Plant Cell Physiol. 46(6): 884–891 (2005)
doi:10.1093/pcp/pci093, available online at www.pcp.oupjournals.org
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Molecular Characterization and Origin of Novel Bipartite Cold-regulated Ice Recrystallization Inhibition Proteins from Cereals

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To understand the molecular basis of freezing tolerance in plants, several low temperature-responsive genes have been identified from wheat. Among these are two genes named *TaIRI-1* and *TaIRI-2* (*Triticum aestivum* ice recrystallization inhibition) that are up-regulated during cold acclimation in freezing-tolerant species. Phytohormones involved in pathogen defense pathways (jasmonic acid and ethylene) induce the expression of one of the two genes. The encoded proteins are novel in that they have a bipartite structure that has never been reported for antifreeze proteins. Their N-terminal part shows similarity with the leucine-rich repeat-containing regions present in the receptor domain of receptor-like protein kinases, and their C-terminus is homologous to the ice-binding domain of some antifreeze proteins. The recombinant TaIRI-1 protein inhibits the growth of ice crystals, confirming its function as an ice recrystallization inhibition protein. The *TaIRI* genes were found only in the species belonging to the *Pooideae* subfamily of cereals. Comparative genomic analysis suggested that molecular evolutionary events took place in the genome of freezing-tolerant cereals to give rise to these genes with putative novel functions. These apparent adaptive DNA rearrangement events could be part of the molecular mechanisms that ensure the survival of hardy cereals in the harsh freezing environments.

Keywords: Antifreeze proteins — Freezing tolerance — Ice-binding proteins — Receptor-like kinase — Wheat.

Abbreviations: AFP, antifreeze protein; CA, cold acclimation; FT, freezing tolerance; IRI, ice recrystallization inhibition; JA, jasmonic acid; LRR, leucine-rich repeat; LT, low temperature; OsPR, *Oryza sativa* phytosulfokine receptor kinase; PR, pathogenesis-related; RI, recrystallization inhibition; RKD, receptor kinase domain; RLK, receptor-like kinase; RLP, receptor-like protein; SA, salicylic acid; TE, transposable element; TH, thermal hysteresis.

The nucleotide sequences reported in this paper have been submitted to GenBank under accession numbers AY968588 (*TaIRI-1*) and AY968589 (*TaIRI-2*).

Introduction

Plants are frequently exposed to unfavorable environmental stresses. To survive in these harsh conditions, they use a variety of avoidance and tolerance mechanisms. In the northern boreal ecosystem, overwintering cereals such as wheat, rye and barley must survive subzero temperatures and long-lasting snow cover, which exposes them to injury caused by freezing (Hiilovaara-Teijo et al. 1999). Upon exposure to cold-acclimating temperatures, plants exhibit many physiological and metabolic changes resulting primarily from changes in gene expression (Thomashow 1999). These changes are required for the plant to achieve its maximal, genetically determined freezing tolerance (FT).

Among the genes induced by low temperature (LT) in plants are several genes encoding antifreeze proteins (AFPs; Jia and Davies 2002). These proteins have an affinity for ice and possess the properties of recrystallization inhibition (RI) and thermal hysteresis (TH). The binding of AFPs restricts the ice crystal growth in the apoplast, enabling plants to survive under freezing conditions (Kuiper et al. 2001). TH activity increases the difference between the freezing and melting temperatures. Animal AFPs exhibit significant differences in the levels of TH, ranging from 1 to 2°C in fishes and from 5 to 10°C in insects (Jia and Davies 2002). In contrast, plant AFPs characteristically have low levels of TH activity (0.1–0.6°C) (Worrall et al. 1998).

It was shown that an AFP from the perennial ryegrass *Lolium perenne* has a higher RI activity and a low TH activity compared with other AFPs (Pudney et al. 2003). In carrot, an LT-up-regulated AFP shows a significant similarity (50–65%) to the polygalacturonase inhibitor family of plant leucine-rich repeat (LRR) proteins, and its overexpression in *Arabidopsis* results in the accumulation of an apoplastic antifreeze activity (Meyer et al. 1999). In winter rye, six AFPs ranging in size from 15 to 35 kDa have been identified from the apoplastic fraction. These AFPs are similar to pathogenesis-related proteins that are normally secreted in response to pathogen infection (Yeh et al. 2000, Yu and Griffith 2001). Such infections release chemical signals that are perceived at the plant cell surface by receptors, among which is a large class of proteins called receptor-like kinases (RLKs). The function of RLKs can be divided into two broad categories. The first includes proteins involved in the control of plant growth and development under normal growth conditions, and the second category includes proteins involved in plant–microbe interactions and stress response (Becraft 2002). RLKs are a class of trans-

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membrane kinases, composed of an extracellular ligand-binding domain linked to a cytoplasmic domain that has intrinsic kinase activity. They are activated by the binding of a ligand to the extracellular domain (Becraft 2002). Kinase domains are relatively conserved among members of RLK families, whereas extracellular domains can be highly diverse. This suggests that different protein domains have been recruited to function as extracellular domains in different families.

Using various approaches, several LT-regulated genes from wheat have been identified. With the aim of understanding their contribution to the enhancement of FT, genes are selected for further characterization based on their sequence homology to known proteins, particularly those involved in the structural adjustment to LT growth and in the LT signal transduction pathway. Among the genes selected are two novel, putative dual function genes which were named *TaIRI* (*Triticum aestivum* ice recrystallization inhibition). The encoded bipartite proteins share homology with RLKs in their N-terminal part and with AFPs in their C-terminal part. The function and evolutionary origin of these proteins in cold-tolerant cereals are discussed.

Results

TaIRI expression is associated with increased FT in various cereals

The *TaIRI-1* and *TaIRI-2* genes were isolated from a cold-acclimated wheat cDNA library. The results in Fig. 1A show that the *TaIRI-1* transcript begins to accumulate following exposure to 4°C and reaches its maximum level after 36 d. A longer exposure to LT does not result in a higher accumulation (data not shown). The *TaIRI-2* transcript also accumulates as soon as LT treatment begins but peaks earlier during the acclimation period. After deacclimation, the transcript levels return to that of non-acclimated control plants. The *TaIRI-1* transcript is found in leaves, crown and roots, whereas *TaIRI-2* strictly accumulates in leaves (Fig. 1B). The accumulation of *TaIRI* transcripts is associated with the FT level of different wheat cultivars (Fig. 1C). The accumulation is greater in winter hardy species such as wheat compared with the less hardy cereal species oat and barley, and there is no detectable accumulation in LT-sensitive species such as rice and corn (data not shown). Other treatments such as salt stress, water stress, exogenous ABA, heat shock and wounding have no detectable effect on *TaIRI* transcript accumulation.

The wheat cold-inducible TaIRI genes encode bipartite ice recrystallization inhibition proteins

Sequencing of the full-length *TaIRI-1* and *TaIRI-2* cDNAs showed that they encode putative proteins of 280 and 409 amino acids with calculated molecular masses of 29 and 43 kDa, respectively (Fig. 2A; accession nos AY968588 and AY968589). Analysis of the TaIRI protein sequences using the PSORT, SignalP and TargetP programs (Nielsen et al. 1997, Nakai and Horton 1999, Emanuelsson et al. 2000) predicted a putative N-terminal cleavage site between amino acids 20 and 21. This suggests the presence of a signal peptide that would target the proteins to the extracellular space or to the membrane via the secretory pathway (Fig. 2A, boxed sequences). The mature, cleaved TaIRI-1 and TaIRI-2 proteins have calculated molecu-

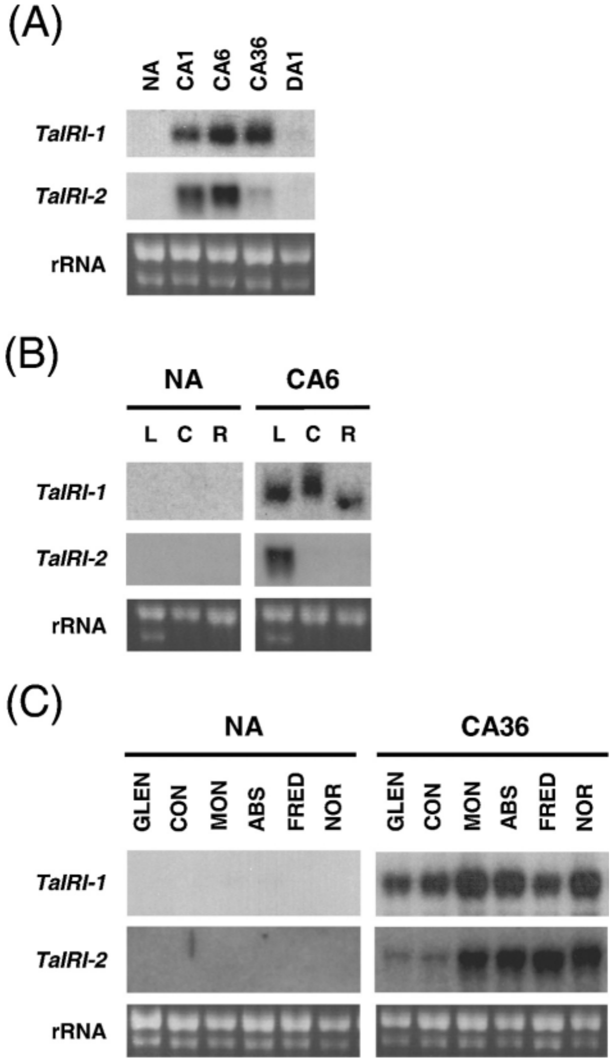

Fig. 1 The *TaIRI* transcripts accumulate during cold acclimation in wheat. (A) Expression in winter wheat cv Norstar. NA, non-acclimated control plants; CA1, CA6 and CA36, plants cold acclimated for 1, 6 and 36 d; DA1, 36-d cold-acclimated plants were de-acclimated for 1 d. (B) Expression in various tissues of wheat cv Norstar. NA, non-acclimated control plants; CA6, plants cold acclimated for 6 d; L, leaves; C, crown; R, roots. (C) Expression in different wheat genotypes. Due to the low abundance of the *TaIRI-2* transcript at 36 d of cold acclimation, the exposure time was increased. GLEN and CON, spring wheats cv Glenlea and cv Concorde; MON, ABS, FRED and NOR, winter wheats cv Monopole, cv Absolvent, cv Fredrick and cv Norstar. NA, non-acclimated control plants; CA36, plants cold acclimated for 36 d. The ethidium bromide-stained rRNA is included to show equal RNA loads.

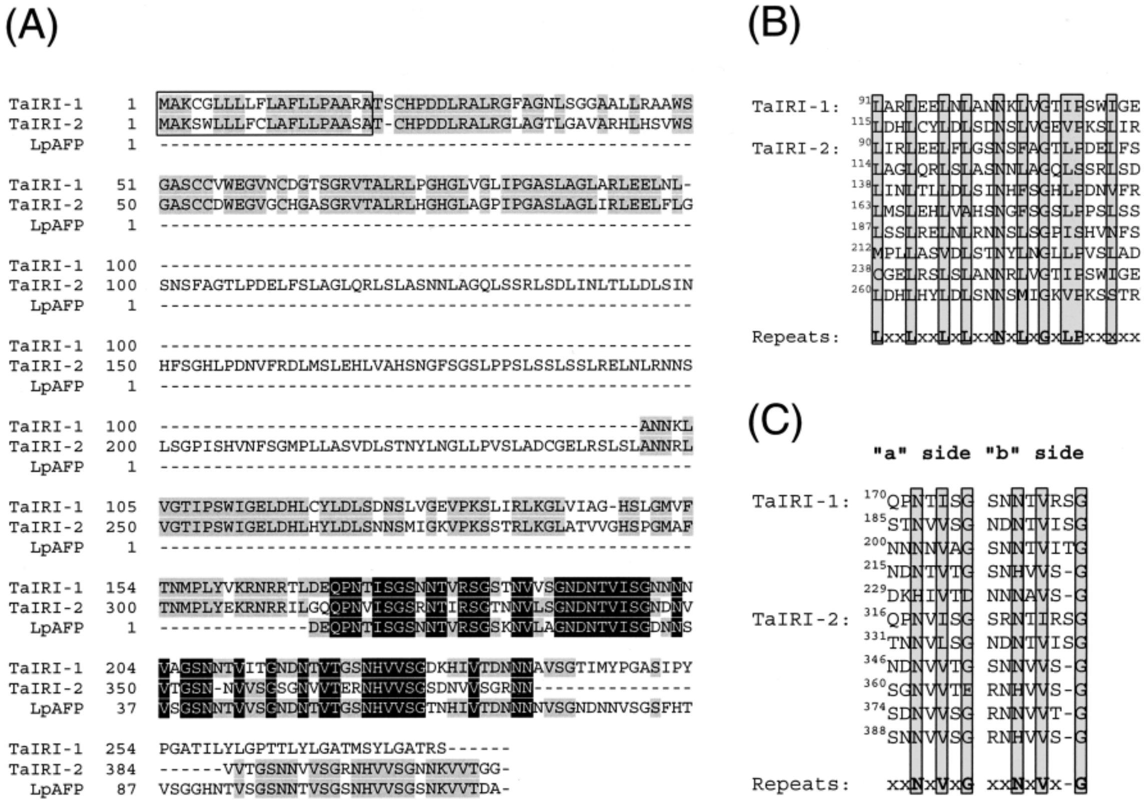

Fig. 2 The wheat *TaIRI* genes encode bipartite proteins. (A) Sequence alignment of the wheat TaIRI proteins with the partial LpAFP, a ryegrass (*Lolium perenne*) antifreeze protein. The predicted signal peptide targeting the proteins to the apoplast is boxed. Residues highlited in black and gray are common to the three or to only two of the proteins, respectively. (B) Alignment of the LRR (leucine-rich repeat) domains of the wheat TaIRI proteins. The highly conserved residues are boxed. (C) Alignment of the ice RI (recrystallization inhibition) domains of the wheat TaIRI proteins. The 'a' side and 'b' side consensus repeated sequences follow the representation suggested by Kuiper et al. (2001) for the ice-binding surfaces of LpAFP. The highly conserved residues are boxed.

lar masses of 26.8 and 40.7 kDa, and share 40.4% identity at the amino acid level. If we do not consider the region between amino acids 99 and 244 of TaIRI-2, which is absent in TaIRI-1, the identity is 74.7 and 54.1% at the 5′ and 3′ ends, respectively. Apart from the signal peptide, the TaIRI proteins possess two distinct domains in a combination not previously reported for AFPs: an LRR-containing domain and an RI domain.

Inter proScan and SMART predicted that TaIRI-1 and TaIRI-2 contain two and eight putative LRR motifs, respectively, located in the N-terminal portion that is homologous to the receptor kinase domain (RKD) of RLKs (Fig. 2B) (Schultz et al. 2000, Mulder et al. 2003). The highest similarity of TaIRI proteins to RLKs is found with a putative *Oryza sativa* phytosulfokine receptor kinase (OsPSR) (accession No. BAC20742), an LRR transmembrane protein kinase and an RLK from *Arabidopsis thaliana* (accession No. NP_200200, BAB10719.1), the putative protein kinase Xa21 from rice (accession No. BAB03621.1), a disease resistance protein family from *A. thaliana* (accession No. NP188952), and putative Cf2/Cf5 disease resistance proteins from rice (accession no. BAB89968).

The RI domain, located at the C-terminus, shows homology with the ice-binding region of the ice recrystallization inhibition (IRI) protein LpAFP (accession no. CAB87814.1) (Fig. 2A, C). The identity between the partial LpAFP and TaIRI-1 (>113 amino acids) is 58.5%, whereas it is 51.7% with TaIRI-2 (>91 amino acids). Since the complete sequence of the N-terminal part of LpAFP was not determined, it is not possible to know if the similarity with the TaIRI proteins extends beyond the RI domain. TaIRI-1, TaIRI-2 and LpAFP possess five, six and eight repeats, respectively, of the xxNxVxG ('a' side) and xxNxVx – G ('b' side) motifs pair forming active ice-binding sites (Fig. 2C). Based on the presence of these motifs, the full-length TaIRI-1 protein and the RKD polypeptide were expressed as His-tagged fusion proteins in *Escherichia coli* (Fig. 3A) and tested for their ability to inhibit ice crystal growth (Fig. 3B). Expression of the soluble recombinant RI domain proved unsuccessful. Fig. 3B shows that bacterial proteins used as a negative control did not prevent the growth of ice crystals. A similar result was obtained with 30% (w/v) sucrose solution (not shown). In contrast, a soluble protein extract from cold-acclimated wheat leaves (positive control), and the recombinant TaIRI-1 protein and RKD polypeptide, clearly demonstrate an inhibition of ice crystal growth.

Because of the homology with RLKs, the affinity-purified recombinant TaIRI-1 protein was tested for kinase activity. Casein kinase II, used as a positive control, transferred 0.062 and 0.021 pmol of phosphate min^{-1} to casein and histone, respectively. However, the recombinant TaIRI-1 protein or the negative control did not show any activity. This indicates that under the conditions used, TaIRI-1 does not autophosphorylate and does not have kinase activity on two commonly used substrates. This result was expected since the homology of TaIRI with RLKs is restricted to the receptor domain of the latter, and not to the kinase domain.

Effect of plant signaling molecules and pathogen infection on TaIRI expression

The sequence homology of TaIRI proteins with proteins involved in disease resistance prompted us to determine the effect of jasmonic acid (JA), ethylene, ABA and salicylic acid (SA) on *TaIRI* gene expression. Results in Fig. 4A indicate that JA and ethylene induce *TaIRI-1*, but not *TaIRI-2*, transcript accumulation in winter wheat cv Norstar. No induction of either *TaIRI* transcript was seen after ABA or SA treatment (data not shown). Since JA and ethylene are hormones known to play important roles in various plant disease pathways, we tested the effect of a biotic stress by inoculation of the plants with common wheat pathogens. Snow molds are a diverse group of parasitic fungi that specifically infect winter cereals under the snow cover. Pink snow mold (*Microdochium nivale*) inoculation does not induce the accumulation of *TaIRI* transcripts (Fig. 4B). In the case of *TaIRI-1*, the transcript accumulation after 42 d of cold acclimation (CA) is so high that it may have masked a possible induction by the pathogen treatment. However, in the case of *TaIRI-2*, the induction by LT is quite low at 42 d CA (see Fig. 1A), therefore allowing for the possible detection of a slight accumulation of the transcript by snow mold infection. Since no signal is observed in the pathogen-challenged tissues, we conclude that *M. nivale* does not induce *TaIRI* expression. Inoculation of wheat with pathogens causing leaf rust (*Puccinia triticina*) and head blight (*Fusarium graminarium*), under non-acclimated conditions, does not induce *TaIRI* transcript accumulation (data not shown). Taken together, these data indicate that biotic stresses do not induce *TaIRI* gene expression under our experimental conditions.

Evolutionary origin of the TaIRI genes

The bipartite structure of TaIRI proteins raises the question as to how these genes have arisen during the course of evolution. As mentioned previously, the LRR-containing region of TaIRI proteins shows the highest similarity with OsPSR, a protein that binds the plant growth factor phytosulfokine (Fig.

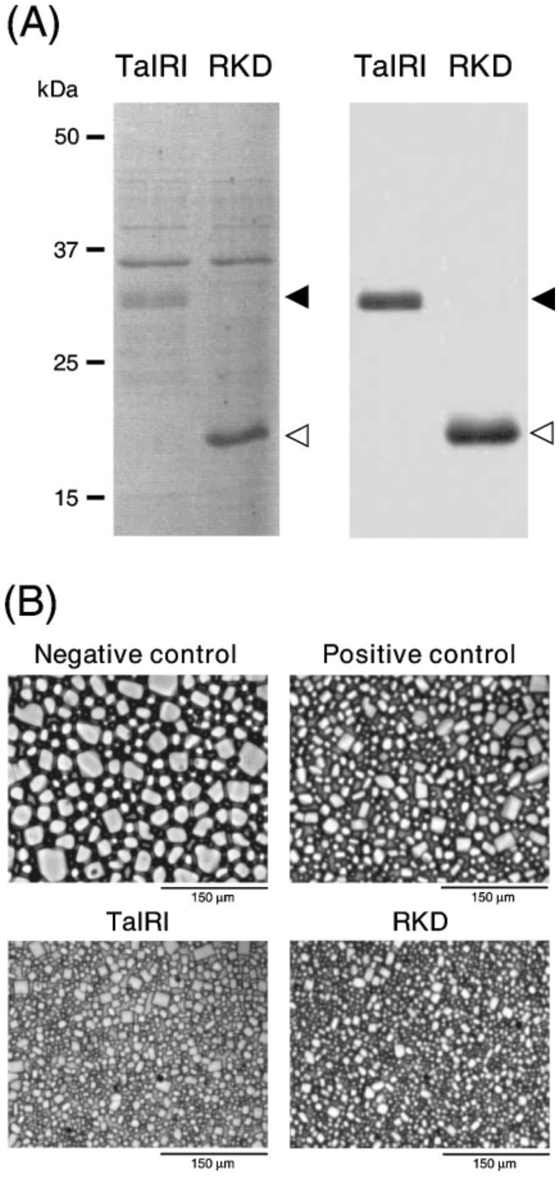

Fig. 3 The recombinant TaIRI-1 protein and RKD polypeptide possess ice recrystallization inhibition activity. (A) SDS–PAGE analysis of bacterial extracts. Recombinant His-tagged proteins were expressed by isopropyl-β-D-thiogalactopyranoside (IPTG) induction of the *E. coli* strains and soluble proteins were extracted. The left panel shows the Coomassie brilliant blue-stained gel and the right panel shows the Western blot obtained with the anti-His antibody. Black arrowhead, His::TaIRI protein; white arrowhead, His::RKD polypeptide. (B) Recrystallization inhibition assays. Protein extracts were added to a sucrose solution and microscopy images were captured over an extended period at freezing temperatures to monitor the effect of the proteins on the growth of ice crystals. Images captured on day 10 are shown. Negative control, proteins extracted from *E. coli* transformed with the non-recombinant plasmid (average crystal size 145.02 µm^2 ± 4.93); positive control, soluble cold-acclimated wheat protein extract (average crystal size 50.77 µm^2 ± 1.52); TaIRI-1, recombinant His:TaIRI-1 protein purified from *E. coli* (average crystal size 40.26 µm^2 ± 3.73); RKD, recombinant His::RKD polypeptide purified from *E. coli* (average crystal size 32.06 µm^2 ± 4.33).

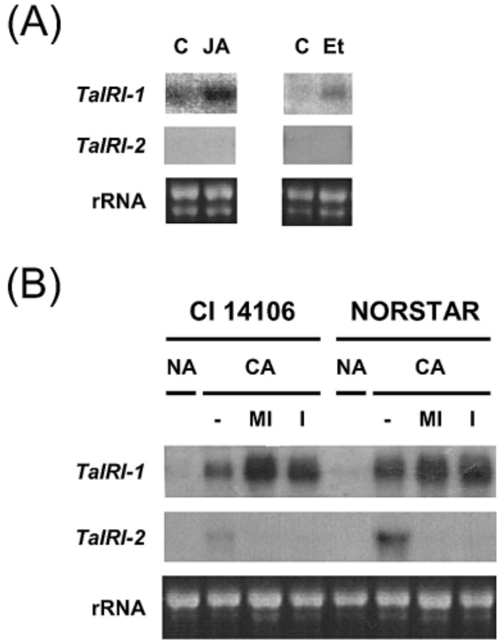

Fig. 4 Accumulation of *TaIRI* transcripts in response to hormone treatments and pathogen infection. (A) Winter wheat cv Norstar was used. C, control plants; JA, jasmonic acid-treated plants; Et, plants treated with ethephon to simulate ethylene treatment. (B) Winter wheats cv CI14106 (snow mold resistant) and cv Norstar (snow mold sensitive) were used. NA, non-acclimated plants; CA, cold-acclimated plants; –, plants collected after 21 d of CA; MI, 21 d cold-acclimated plants were mock-inoculated and cold acclimated for an additional 21 days; I, 21 d cold-acclimated plants were inoculated with snow mold LTB #013 and cold acclimated for an additional 21 d.

5A). A careful sequence homology analysis of the 3′-untranslated region of both *TaIRI* transcripts revealed that they possess short sequences that would encode peptides homologous to OsPSR (Fig. 5B). When the stop codon of the *TaIRI-1* nucleotide sequence is removed and a frameshift is introduced, the amino acid sequence TaIRI-1a is obtained. For TaIRI-2a, nucleotides were substituted to remove two consecutive stop codons at the end of the coding region of *TaIRI-2*. The high similarity of the portions of TaIRI flanking the RI domains to OsPSR suggests that the DNA region encoding the RI domain has been inserted in the coding region of a wheat ortholog of *OsPSR* (Fig. 5C).

Discussion

Two related genes encoding IRI proteins from wheat were identified and characterized. The expression data indicate that the accumulation of *TaIRI* transcripts is associated with the capacity of cereal species to cold acclimate and develop FT. TaIRI proteins share homology with two subsets of proteins: their N-terminal part has similarity with the LRR-containing regions present in the receptor domain of RLKs, while their C-terminus is homologous to the RI domain of AFPs. The up-regulation of *TaIRI* gene expression by LT, the homology of the TaIRI proteins to a known AFP and the RI activity strongly suggest a role for these proteins in CA and/or FT.

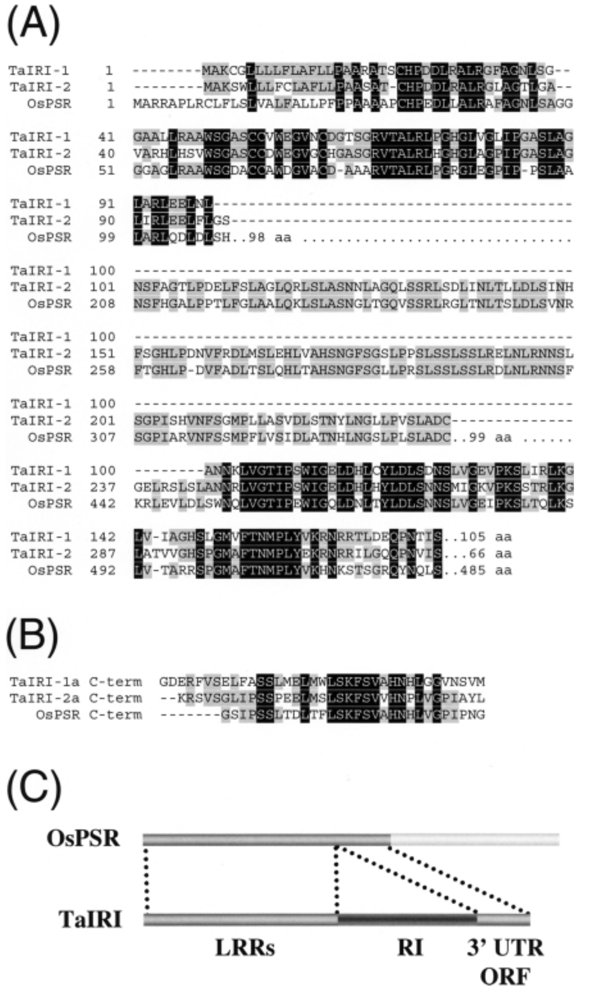

Fig. 5 Evolutionary origin of the wheat *TaIRI* genes. (A) The N-terminal LRR-containing region of TaIRI proteins is highly homologous to OsPSR, a putative phytosulfokine receptor kinase from rice. The sequences are shown for the homologous regions only to simplify the alignment. The length (...x aa...) of the hidden portions is indicated. (B) Open reading frames predicted for the 3′-untranslated regions of *TaIRI*s encode a short region highly homologous to OsPSR. Since these peptides do not exist in the TaIRI-1 and TaIRI-2 native proteins, the names were changed to TaIRI-1a and TaIRI-2a. The portion showing high similarity is shown. (C) Schematic representation of the RI domain insertion in OsPSR. The *TaIRI* genes may have evolved from the insertion of an ice-binding domain in a wheat ortholog of OsPSR. Residues in black and gray are common to the three or to only two of the hypothetical proteins, respectively.

The relatively short LRR motifs (22–28 amino acids) can be found up to 35 times in a variety of cytoplasmic, membrane and extracellular proteins, and it is widely accepted that they are involved in protein–protein interactions (Meyer et al. 1999, Becraft 2002). Preliminary analysis using the yeast two-hybrid assay revealed that the TaIRI-1 protein, its RKD and its RI domain do not show any homotypic interaction capacity. This result does not rule out, however, that the protein could have the capacity to heterodimerize. The homology with the receptor domain of RLKs and the lack of kinase activity suggest that TaIRI proteins could be receptor-like proteins (RLPs), a subclass of RLKs. Some RLPs are hypothesized to be secreted proteins, whereas others are membrane bound (Shiu and Bleecker 2001). The *Arabidopsis* CLV2 protein is an RLP with a predicted LRR-containing ectodomain and a transmembrane domain, but no cytoplasmic kinase domain (Jeong et al. 1999). It is unknown if or how LRR-containing RLPs can transmit signals across the plasma membrane. Studies on CLV2 showed that heterodimer formation with CLV1 provides a possible mechanism by which this class of RLPs may function as signaling components. Heterodimerization between an RLP and an RLK could form a functional complex with both a receptor domain and a cytoplasmic signaling domain (Jeong et al. 1999). We hypothesize that TaIRI proteins could, in a similar fashion, heterodimerize with another protein containing a kinase domain to form a functional transduction unit.

The presence of LRR motifs in TaIRI-1 and TaIRI-2 suggested a possible role in pathogen resistance. In winter rye, AFPs that belong to the glucanase-like, chitinase-like and thaumatin-like proteins are also known as pathogenesis-related (PRs) proteins (Yu and Griffith 1999, Yu and Griffith 2001). Resistance to both freezing and pathogens is a desirable trait for overwintering cereals because the combined detrimental effects of these stresses may decrease plant survival, leading to decreases in yield (Hiilovaara-Teijo et al. 1999). Although plants growing under stressful conditions often become more susceptible to fungal diseases, some plants exhibit a phenomenon of cross-adaptation whereby exposure to one stress provides tolerance to others. For example, perennial grasses become more resistant to fungal diseases such as snow molds, powdery mildews, leaf spots and leaf rust once the plants have undergone acclimation to LT (Tronsmo et al. 1993, Hiilovaara-Teijo et al. 1999). One interpretation of this result is that metabolic responses of overwintering plants to LT pathogens may be too slow at subzero temperatures to provide an adequate defense. In the present work, the inoculation of wheat by snow mold (*M. nivale*), head blight (*F. graminarium*) and leaf rust (*P. triticina*) did not result in a higher accumulation of the *TaIRI* transcripts in LT-treated plants. We hypothesize that TaIRI proteins may have lost their function in pathogen resistance because of the reduction of the number of LRR motifs as a result of the insertion of an RI domain in the LRR-containing ancestral gene.

The C-terminal part of TaIRI is homologous to LpAFP, a partial gene coding for an AFP from the rye grass *L. perenne*. The encoded, incomplete 118 residue LpAFP has an IRI activity higher than that of other AFPs (Sidebottom et al. 2000). Its primary structure shows a series of highly conserved repeated motifs with regularly spaced serine and threonine residues that may form hydrogen bonds with the ice surface (Sidebottom et al. 2000). Kuiper et al. (2001) have suggested a model that predicts two ice-binding sites, one on either side of the LpAFP molecule, with the xxNxVxG ('a' side) and xxNxVx – G ('b' side) consensus motifs. This unusual duplication of putative ice-binding sites on opposite sides of the protein could be responsible for the high RI activity of LpAFP and possibly of TaIRIs. Homology search with the RI domain (113 C-terminal amino acids) showed that this region exists only in four species that are known to be cold-tolerant cereals from the *Pooideae* subfamily of the *Poaceae* (*Gramineae*) family: wheat, *L. perenne*, rye and barley. Thus, it is likely that *TaIRI* genes may have arisen from the insertion of an ice-binding domain in a gene encoding an RLK protein. Because *TaIRI* genes have not been identified in rice so far, the insertional event must have taken place after the divergence of the *Pooideae* (comprising wheat, a cold-tolerant cereal) and *Pharoideae* (comprising rice, a cold-sensitive cereal) subfamilies of the *Poaceae* family.

The fact that the protein produced in *E. coli* possesses an RI activity, and knowing that this prokaryote does not allow for protein glycosylation, is in line with the finding of Pudney et al. (2003) that this post-translational modification plays no role in the interaction with ice. Structure analysis of several AFPs revealed that they represent a diverse array of proteins (Griffith and Yaish 2004). The existing evidence suggests that AFPs have evolved independently in a wide range of cold- and freezing-tolerant organisms in their natural habitats (Griffith et al. 1997). Some AFPs like DcAFP possess antifreeze activity but do not have an RI domain with the a–b motifs pairs (Worrall et al. 1998, Meyer et al. 1999, Smallwood et al. 1999). The fact that repeats of the very small a and b motifs like the ones present in LpAFP and TaIRIs is sufficient for ice-binding activity may offer an explanation for the evolution of those repeats in AFPs. It was suggested that continuing selection would lead to the appearance of AFPs containing additional copies of this short repeat (Ewart et al. 1999). Comparison of RI activity between TaIRIs and other AFPs under the same experimental conditions remains to be determined. However, the presence of multiple ice-binding domains in TaIRIs and the fact that the RKD also possesses RI activity suggest that TaIRI proteins could prove to be potent AFPs.

Insertion of transposable elements (TEs) could explain the peculiar structure of the *TaIRI* genes from an evolutionary perspective. It is possible that TE-induced gene alterations have led to the emergence of a novel PR protein containing an RI domain, or conversely a novel IRI protein containing LRRs. Since TE insertion/excision leaves a footprint at the genome level, we analyzed the *TaIRI* genes for such sequences using the Triticeae Repeat Sequence Database (http://wheat.pw.usda.gov/ggpages/ITMI/Repeats/index.shtml). This analysis revealed the presence of a dozen signature sequences distributed in the N-terminal portion homologous to RLPs and in the 3′-untranslated region. Interestingly, no TE footprint is found within the RI domain itself, suggesting that the flanking TE signatures may indicate the integration sites of the RI domain.

In support of the TE hypothesis, it was suggested that insertion of TEs at the *Hm1*, *L6* and *Xa21* loci may result in a loss of function or impaired function of the encoded proteins (Ronald 1998). It has been suggested that, in yeast, genetic variation is higher and natural selection is more intense when environmental conditions become less favorable (Szafraniec et al. 2001). It could be speculated that tolerant plants arise from the acquisition of new genetic information through TE insertion or natural mutations that occur during the process of adaptation to cold environments. The appearance of genes such as *TaIRI* in the cold-tolerant cereals supports this hypothesis. TE insertion in the regulatory or coding regions of *TaIRI* could have changed their expression pattern or their encoded products, hence the function of the encoded proteins. This hypothesis has some merit in that it is supported by observations, described mainly in microorganisms, showing that selective environments (such as those with limited nutrients) cause increases in double strand breaks and mutation rate (Dong 2004).

The availability of additional genome sequences and cross-species comparisons will help to determine the origin of the *TaIRI* genes. During the course of evolution, genomes are subjected to many modifications that give rise to new genes and gene families. Comparing various human sequences with those of primates has revealed events that have sculpted our genome throughout evolution (Thomas et al. 2003), and it is likely that a similar phenomenon has occurred in plants. It is not known when or how the FT cereals have acquired the *TaIRI* genes. However, these genes could represent valuable tools to study the molecular evolutionary basis of antifreeze proteins in plants.

Materials and Methods

Plant materials and growth conditions

In this study, we used spring wheat genotypes [*T. aestivum* L. cv Glenlea, LT$_{50}$ (lethal temperature that kills 50% of the seedlings) −8°C; cv Concorde, LT$_{50}$ −8°C and cv Quantum, LT$_{50}$ −8°C], and winter wheat genotypes (*T. aestivum* L. cv Monopole, LT$_{50}$ −15°C; cv Fredrick, LT$_{50}$ −16°C; cv Absolvent, LT$_{50}$ −16°C and cv Norstar, LT$_{50}$ −19°C).

Wheat plants were grown and treated as previously described (Danyluk et al. 1998). Briefly, control plants were grown at 20°C, CA was performed at 4°C, and deacclimation was performed by returning cold-acclimated plants to 20°C. For hormone treatments, 6-day-old Norstar seedlings were sprayed daily for 3 d with 50 μM JA or 40 μM SA prepared in 0.025% ethanol and 0.005% Tween-20. ABA treatment was performed by watering the plants with a nutrient solution containing $10^{-4\,\mathrm{M}}$ ABA with concomitant foliar spraying with $10^{-4\,\mathrm{M}}$ ABA prepared in 0.02% Tween-20 for 18 h. Control plants were sprayed with 0.025% ethanol and 0.005% Tween-20 (JA and SA), or with 0.02% Tween-20 alone (ABA). For ethylene treatment, Norstar leaves were sprayed daily with 10 mM ethephon dissolved in water and 0.005% Tween-20 at the end of the day. Since ethephon splits up into ethylene, HCl and H_3PO_4 in the plant, control plants were sprayed daily for 3 d with 2 mM HCl, 2 mM H_3PO_4 and 0.005% Tween-20 (Griffith et al. 1997). For pathogen infection, crowns of Norstar and CI14106 wheat cold-acclimated for 3 weeks were mock-inoculated or inoculated with snow mold LTB #013 and grown for an additional 3 weeks under CA conditions.

Molecular analyses

Clones were randomly picked from a cold-acclimated Norstar wheat cDNA library (Houde et al. 1992) and submitted to virtual subtraction screening to identify genes expressed at low levels. Briefly, the clones were screened with digoxigenin-labeled cDNA probes (Roche) from control and cold-acclimated Norstar wheat. Clones showing no or low hybridization signal with both probes were sequenced to identify those with potential functions in cell signaling. Selected clones were analyzed by Northern blot to identify those regulated by abiotic stresses. Two of these clones named *TaIRI-1* and *TaIRI-2*, for *T. aestivum* ice recrystallization inhibition, were analyzed to determine homology to known genes (Altschul et al. 1990). Protein sequence analyses were carried out with programs available on the ExPASy Molecular Biology Server (http://ca.expasy.org). For Northern analyses, total RNA was isolated using Tri-reagent (Molecular Research Center) and analyzed by electrophoresis on formaldehyde–agarose gels, transfer to nitrocellulose membranes and hybridization with the ^{32}P-labeled *TaIRI* cDNA inserts. Washes were performed at the stringency required to prevent cross-hybridization of the probes.

Biochemical analyses

For protein kinase and IRI activity assays, the *TaIRI-1* open reading frame (amino acids 1–280) and RKD (amino acids 1–170) were expressed in *E. coli* as (His)$_6$ tag fusions using standard methods (pTrcHis vector; Invitrogen). The recombinant His-tagged TaIRI-1 protein and RKD polypeptide were purified by affinity chromatography (Novagen). Bacterial proteins extracted from non-transformed *E. coli* cells were used as a negative control in the assays. For autophosphorylation experiments, 5 μg of affinity-purified TaIRI-1 recombinant protein was incubated with [γ-^{32}P]ATP in kinase buffer [50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM dithiothreitol (DTT) and 10 μM ATP] in a 200 μl total volume, for 15 min at 30°C. For transphosphorylation experiments, histone and casein (Sigma) were used as substrates. Reactions were stopped with 600 μl of 25% trichloroacetic acid (TCA) and filtered on glass microfiber filters (Whatman). Filters were washed twice with 3 ml of 5% TCA and twice with 5% methanol, dried and the incorporated label was determined using a liquid scintillation counter. Casein kinase II (Sigma) was used as a positive control. Bacterial proteins extracted from non-transformed *E. coli* cells were used as a negative control.

Antifreeze activity assays were kindly performed by Ice Biotech Inc. (Flamborough, Ontario, Cananda) as described (Pudney et al. 2003). Desalted recombinant protein samples were spun to precipitate the insoluble materials, then diluted with 40% sucrose to a final concentration of 20% (w/v) The sample droplets (5.5 μl; 3 μg/μl) were applied to clean microscope slides and covered with coverslips avoiding the trapping of air bubbles. The slides were frozen in liquid nitrogen and laid flat in the freezer. Microscopy images of the crystals' growth were captured over a period of at least 14 d after samples were frozen. A positive control with known IRI activity, consisting of total soluble proteins from cold-acclimated wheat leaves, and a negative control containing bacterial proteins were also tested.

Acknowledgments

We thank Sylvie Cloutier (Agriculture and Agri-Food Canada, Winnipeg MB, Canada), André Laroche (Agriculture and Agri-Food Canada, Lethbridge AB, Canada) and Thérèse Ouellet (Agriculture and Agri-Food Canada, Ottawa ON, Canada) for pathogen-challenged wheat materials. We also thank Ice Biotech Inc. (Flamborough ON, Canada) for the RI activity assays. This work was supported by the Natural Sciences and Engineering Research Council of Canada and by Genome Canada/Genome Quebec.

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(Received October 13, 2004; Accepted March 21, 2005)