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Presence and organization of an osmotic stress response domain in wild *Triticeae* species

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Abstract Sixteen *Triticeae* species of the genera *Aegilops* L., *Pseudoroegneria* (Nevski) Löve, *Taeniatherum* Nevski and *Thinopyrum* Löve were investigated by PCR amplification for the presence of a wheat germin gene internal domain involved in osmotic stress resistance. In all of the species studied a single band of identical or very similar size was detected, After cloning and sequencing of these fragments, different degrees of homology were found with the original wheat domain, which suggested that in these species there are functional differences in the osmotic response involving the germin core.

Key words *Triticeae* · Osmotic stress · PCR · DNA sequence

Introduction

Many wild relatives of the cultivated *Triticeae* possess interesting traits that can be successfully transferred to cultivars through modern plant breeding techniques. The genera *Aegilops*, *Pseudoroegneria*, *Taeniatherum* and *Thinopyrum* used in this study are highly interesting potential donors of a wide range of traits that have already been studied. Although many traits affecting the agronomic yield of cultivars have been successfully enhanced, others such as environmental stress resistance have remained difficult to alter. This is mainly due to the complexity of the genetic mechanisms associated with these responses in which many genes acting in signal transduction cascade patterns are probably involved. Therefore, if genetic improvement of these traits is to be possible, a better understanding is required of how these responses occur.

Germins are a family of homopentameric proteins in wheat that are encoded by a multigene family expressed during the cellular hydration of embryos during germination (Lane 1985; Lane et al. 1991). Both germin mRNAs and germins themselves accumulate during the "water growth" step. This suggests that germins play a preponderant or even leading role in germination. Hurkman et al. (1990) identified as a germin a protein implicated in the osmotic stress response of salt-resistant barley cultivars. Lane et al. (1991) proposed that germins may alter the properties of the cell wall during cell growth and that their expression could, in some cases, be induced by auxin-mediated signals since auxin-response elements have been detected in the 5′-flanking regions of some germins. These authors isolated two germin clones and found both to be 224 amino acids long. The mature proteins also showed about 90% similarity. They also found a completely conserved internal core of 91 amino acids that showed about 50% similarity to the corresponding region of spherulins, proteins that accumulate during spherulation of the *Physarum polycephalum* plasmodium. Spherulation is a process of encystment, desiccation and developmental arrest that can be induced by a wide variety of environmental stress (Lane et al. 1991). Therefore, it seems likely that germinative hydration, spherulation and osmotic stress responses of crops, all of which affect the structure of the cell wall, may share similar molecular mechanisms associated with the internal, highly conserved germin cores.

When gene sequence information is available, the combination of polymerase chain reaction (PCR) and automatic DNA sequencing is a powerful tool that may be used for isolating and characterizing homologous genes, or particular gene regions, in a short time. In the study presented here, the information provided by the wheat and *Physarium* DNA sequence of the germin genes were used to analyze wild *Triticeae* species of

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Aegilops, *Pseudoroegneria*, *Taeniatherum* and *Thinopyrum* in order to evaluate the distribution and variation of the internal germin core and to search for possible alterations that might suggest qualitative and/or quantitative differences in the osmotic stress responses of wild *Triticeae*.

Materials and methods

Plant material

Sixteen species representing four genera and eight genomes of the *Triticeae* tribe were used in this study (Table 1). Seeds from 15–30 individuals per population and up to ten populations per species were germinated in darkness and grown in greenhouses. The material was provided by the germplasm collection of the Plant Breeding Department, Instituto Nacional de Investigaciones Agrarias (I.N.I.A.).

DNA extraction and PCR amplification

Fresh, young leaves from the individuals and populations of each species were mixed for DNA extraction following the method of Dellaporta et al. (1983). The oligonucleotides 5′-TTCTCGTCCAAGTTGGCC-3′ and 5′-CTGGCTGTTGAAGGAGAC-3′ flanking the conserved internal germin core in wheat an *Physarium* were designed and synthesized for PCR amplification. PCR assays were carried out in a PTC-100 MJ Research thermocycler using the Bio Taq polymerase of Bioprobe Systems. The PCR conditions for a final reaction volume of 60 ul of 1 × Bio Taq buffer were: genomic DNA 200 ng, primers 100 ng each, dNTPs 1 m*M* each, MgCl 2.5 m*M*, 2.5 U Bio Taq.

DNA purification, cloning and sequencing

Amplified DNA fragments were separated by electrophoresis on 1% agarose gels, and phenol-extracted from gel slides with vortex agitation, exposure overnight at − 80°C, phenol/chloroform cleaning and ethanol precipitation. Purified fragments were cloned into the PCR™II vector included in the TA Cloning Kit (Invitrogen) following the TA cloning procedure. The *E. coli* strand INVaF′ was transformed, and recombinant clones were selected on LB-agar plates containing ampicillin. Selected clones were grown in LB media containing ampicillin for 18 h at 37°C with agitation. Recombinant plasmids were recovered with the Kristal Plasmid Miniprep Kit of Cambridge Molecular Technologies. Eight clones were sequenced per species, and bidirectional sequencing was achieved using a Perkin-Elmer ABI PRISM 377 DNA Sequencer employing the ABI PRISM™Dye Terminator Cycle Sequencing Kit protocol with ampliTaq Polymerase FS. Eight PCR clones were sequenced per species.

Sequence analysis

DNA sequences were aligned with the PILEUP program of the GCG (Genetic Computer Group) software package (Deveroux et al. 1984) using a DEC-Alpha computer. A gap weight of 5.0 and gap length of 0.3 were used. The programs TRANSLATE and MOTIFS of the GCG were used to search for conserved protein motifs in sequence data bases. The program DNASTAR megalink was used for further statistical comparison between sequences.

Results and discussion

In the PCR amplification assays, a single, discrete band of about 350 base pairs (bp) in length was detected for all species tested. This indicates that the positions of the two primers are highly conserved in the *Triticeae* tribe. This observation is in agreement with the important physiological role that some authors have attributed to germins (Hurkman et al. 1990; Lane et al. 1991). After sequencing for eight clones per species, a single sequence was found. This result suggests that the primer positions are not conserved in other genes of the germin family. The amplified fragments showed variable sizes ranging from 317 to 357 bp. When the sequences

Table 1 The 16 *Triticeae* species used in this study

Species	Genomic constitution[a]	Growth habit
Aegilops caudata L.	CC	Annual
Aegilops tauschii Coss.	DD	Annual
Aegilops comosa Sibth. and Sm	MM	Annual
Aegilops umbellulata Zhuck.	UU	Annual
Aegilops uniaristata Vis.	NN	Annual
Aegilops ventricosa Tausch Tausch	DDNN	Annual
Aegilops ovata L.	$UUM^{o}M^{o}$	Annual
Aegilops binucialis Vis.	$UUM^{b}M^{b}$	Annual
Aegilops triuncialis L.	UUCC	Annual
Thinopyrum bessarabicum (Savul. & Rayss) Löve	$E^{b}E^{b}$	Perennial
Thinopyrum elongatum (Hochst) Dewey	$E^{e}E^{e}$	Perennial
Thinopyrum junceum (L.) Löve	$E^{b}E^{b}E^{b}E^{b}E^{e}E^{e}$	Perennial
Thinopyrum intermedium (Hochst) Barkworth Dewey	$E^{e}E^{e}E^{e}E^{e}StSt$	Perennial
Thinopyrum ponticum (Podp.) Barkworth Dewey	$E^{e}E^{e}E^{e}E^{e}E^{e}E^{e}StSt$	Perennial
Pseudoroegneria spicata (Pursh) Löve	StSt	Perennial
Taeniatherum caput-medusae (L.) Nevski	TT	Perennial

[a] Wang et al. (1994)

were aligned, greatest homology was recorded at 362 characters (Fig. 1). Most of the amplified germin core regions showed high levels of homology (75.3–99.4% similarity) with point mutations and single deletions being the main differences observed between sequences. However, in the cases of *Thinopyrum bessarabicum* (Savul & Rayss) Löve and *Th. junceum* (L.) Löve, two extremely diverged, unrelated sequences were detected that showed complete similarity only in the primer domain regions (< 25% of the total length). The putative fragment of *Th. ponticum* (Podp.) Barkworth Dewey presented the highest level of variation within the germin-related sequences, showing 65.3–75.4% similarity with the rest of the core DNA sequences.

The (G+C) content of the germin fragments varied from 60.6% to 63.3%. This relatively high (G+C) content is typical of coding regions of genes that are expressed in developing or mature cereal seeds in which the average genomic (G+C) content is normally higher (Salinas et al. 1988). In addition, after translation (Fig. 2) the peptide codon region showed a strong (G+C) preference for the third position (> 70%), something commonly found in monocots (Murray et al. 1989). As expected, the transition-transversion rates (ns/nv) from pairwise comparisons of the *Th. bessarabicum* and *Th. junceum* fragments with the other sequences were low (0.403–0.645), indicative of highly saturated substitution sequences (Holmquist 1983). Low ratios were especially obvious in the case of the *Th. ponticum* germin-related sequence, ranging from 0.278 to 0.647 when compared with other species. The pairwise comparisons for the rest of the species generally showed relatively high values (close to or even higher than 2.0). This suggests recent evolutionary divergence between germin sequences since transitions are more likely to occur than transversions (Quicke 1993). Data base searches were performed for a signature sequence of 20 highly conserved peptides within the germin core. GGTNP PHxHPRATEI GIVMK, of which the internal decapeptide is totally conserved in spherulins. Positive identification was made for all species except *Th. bessarabicum* and *Th. junceum*, with x = Valine or Isoleucine in the eighth position (Fig. 2). The most distinct modification was observed in *Th. ponticum*, with two deletions and four amino acid substitutions including the change of three hydrophobic amino acids (HTI) for non-hydrophobic residues (PPN). *Th. intermedium* (Host) Barkword Dewey showed three substitutions of which two were seen to be hydrophobic

Fig. 1 Aligned DNA sequences of the internal germin core domain of the 16 wild *Triticeae* species and wheat (*Triticum aestivum* L.). The *top three rows* indicate the positions of the aligned sequences. *Dashes* denote gaps; *dots* denote identity

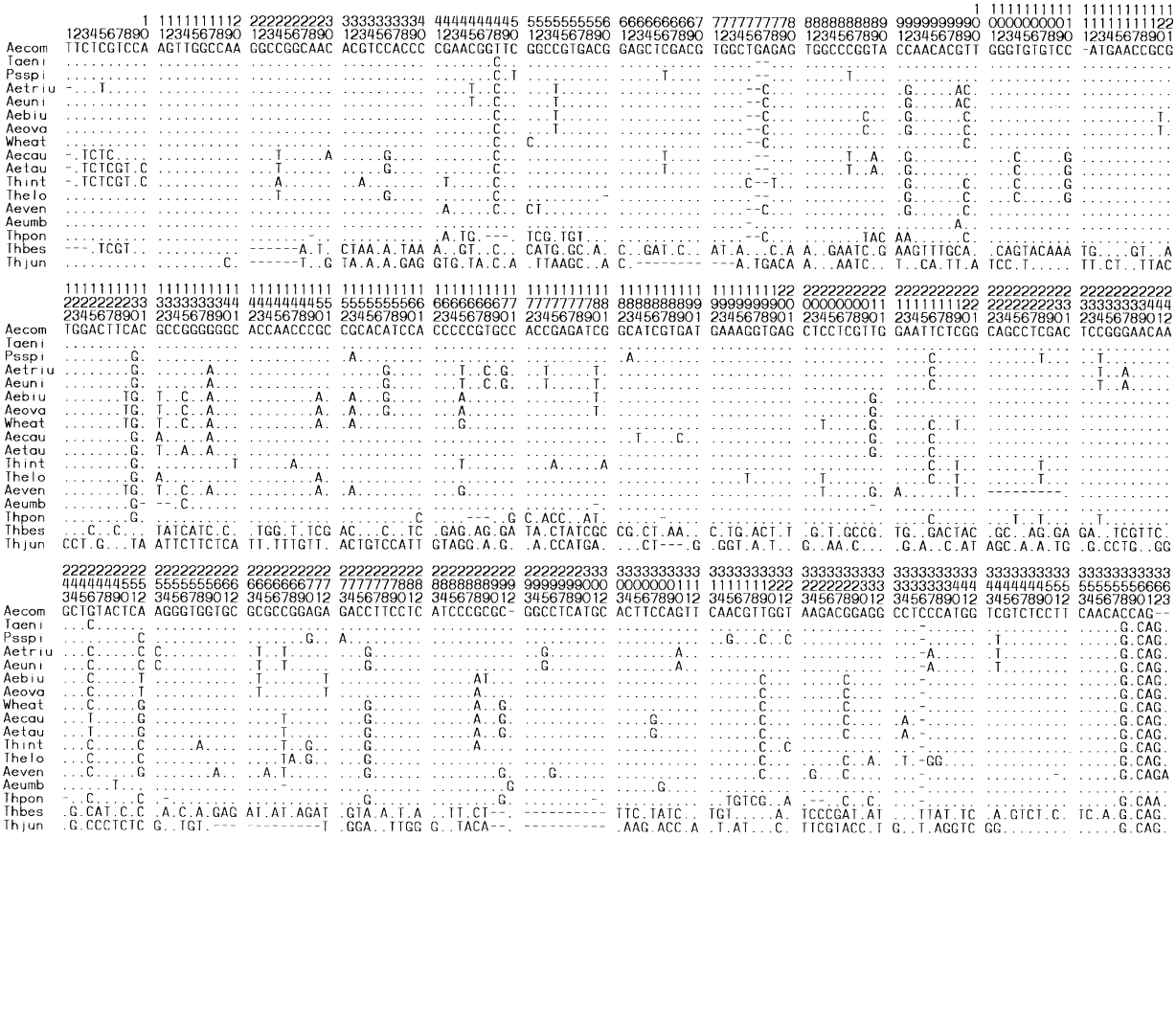

Fig. 2 Comparison of the amino acid sequences as deduced from the DNA amplified germin core sequences. The *box* defines the highly conserved decapeptide in germins and spherulins. The *top three rows* indicate the positions of the aligned sequences *Dashes* denote gaps; *dots* denote identity

amino acids (K) introduced into non-hydrophobic positions (NE). *Aegilops unbellulata* Zhuck. also showed important alterations with two deletions in positions 15 and 20. Finally, *Ae. triuncialis* L. and *Ae uniaristata* Vis. shared the same change of alanine for glycine in the 12th position, a minor alteration since these amino acids have similar biochemical properties. Highly conserved domains in protein cores like the germin signature 20-mer amino acid motif are thought to be functionally relevant. Substantial alterations in the biochemical nature of the amino acid composition, as observed in *Th. ponticum*, *Th. intermedium* and *Ae. umbellulata*, could be related to either important differences in enzyme activity or complete suppression of activity. Furthermore, the variability observed along the complete germin core sequence probably also accounts for functional differences between the species. Future field studies on osmotic stress responses might detect positive correlations between germin core alterations and the functional constraints of such responses.

Further characterization of the germin genes at the structural and regulatory levels, combined with osmotic stress testing in the field, could lead to important advances in the improvement of the genetic response to salt and drought stresses in the cultivars of the *Triticeae*.

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