damage plants, causing changes in cell volume and membrane shape, disruption of water potential gradients, physical damages to the membranes and protein degradation.

While it is becoming increasingly clear that plants can efficiently sense cold stress and mobilise appropriate responses, it is not so clear how these information are obtained and transduced to the cell nucleus to induce freezing tolerance. The molecular mechanisms leading to the cold-induced plant response involve three steps: (a) perception of external changes; (b) transduction of the signal to the nucleus; (c) gene expression. Although in recent years a number of genes have been cloned whose expression is induced or enhanced by low temperatures (Hughes and Dunn, 1996; Cattivelli *et al.*, 2000), little is known about the regulatory mechanism controlling the stress responses. This is mainly due to the fact that cold stress response is a multigenic trait involving genes that may have either redundant or additive effects and may interact with each other in different and complex ways. One of the primary targets is to clarify the functions of these genes and how they are regulated by external changes to better understand plant cold stress responses. Most of these studies have been carried out using either barley or *Arabidopsis* as model plants, although it is generally believed that most of the molecular pathways controling the plant response to low temperatures are well conserved in all plant species.

Signal perception

The exact nature of the primary sensor of cold stress is still not known. Nevertheless, all receptors identified so far in plants (i.e. receptors for ethylene, red and blue light) are located in membranes. One of the best-characterised classes of membrane receptors is formed by protein kinases. The binding of the ligand causes a conformational change of the receptor triggering the kinase activity and the subsequent signal transduction through protein kinase cascades. These receptor-like protein kinases (RLKs) contain characteristic domains: the extracellular leucine-rich repeat

(LRR) motif, the single membrane-spanning domain and the cytoplasmic protein kinase domain (Braun and Walker, 1996). Recently, an *Arabidopsis* gene induced early after dehydration, low temperature and high salt, and coding for a RLK was isolated, suggesting a possible role of this class of genes in the perception of cold stress (Hong *et al.*, 1997).

In recent years an increased number of evidences has pointed out the potential role of the chloroplast as a primary cold stress sensor. In fact plants must constantly balance energy absorbed through the photosynthetic apparatus with energy utilised through metabolism. Cold stress has the potential to upset this balance, leading to an overproduction of free electrons which in turn can damage the reaction centre of the photosystem II (PSII), and in particular the D1 protein, and may lead to the perturbation and inhibition of the photosynthetic electron transport. This phenomenon is known as photoinhibition of photosynthesis (Andersson *et al.*, 1992). The mechanisms leading to an inhibition of electron transport through PSII are not known, but the multiple reduction/oxidation (redox) reactions in PSII have a potential to create AOS (activated oxygen species) leading to the degradation of D1 protein (Russell *et al.*, 1995). Some authors suggested that the redox state of PSII might be the first component in a redox sensing/signaling pathway, acting synergically with other signal transduction pathways to integrate stress response. Gray *et al.* (1997) found that the expression of a cold-induced gene (*Wcs19*) is correlated with the reduction state of PSII in wheat.

Mutations affecting chloroplast development modify the expression of cold-regulated genes. In barley, the cold induced expression of *cor14b, cor tmc-ap3* (genes coding for chloroplastic localised proteins) and of the *blt14*-gene family was strongly reduced in plants carrying the albino mutation a_n, further supporting the role of the chloroplast in the perception of stress signals. It has also been reported that light modulates the cold stress response; in fact the expression of the cold-regulated barley gene *cor14b* is enhanced by red or blue light, suggesting an involvement of a phytochrome or blue light photo-receptors in the signal cascade events leading to cold-induced gene expression (Crosatti *et al.*, 1999).

Signal transduction

A number of molecules have been proposed to be involved in cold stress signaling. This is probably due to the fact that low temperature activates different pathways in the cell, at the same time. The interaction and reciprocal modulation between these signaling cascades, determine the specific stress response of the cell.

One of the molecules involved in stress signaling is the plant hormone abscisic acid (ABA). It is well known that low temperature induces a temporary increase in endogenous ABA levels in plants. During the first day of hardening, ABA concentration reaches a peak in 24 h; then the amount of ABA returns to basal levels for the remaining time of hardening (Murelli *et al.*, 1995; Lång *et al.*, 1994). Exogenous application of ABA at room temperature results in some degree of protection against freezing stress (Heino *et al.*, 1990). ABA, therefore, has been proposed as a necessary mediator in triggering most of the physiological and adaptive cold-stress responses.

A number of stress-inducible genes are also known that are not under the control of ABA, indicating that different stress signaling pathways exist. In barley, in addition to a number of ABA regulated *cor* genes (the most common are the dehydrins, Close *et al.*, 1989) there are cold (Cattivelli and Bartels, 1990) responsive genes not affected by ABA. A comparative study between the drought and the cold responses showed that, while ABA is the key hormone in drought response (application of exogenous ABA induces the expression of most, but not all, genes induced by dehydration), its role in cold response is limited since only few cold-regulated genes are controlled by ABA (Grossi *et al.*, 1992).

In recent years Ca^{2+} was found to be involved in the regulation of many plant responses to environmental stimuli as a second messenger (Webb *et al.*, 1996). In alfalfa as well as in *Arabidopsis* it has been demonstrated that Ca^{2+} influx acts as a signal transduction component in gene activation at low temperature. Addition of Ca^{2+} chelators prevents cold acclimation and the expression of cold-regulated genes, while addition of Ca^{2+} ionophore induces the expression of cold acclimation-specific genes at 25°C (Monroy and Dhindsa, 1995; Tähtiharju *et al.*, 1997).

Protein phosphorylation and dephosphorylation are key steps in cold stress signaling. A number of genes encoding protein kinases and phosphatases induced by cold stress have been cloned. Several Ca^{2+}-dependent protein kinases (CDPKs) have been identified in *Arabidopsis* and alfalfa (Hrabak *et al.*, 1996). These proteins have a serine/threonine protein kinase catalytic domain, an autoinhibitory domain and a Ca^{2+}-binding domain. Thus, CDPKs in plants seem to be capable to detect the changes in the cytoplasmic concentration of free Ca^{2+} and to induce the cellular response.

Gene activation

Signal transduction pathways induced by cold stress lead to gene activation. One of the approaches to understand how stress-activated genes are regulated is to analyse their promoters in order to characterise the sequences (*cis*-acting elements) essential for gene activation, and to identify transcription factors which recognise and bind to these sequences. One of the best-characterised *cis*-acting element is the ABA-responsive element (ABRE), which contains the palindromic motif CACGTG with the ACGT core element or the so-called G-box. ACGT-like elements have been observed in a number of *cor* genes induced by low temperature following increase in intracellular ABA concentration. In many cases ABRE alone is not sufficient for gene activation and additional elements (coupling elements) are also required.

Other *cis*-acting elements involved in the activation of cold stress-induced, ABA-independent genes have also been identified. The drought response element (DRE, sequence: TACCGACAT) is present in the promoter of several *cor* genes that are also induced by drought stress, such as the *Arabidopsis* genes *lti78/rd29a* and *lti76/rd29b* (Yamaguchi-Shinozaki and Shinozaki, 1994). In barley the low temperature-induced expression of the *blt4.9* gene coding for a non-specific Lipid Transfer Protein (CTP), was shown to be mediated by the novel *cis*-acting element CCGAAA (Dunn *et al.*, 1998).

Post-transcriptional regulation of cold-responsive genes is also known. In barley, particularly, the low temperature responsive genes are regulated either transcriptionally or at the level of mRNA stability (Dunn *et al.*, 1994), and low temperature-dependent protein factors are involved in modulation of mRNA stability (Phillips *et al.*, 1997). The activity of the low temperature-dependent protein factors can also be modified by the presence of green chloroplasts. Indeed, at 22°C etiolated barley plants accumulate at detectable level mRNAs corresponding to the cold-regulated gene *blt14*. When the same plants are exposed to cold in absence of light an increased mRNA accumulation above the level present in green cold-treated plants can be detected (Grossi *et al.*, 1998). Several low temperature-induced RNA-binding proteins, which might stabilize or activate mRNA, have been identified in *Arabidopsis* and barley (Carpenter *et al.*, 1994; Dunn *et al.*, 1996).

BARLEY COLD-REGULATED GENES

Cold acclimation leading to increased frost tolerance requires the expression of a number of *cor* genes (Cattivelli and Bartels, 1989). In barley more than 20 cDNA clones, which expression is affected by low temperatures, have been isolated (see Table 1). The accumulation of *cor* mRNAs depends primarily on the low temperature stimulus, but can also be modulated by other factors such as application of

Table 1. Cold stress related genes isolated in barley.
1. tafla. Erfðavísar sem hafa verið einangraðir í byggi og tengjast kuldaálagi.

Gene	Stress-related expression	Observation	Reference
blt4 gene family	Expressed in leaves during cold, drought and ABA treatment *Blt4.9* is strongly expressed in epidermal cells of cold-acclimated emerging leaves	Codes for non specific lipid transfer protein, possible involved in wax synthesis or secretion. Mapped on chr. 2H	Hughes *et al.*, 1992 Dunn *et al.*, 1991 White *et al.*, 1994
blt14 gene family	Expressed during cold treatment only		Dunn *et al.*, 1990 Cattivelli and Bartels, 1990 Grossi *et al.*, 1998
Blt63	Expressed during cold treatment only	Codes for elongation factor 1α	Dunn *et al.*, 1993
blt101 gene family	Expressed during cold acclimation in the perivascular layers of the transition zone of the crown	The protein has a laeder sequence for the secretory pathway	Goddard *et al.*, 1993 Pearce *et al.*, 1998
Blt801	Expressed during cold and ABA treatment	Codes for a RNA binding protein	Dunn *et al.*, 1996
Cor14b	Expressed during cold treatment only and enhanced by light	Expressed only in leaves, encodes for a chloroplast localised protein. Mapped on chr. 2H	Cattivelli and Bartels, 1990 Crosatti *et al.*, 1995, 1999
Tmc-ap3	Constitutively expressed, enhanced by cold	Expressed only in leaves, encodes for a chloroplast localised protein. Mapped on chr. 1H	Baldi *et al.*, 1999
Dhn 5	Expressed during drought, cold and ABA treatment	Lea group 2. Mapped on chr. 6H	van Zee *et al.*, 1995
Paf93	Expressed during drought, and cold treatment	Lea group 2. Mapped on chr. 6H	Cattivelli and Bartels, 1990 Grossi *et al.*, 1995
Aba2 and *Aba3*	Expressed in leaves during cold, drought and ABA treatment	Lea group 2. Mapped on chr. 5H	Gulli *et al.*, 1995
Hva1	Expressed in seedlings during cold, salt, ABA and drought treatment (depending from developmental stage)	Lea group 3. Expressed in the aleurone during development of embryo desiccation tolerance	Hong *et al.*, 1988 Sutton *et al.*, 1992 Hong *et al.*, 1992

ABA, drought stress or light. The analysis of the expression pattern reveals that *cor* mRNAs reach their maximum levels within 2–3 days after exposure to cold. When the plants are returned from low temperatures to 20°C the level of *cor* mRNAs falls dramatically in few hours (Cattivelli and Bartels, 1990). Their expression and the accumulation of the corresponding proteins have been correlated with increased frost tolerance (Crosatti *et al.*, 1996; Pearce *et al.*, 1996).

Sequence and expression analysis of the *cor* genes allowed the identification of several gene classes. The expression of members of the *blt14* gene family is post-transcriptionally up-regulated (Dunn *et al.*, 1994) in response to cold, but not to drought or ABA. So far five *blt14*-related genes have been isolated;

they are expressed in different tissues and show both different threshold induction temperatures and genotype-dependent induction kinetics (Grossi *et al.*, 1998). *In situ* hybridisation analysis of *cor* gene expression showed that *blt14* genes (as well as another *cor* gene, *blt101*) are strongly expressed in perivascular cell layers in the vascular-transition zone of cold-acclimated barley crown, although also present in other organs and tissues (Pearce *et al.*, 1998).

A putative function can be assigned to several barley *cor* genes based on their sequence similarities. The *blt4 cor* gene family encodes non-specific LTP's. Although non-specific LTPs do not bind specifically to lipids, they are known to transfer lipids between donor and acceptor membranes *in vitro*. All members of the *blt4* gene family have a consensus signal peptide in the N-terminus for an extracellular transport suggesting a possible involvement in wax synthesis or secretion (White *et al.*, 1994). The mRNAs corresponding to *blt4.9* were strongly expressed in epidermal cells of cold-acclimated emerging leaves of barley (Pearce *et al.*, 1998), suggesting a possible role in reducing stress-induced water loss. The *cor*-gene *blt63*, is a member of a multigene family encoding the protein synthesis elongation factor-1a (Dunn *et al.*, 1993), while the *blt801* clone encodes a RNA-binding protein. BLT801 has a N-terminal amino acid stretch with a consensus RNA-binding domain and a C-terminal domain with repeated glycine residues interspersed with tyrosine and arginine (Dunn *et al.*, 1996).

Specific COR proteins have been found to be localised in the chloroplasts. The first *cor* gene isolated with such characteristics was *cor14b* (formerly *pt59* – Crosatti *et al.*, 1995). The accumulation of *cor14b* mRNA occurs only at low temperatures, but is enhanced after even brief exposure of the plant to light. The *cor14b* mRNAs are accumulated only slightly more in the more tolerant cultivars than in the less tolerant ones, although the former has a higher induction-temperature threshold of *cor14b* expression than the latter (Crosatti *et al.*, 1996). This may represent an evolutionary advantage enabling the more tolerant varieties in the field to prepare for the cold well ahead of the less tolerant ones. The expression of *cor14b* is also affected by light and light quality. Red or blue but not far-red or green light are able to promote COR14B accumulation in etiolated plants, suggesting that phytochrome and blue light photoreceptors may be involved in the control of *cor14b* gene expression (Crosatti *et al.*, 1999). The *cor* gene *tmc-ap3* encodes a putative chloroplastic amino acid selective channel protein, and is expressed at low level under normal growing temperature, although its expression is strongly enhanced after cold treatment. A positive correlation between the expression of *tmc-ap3* and frost tolerance was found among barley cultivars and among cereals species. Western blot analysis in cereals shows that the *cor tmc-ap3* gene product is localised in the chloroplastic outer envelope membrane, supporting its putative function. Notably, frost-tolerant cultivars accumulate COR TMC-AP3 protein more rapidly and at higher level than frost-sensitive ones. These results suggest that an increased amount of a chloroplastic amino acid selective channel protein could be required for cold acclimation in cereals (Baldi *et al.*, 1999).

Several genes induced by cold stress belong to the Late Embryogenesis Abundant (LEA) gene family. The LEA proteins accumulate in the embryo during desiccation; their expression is also regulated by drought stress and ABA treatment. The dehydrins represent the class 2 of the *Lea* gene family. Dehydrins are the main group of proteins induced by drought, salt stress, cold acclimation, embryo development and ABA in barley as well as in many other species. Dehydrins have highly conserved, lysine-rich stretch of 15 amino acids (EKKGIMDKIKEKLPG) known as the "K segment". In addition many dehydrins contain a stretch of serines, the "S segment", which can be phosphorylated, and a further consen-

sus amino acid sequence (-V/T-DEYGNP), the "Y segment" (Close, 1996).

The class 3 of the *Lea* genes contain sequences homologous to the cotton *D11* gene isolated from dormant seeds. Proteins of this family contain a tandem repeats motif of 11 amino acids that may form an amphiphilic α-helix structure (Dure *et al.*, 1989; Dure, 1993). In barley a single gene (*HVA1*) belonging to this class has been isolated. The *HVA1* gene is expressed in aleurone layers during embryo desiccation, although its expression is rapidly induced in young seedlings by ABA, dehydration, salt and cold (Hong *et al.*, 1992).

To investigate the genetic relationship between frost tolerance and the expression of cold-regulated genes, the expression and regulation of the wheat gene homologous to the barley cold-regulated *cor14b* gene was compared in frost sensitive and frost tolerant wheat genotypes at different temperatures. At 18/15°C (day/night temperatures) frost tolerant plants accumulated *cor14b* homologous mRNAs and expressed COR14b proteins whereas the sensitive plants did not. This indicates that the threshold induction-temperature of the wheat gene homologous to *cor14b* is higher in frost resistant plants. Studies made with chromosome substitution lines show that the threshold induction-temperature polymorphism of *cor14b* gene in wheat is controlled by locus(i) located on the chromosome 5A, while *cor14b* gene was mapped onto the long arm of the chromosome 2A. The analysis of single chromosome recombinant lines derived from the cross between Chinese Spring/*Triticum spelta* 5A and Chinese Spring/Cheyenne 5A identified two loci with additive effect involved in the genetic control of *cor14b* homologous mRNAs accumulation. The first locus was positioned tightly linked with marker *psr911*, while the second one was located between marker *Xpsr2021* and Frost resistance gene *Fr1* (Vágújfalvi *et al.*, 2000) Because of the high degree of sinteny among the *Triticeae* homeologous group 5 in the *Vrn-A1-Fr1* region (Galiba *et al.*, 1995) orthologous loci controlling the expression of *cor* genes are likely to be present in all *Triticeae* genomes.

CONCLUSIONS

Recent advances in the molecular understanding of cold tolerance have produced a number of molecular tools that will contribute to the improvement of barley adaptation to cold environments. It is generally accepted that the expression of stress related genes is an essential part of the plant adaptation processes, although a direct evidence of their function is still lacking for many genes. The use of transgenic plants, the development of new genomic tools such as expressed sequence tags databases, and the identification of mutants with altered response to cold will contribute to further understanding of the molecular bases of frost tolerance.

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