Identification and characterization of an expansin gene AsEXP1 associated with heat tolerance in C₃ Agrostis grass species

Jichen Xu¹,², Jiang Tian², Faith C. Belanger² and Bingru Huang²,*

¹ Beijing Forestry University, Beijing 100083, China
² Department of Plant Biology and Pathology, Rutgers, the State University of New Jersey, New Brunswick, NJ 08901, USA

Received 8 July 2007; Revised 24 August 2007; Accepted 30 August 2007

Abstract

Plant tolerance of heat stress involves various changes at physiological and molecular levels. The objective of this study was to examine the expression of a gene encoding expansin protein in relation to heat tolerance in two C₃ grass species and genotypes differing in heat tolerance. Heat-tolerant, thermal Agrostis scabra, adapted to high temperatures in geothermal areas in Yellowstone National Park, was subjected to 20 °C (control) or 40 °C (heat stress) for 7 d in a growth chamber. Differential display analysis identified that a gene, AsEXP1, encoding an expansin protein, was strongly up-regulated in leaves exposed to heat stress in thermal A. scabra. Virtual northern hybridization and RT-PCR confirmed that AsEXP1 was a heat-inducible gene in leaves. The expression of AsEXP1 was induced at 1 h of plant exposure to heat stress and reached the highest level of expression at 4 h of treatment. A 1.3 kb full-length cDNA of AsEXP1 was isolated, which encodes a 251 amino acid protein. Two ecotypes of thermal A. scabra and 10 genotypes of Agrostis stolonifera (creeping bentgrass), a widely used turfgrass species in cool climatic regions, varying in the level of heat tolerance, were exposed to 40 °C for 7 d to examine the level of AsEXP1 expression in relation to heat tolerance. Genetic variation in heat tolerance was evaluated by measuring cell membrane stability, photochemical efficiency, and leaf growth. RT-PCR analysis revealed that the level of AsEXP1 in different genotypes was positively correlated with the level of heat tolerance in both grass species. The results first identified a heat-related expansin gene in grass species and suggest that AsEXP1 may be useful as a molecular marker to select for heat-tolerant grass germplasm.

Key words: Agrostis scabra, Agrostis stolonifera, high temperature, turfgrass.

Introduction

Expansins are a family of proteins in plant cell walls that are involved in cell wall disassembly, cell separation, and cell expansion (Cosgrove, 2000). Although the mechanism of expansin action is still unknown, it has been suggested that expansins may play a role as cell wall-loosening agents and thus induce cell expansion or elongation by increasing cell wall extensibility (McQueen-Mason et al., 1992; Cosgrove, 2000; Zenoni et al., 2004). Two families of expansin genes have been identified, α-expansins and β-expansins, and both have similar wall-loosening effects (Cosgrove, 1997). Expansin cDNAs were cloned and gene expression was characterized in various plant species, such as seedlings of cucumber (Cucumis sativus), rice (Oryza sativa), and Arabidopsis thaliana (Shcherban et al., 1995), pea (Pisum sativum) petals (Michael, 1996), and ripening tomato (Solanum lycopersicum=Lycopersicon esculentum) fruit (Rose et al., 1997). Expansin gene expression has been associated with cell growth in actively growing tissues, such as in hypocotyls in tomato (Reinhardt et al., 1998), stem internodes in rice (Cho and Kende, 1997), and adventitious roots in pine (Pinus taeda) (Greenwood et al., 2006).

* To whom correspondence should be addressed. E-mail: huang@aesop.rutgers.edu

© 2007 The Author(s).
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.0/uk/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.
Although various expansin genes are identified and characterized for cell growth and development, little is known of expansin involvement in plant tolerance to environmental stresses. Limited research suggests that expansin expression may be associated with plant adaptation to environmental stresses through its regulation of cell growth and expansion. Studies with primary roots of maize (Zea mays) subjected to low soil water potential have found that the expression of at least three expansin genes was up-regulated in the apical regions of the elongation zone at low water potential and roots at low water potential had more expansin protein than roots in well-watered soils (Wu et al., 1996; Wu and Cosgrove, 2000). Wu and Cosgrove (2000) suggest that expansins may play a role in the maintenance of root growth by increasing cell wall extensibility. A study with a resurrection plant, Craterostigma plantagineum, found that the maintenance of high cell wall extensibility of leaves during dehydration was correlated with increased activity of expansin proteins and transcript level of \( \alpha \)-expansins in cell walls of leaves, suggesting a role of expansins in the regulation of leaf growth during dehydration (Jones and McQueen-Mason, 2004). Expression of \( \alpha \)-expansin genes has recently been reported to be up-regulated during root acclimation to oxygen deficiency in a wetland plant, Rumex palustris, suggesting the importance of expansins in plant adaptation to oxygen deficiency (Colmers et al., 2004).

Heat stress is a major factor limiting plant growth of C\(_3\) plant species. Most C\(_3\) turfgrass or forage grasses grow most actively at temperatures up to 24 °C. A C\(_3\) grass species, thermal A. scabra, has recently been found to grow actively in soils with temperatures up to 45 °C in geothermal areas in the Yellowstone National Park (Tercek et al., 2003). Thermal A. scabra exhibits superior whole-plant tolerance to heat stress over a grass species in the same genus, A. stolonifera, widely used as a turfgrass in cool climatic regions (Lyons et al., 2007; Xu and Huang, 2007). Using differential display, some heat-inducible genes have been identified in thermal A. scabra, including an expansin-like gene fragment (AsEXP1) (B Huang, unpublished data). Growth maintenance under heat stress is an important component of plant heat tolerance (Hong and Vierling, 2000; Karim et al., 2000). However, how expansin expression is related to plant tolerance to heat stress has not been investigated, despite the well-accepted role of expansin in controlling cell growth. Characterization of expansin expression for heat-tolerant plants adapted to long-term high temperatures may provide insights into the role of expansins in plant response to heat stress.

The objectives of this study were to isolate and characterize the expression of AsEXP1 in thermal A. scabra and to examine whether expression of AsEXP1 is associated with heat tolerance in thermal A. scabra and genotypes of A. stolonifera adapted to different temperature regimes.

**Materials and methods**

**Plant materials and growth conditions**

Seeds of two ecotypes (‘NTAS’ and ‘FTAS’) of thermal Agrostis scabra were collected from two geothermal sites in Yellowstone National Park. Tillers of 10 genotypes (‘Penn A-4’, ‘Independence’, ‘Declaration’, ‘Shark’, ‘L-93’, ‘Pennlinks’, ‘Putter’, ‘Kingpin’, ‘Century’, and ‘Backspin’) of A. stolonifera were collected from the turfgrass research farm at Rutgers University. Plants of both species were propagated vegetatively in a greenhouse. Tillers of clonal plants for each genotype were transplanted to plastic pots (15 cm in diameter and 14 cm in depth) filled with sand in a greenhouse for 60 d to allow root and shoot establishment. All plants were then moved into a growth chamber with a day/night temperature of 20 °C, a 14 h photoperiod, and photosynthetically active radiation of 500 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) for 14 d before treatments were imposed. Plants were watered once per day and fertilized weekly with 40 ml Hoagland’s nutrient solution (Hoagland and Arnon, 1950).

**Heat stress treatment**

Four replicates of each genotype were exposed to normal growth temperature of 20 °C or heat stress (40 °C) for 7 d in four growth chambers. Plants were watered twice a day during the heat treatment to prevent water deficit, and fertilized weekly with 40 ml Hoagland’s nutrient solution. The 12 grass ecotypes/genotypes were arranged randomly inside each growth chamber.

At 7 d of treatment, fresh leaves were sampled to determine physiological responses of both A. scabra and A. stolonifera to heat stress and to compare genetic variation in heat tolerance among ecotypes of A. scabra and different genotypes of A. stolonifera. Root tissues of ‘NTAS’ and leaf tissues of all the genotypes tested were frozen in liquid nitrogen immediately on harvest, and stored at −80 °C for molecular analysis.

For the time-course experiment, four replicates of ‘NTAS’ genotype were exposed to 20 °C or 40 °C (heat stress) in four growth chambers. Leaf tissues were harvested at 0, 1, 2, 4, 6, 24, and 48 h, and 7 d after initiation of heat stress treatment for molecular analysis.

**Physiological analysis**

Leaf electrolyte leakage (EL) was measured to evaluate cell membrane stability (Marcum, 1998). For EL analysis, 0.1 g fresh leaf segments (approximately 0.5 cm long) from each sample were incubated in 15 ml deionized water on a shaker for 24 h. The leaf segments (approximately 0.5 cm long) from each sample were incubated in 15 ml deionized water on a shaker for 24 h. The conductance of the incubation solution with killed tissues \( \left( \frac{F_i}{F_{\text{m}}(i)} \right) \) was determined using a fluorescence induction monitor (ADC BioScientific Ltd, Hoddesdon, UK).

Leaf growth was evaluated using relative leaf area. It was calculated as \([\text{leaf area of heat-stressed plants} - \text{leaf area of control plants}] / \text{leaf area of control plants}] \times 100\). Leaf area was measured on five leaves per plant.
Differential gene expression

Differential display was used to identify the heat-inducible gene in thermal A. scabra. Total RNA was extracted (RNaseasy Plant Mini Kit, Qiagen Inc.) from leaves of thermal A. scabra exposed to a normal growth temperature (20 °C) or to 40 °C for 7 d. cDNA was prepared following the SUPER SMART cDNA Synthesis Protocol (Clontech Inc.). Twelve pairs of primers (Delta Differential Display Kit, Clontech Inc.) were tested for differential gene expression. The PCR products were separated on a non-denaturing 6% polyacrylamide gel. After silver staining, the polymorphic fragments were recovered, cloned into the pGEM-T Easy vector (Promega Inc.) and sequenced (Integrated DNA Technologies Inc.).

Hybridization

Dot blot hybridization was used to screen for up-regulated or heat-inducible gene fragments in heat-stressed plants. Plasmid DNA of the clones generated from the differential display was loaded on a nylon membrane (Osmonics Inc.). Total cDNA and the 570 bp AsEXP1 gene fragment in the 3’ end of the coding region were labelled with a random primer DNA labelling system (Invitrogen Inc.) and used as a probe for the dot blot and the virtual northern blot, respectively.

Virtual northern hybridization (Franz et al., 1999) was performed to confirm the expression of AsEXP1, which was identified as a heat-inducible gene through differential display. For virtual northern hybridization, cDNA was prepared from the total RNA, separated in 1.5% agarose gel, and blotted onto a nylon membrane. The amount of cDNA was measured using a spectrophotometer (GENESYS, Spectronic Instruments, Rochester, NY, USA). Hybridization conditions were as follows: 5× SSC, 1× Denhardt’s solution, 20 mM sodium phosphate, 1% SDS, 100 μg ml⁻¹ calf thymus DNA, and 5% dextran sulphate, at 65 °C overnight. The membrane was washed twice for 20 min each time with 2× SSPE/1% SDS and 0.1× SSPE/1% SDS at 65 °C.

RT-PCR analysis

RT-PCR was performed to compare the level of AsEXP1 expression in shoots and roots in two ecotypes of thermal A. scabra and 10 genotypes of A. stolonifera exposed to heat stress, and to examine the time-course of AsEXP1 expression in shoots during heat stress.

First-strand cDNA generated with Super Smart cDNA Synthesis Kit (Clontech, USA) was used as template. The amount of cDNA used for RT-PCR was adjusted to the same level based on the amount of transcripts of an actin gene which was determined in each sample. cDNA amounts generating equal actin amplification were used as template. The PCR cycling conditions were denaturation at 94 °C for 1 min, 55 °C for 1 min, 55 °C or 60 °C for 1 min, and extension at 72 °C for 1 min. The PCR products were checked on a 1.5% agarose gel stained with ethidium bromide. The expression level of each gene was estimated based on the intensity of the band.

Isolation of full-length cDNA of AsEXP1

Fragments containing the 3’ and 5’ ends of the cDNA were isolated by using the SMART™ RACE cDNA Amplification Kit (Clontech, USA). Amplified cDNA fragments were then purified, cloned into the pGEM-T vector, and sequenced. Primers for amplification of the genes (AsEXP1) were as follows: forward, 5’-GAACCTGGCAGAG-GACGGTGCTGA-3’; reverse, 5’-GAAGGGAAGGCCTAGAGCGGTTGCTGA-3’. Alignment of amino acid sequence and phylogenetic analysis were conducted with ClustalX 1.81. Subcellular localization of AsEXP1 was predicted by TargetP1.1 (http://www.cbs.dtu.dk/services/TargetP).

Results

Identification of heat-inducible genes in thermal A. scabra by differential gene expression analysis and dot hybridization

Twelve pairs of primers were tested for differential gene expression in leaves of thermal A. scabra exposed to 20 °C or 40 °C for 7 d. Bands identified as being unique to the heat-treated samples were cloned and dot blotted onto nylon membranes. The blot analyses may represent expression of multiple expansin genes, since the probe used was not tested for gene specificity. Two copies of the membrane were prepared for the evaluation of gene expression pattern in heat-stressed and control plants. One membrane was hybridized with labelled cDNA from the control samples and the other membrane was hybridized with labelled cDNA from the stressed samples. The hybridization result is shown in Fig. 1. One of the clones exhibited much stronger hybridization with cDNA from the plants exposed to heat stress, indicating it was a heat-induced gene. Sequence analysis of the clone indicated it encoded an expansin gene, designated AsEXP1.

Confirmation by virtual northern hybridization and RT-PCR

Virtual northern hybridization confirmed the up-regulation of AsEXP1 by heat stress in leaves of A. scabra. The cDNA prepared from leaves of the control and heat-stressed plants was separated on agarose gels and blotted on nylon membranes. Figure 2A shows the hybridization signal of cDNA by probing with the AsEXP1 DNA fragment. Figure 2B is the gel stained with ethidium bromide, illustrating that the same amounts of cDNA were loaded for both samples. A strong signal of AsEXP1 was displayed in leaves of thermal A. scabra exposed to heat stress (Fig. 2A, lane 2). Leaves of plants exposed to 20 °C

![Fig. 1. Dot blot hybridization of clones identified from differential display probed with labelled cDNA from unstressed (A) or heat-stressed (B) thermal A. scabra.](image-url)
had a weak expression of \textit{AsEXP1} (Fig. 2A, lane 1). RT-PCR revealed that expression of \textit{AsEXP1} was detected in roots of thermal \textit{A. scabra} exposed to heat stress or grown under normal conditions (Fig. 2C). \textit{AsEXP1} in roots was not up-regulated when the whole-plant was exposed to heat stress.

\textbf{Isolation of full-length cDNA of AsEXP1}

Based on the sequence of the fragment, RACE-PCR was used to obtain the full-length coding sequence of \textit{AsEXP1}. The 1329 bp cDNA sequence contained an untranslated 5' upstream sequence of 103 bp, an open reading frame of 756 bp, and an untranslated 3'-sequence of 470 bp. A 251 amino acid protein with a molecular mass of 26 376 Da was predicated from the cDNA sequence. A signal peptide cleavage site was predicted at position 23 (Fig. 3). A comparison of the deduced amino acid sequence of the \textit{A. scabra} expansin with similar sequences from other species is shown in Fig. 3. \textit{AsEXP1} protein showed high homology with other expansin proteins from maize and \textit{Festuca pratensis} (Fig. 3). An unrooted phylogenetic analysis of the \textit{AsEXP1} protein sequence and other \textit{\alpha}-expansin proteins in \textit{A. thaliana}, \textit{F. pratensis}, and \textit{Z. mays} was conducted. The phylogenetic analysis showed the existence of three expansin clusters (Fig. 4). \textit{AsEXP1} protein was grouped in cluster II and was most related to an \textit{\alpha}-expansin protein from \textit{F. pratensis}.

\textbf{Correlation of AsEXP1 expression and heat tolerance in Agrostis}

Three physiological parameters, leaf electrolyte leakage (EL), photochemical efficiency (\(F_v/F_m\)), and relative leaf area, were measured to evaluate genetic variation in heat tolerance in two ecotypes of thermal \textit{A. scabra} and 10 genotypes of \textit{A. stolonifera} (Table 1). Physiological responses of two ecotypes of thermal \textit{A. scabra}, ‘NTAS’ and ‘FTAS’, and 10 genotypes of \textit{A. stolonifera} to heat stress were examined by exposing plants to 40 °C for 7 d.


\begin{itemize}
\item \textit{AsEXP1} (FpEXP2)
\item \textit{ZmEXP1}
\end{itemize}

\textbf{Fig. 2.} Virtual northern hybridization and RT-PCR of expression of \textit{AsEXP1} in shoots or roots of unstressed (1) and heat-stressed (2) \textit{A. scabra}: (A) hybridization pattern in shoots; (B) gel containing cDNA samples stained with EB; (C) RT-PCR result in roots.

\textbf{Fig. 3.} Alignment of the deduced amino acid sequence of the \textit{AsEXP1} gene with other \textit{\alpha}-expansin proteins. Identical residues are indicated with asterisks, and well-conserved residues are indicated with double dots. The predicated signal peptide cleavage site is indicated with an arrow. Accession numbers of expansin proteins from \textit{Zea mays}: ZmEXP1, AF332169; \textit{Festuca pratensis}: FpEXP2, CAC06433.
"FTAS" and "NTAS" were the most heat tolerant, followed by a group including "Declaration", "Penn A-4", "Independence", and "Shark", and with "L-93" and "Pennlinks" being intermediate in tolerance, and "Kingpin", "Backspin", "Century", and "Putter" being the most heat sensitive. There was also significant variation in leaf growth among all the genotypes tested: "NTAS" had the highest relative leaf area, followed by "Shark", "Century", "L-93", "Declaration", "Penn A-4", "Pennlinks", "FTAS", "Putter", "Independence", "Backspin", and "Kingpin".

Virtual northern hybridization was performed to compare AsEXP1 gene expression in genotypes differing in heat tolerance that were exposed to heat stress. Equal amounts of total cDNA from each sample were separated on an agarose gel and hybridized with the AsEXP1 cDNA (Fig. 5A). Most heat-tolerant genotypes, "NTAS", "FTAS", "Declaration", and "Penn-A4" exhibited strongest AsEXP1 expression. The expression signal of AsEXP1 was undetectable or weakly expressed in more heat-sensitive "Pennlinks", "Backspin", "Kingpin", and "Putter".

### Table 1. Physiological responses of two ecotypes of thermal *A. scabra* and 10 genotypes of *A. stolonifera* to heat stress

Leaf electrolyte leakage (EL), photochemical efficiency ($F_v/F_m$), and leaf area were measured after 7 d of exposure to 40 °C.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>EL (%)</th>
<th>$F_v/F_m$</th>
<th>Relative increase of leaf area (% change)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. scabra</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>'NTAS'</td>
<td>22.3 C</td>
<td>0.86 A</td>
<td>81.0</td>
</tr>
<tr>
<td>'FTAS'</td>
<td>23.8 C</td>
<td>0.82 A</td>
<td>33.0</td>
</tr>
<tr>
<td><em>A. stolonifera</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>'Backspin'</td>
<td>47.8 A</td>
<td>0.31 C</td>
<td>11.5</td>
</tr>
<tr>
<td>'PennA-4'</td>
<td>27.2 B</td>
<td>0.75 AB</td>
<td>38.5</td>
</tr>
<tr>
<td>'Independence'</td>
<td>26.6 B</td>
<td>0.76 AB</td>
<td>17.6</td>
</tr>
<tr>
<td>'Declaration'</td>
<td>29.0 B</td>
<td>0.73 AB</td>
<td>38.9</td>
</tr>
<tr>
<td>'Shark'</td>
<td>25.9 BC</td>
<td>0.74 AB</td>
<td>47.1</td>
</tr>
<tr>
<td>'L-93'</td>
<td>33.2 B</td>
<td>0.61 B</td>
<td>40.6</td>
</tr>
<tr>
<td>'Putter'</td>
<td>43.1 A</td>
<td>0.41 C</td>
<td>27.1</td>
</tr>
<tr>
<td>'Kingpin'</td>
<td>48.2 A</td>
<td>0.32 C</td>
<td>4.0</td>
</tr>
<tr>
<td>'Century'</td>
<td>47.6 A</td>
<td>0.35 C</td>
<td>41.4</td>
</tr>
<tr>
<td>'Pennlinks'</td>
<td>35.1 B</td>
<td>0.62 C</td>
<td>37.5</td>
</tr>
</tbody>
</table>

* Means within a column for EL and $F_v/F_m$ followed by the same letters were not significantly different based on LSD test at $P=0.05$. 

"FTAS" and "NTAS" were the most heat tolerant, followed by a group including "Declaration", "Penn A-4", "Independence", and "Shark", and with "L-93" and "Pennlinks" being intermediate in tolerance, and "Kingpin", "Backspin", "Century", and "Putter" being the most heat sensitive. There was also significant variation in leaf growth among all the genotypes tested: "NTAS" had the highest relative leaf area, followed by "Shark", "Century", "L-93", "Declaration", "Penn A-4", "Pennlinks", "FTAS", "Putter", "Independence", "Backspin", and "Kingpin".

Virtual northern hybridization was performed to compare AsEXP1 gene expression in genotypes differing in heat tolerance that were exposed to heat stress. Equal amounts of total cDNA from each sample were separated on an agarose gel and hybridized with the AsEXP1 cDNA (Fig. 5A). Most heat-tolerant genotypes, "NTAS", "FTAS", "Declaration", and "Penn-A4" exhibited strongest AsEXP1 expression. The expression signal of AsEXP1 was undetectable or weakly expressed in more heat-sensitive "Pennlinks", "Backspin", "Kingpin", and "Putter".
Correlation coefficient analysis was performed to determine the relationship between the level of AsEXP1 expression and the level of heat tolerance (as indicated by EL and $F_v/F_m$). The hybridization signal strength of AsEXP1 for each genotype was scored using a 1–9 (weakest to strongest signal) scale. ‘NTAS’, ‘Backspin’, ‘Penn-A4’, ‘Independence’, ‘FTAS’, ‘Declaration’, ‘Shark’, ‘Putter’, ‘Kingspin’, ‘Century’, and ‘Pennlinks’ were scored at 9, 1, 9, 3, 9, 3, 1, 1, 3, 1, 3, and 1, respectively (Fig. 5). The correlation coefficient between AsEXP1 expression level and $F_v/F_m$ was 0.749, implying a positive correlation between AsEXP1 expression level and heat tolerance ability as indicated by photochemical efficiency in thermal A. scabra and A. stolonifera. The correlation coefficient between AsEXP1 expression level and EL was −0.712, implying a negative correlation of AsEXP1 expression level with cellular leakage or a positive relationship with cell membrane stability or heat tolerance. The expression of AsEXP1 was positively correlated with leaf expansion, with a correlation coefficient of 0.443.

AsEXP1 expression in different genotypes differing in heat tolerance was further tested using RT-PCR. The two thermal A. scabra ecotypes, ‘NTAS’, ‘FTAS’, and two genotypes of A. stolonifera, ‘Declaration’ and ‘Penn-A4’, showed strong amplification of AsEXP1, whereas no AsEXP1 was detected in other genotypes that were relatively more sensitive to heat stress (Fig. 6). The RT-PCR result was consistent with the virtual northern hybridization test in Fig. 5, confirming the positive relationship of AsEXP1 expression and heat tolerance.

The time-course of AsEXP1 expression in two Agrostis species during heat stress was evaluated using RT-PCR. Thermal A. scabra plants were exposed to 40 °C and sampled at 1, 2, 4, 8, 24, and 48 h, and 7 d of treatment. RT-PCR was conducted to determine the time-course of AsEXP1 expression (Fig. 7). AsEXP1 expression was induced following 1 h of exposure to heat stress. The expression level reached the highest level within 4 h of heat stress and remained high during the treatment period of 7 d (Fig. 7).

Discussion

Stress-tolerant plants develop various defence mechanisms in order to survive in unfavourable environmental conditions, including induction or up-regulation of proteins or genes controlling cell growth and metabolism. Through differential display analysis, a gene encoding an expansin protein, AsEXP1, was identified which was induced in leaves of thermal A. scabra when the whole plant was exposed to heat stress (40 °C) for 7 d. An unrooted phyllogenetic analysis between AsEXP1 protein and other α-expansin proteins in Arabidopsis, F. pratensis, and Z. mays revealed three expansin clusters. In cluster I, there were four α-expansin proteins in Arabidopsis and one α-expansin protein from Z. mays. The functions of α-expansin proteins in this cluster are unknown. AsEXP1 protein was grouped in cluster II and was closely related to two α-expansin proteins from Z. mays, ZmEXP1 and ZmEXP5, which had been found to be associated with increasing cell wall extensibility and maintaining root elongation under water deficit (Wu et al., 2001). Cluster III was composed of 13 α-expansin proteins in Arabidopsis and one α-expansin protein from Z. mays. Among them, AtEXP18 and AtEXP7 have been reported to be involved in root hair initiation (Cho and Cosgrove, 2002).

RT-PCR results showed that AsEXP1 expression was induced in shoots following 1 h of exposure to heat stress, and the expression level reached the highest level within 4 h of treatment. Virtual northern hybridization analysis confirmed that AsEXP1 exhibited strong expression in
leaves only in plants exposed to heat stress, but was barely detectable in plants exposed to optimum growth temperature (20 °C) for thermal *A. scabrum*. These results indicate that *AsEXP1* was highly up-regulated in shoots under heat stress. However, *AsEXP1* was expressed constitutively in roots of thermal *A. scabrum* at low and high temperatures. Expansins have been shown to be involved in a number of developmental processes, particularly cell growth through alteration of cell wall extensibility (Cosgrove, 1997, 2000). In a study examining the effects of temperature on cell wall properties in relation to the growth rate of *Oryza sativa* coleoptiles and azuki bean (*Phaseolus angularis*) epicotyls, it was suggested that temperature modulates the growth rate of plant shoots by affecting mainly the mechanical properties of the cell wall (Nakamura *et al.*, 2003). While heat stress is a major factor limiting cool-season grass growth, as such it is reasonable to envisage that the induction or enhancement of expansin gene expression may play a role in the modification of cell walls in thermal *A. scabrum* to allow for continued leaf growth during heat stress. Expansins are believed to be able to fully restore cell extension in heat-denatured cell walls (Wu and Cosgrove, 2000). Among the 12 genotypes examined, ‘NTAS’ which had most leaf growth following 7 d of heat stress also exhibited strongest *AsEXP1* expression. The correlation coefficient between the level of *AsEXP1* expression and the changes in leaf area during heat stress was 0.443, suggesting a positive relationship between expansin expression and leaf expansion.

The level of *AsEXP1* expression in shoots varied with *Agrostis* species and genotypes differing in heat tolerance. Cell membrane stability and photochemical efficiency of leaves are two commonly used physiological indicators of plant tolerance to heat stress (Marcum, 1998; Rachmilevitch *et al.*, 2006). The responses of both physiological parameters to heat stress indicated that the genotypes examined were ranked into three groups in heat tolerance, with the two ecotypes of thermal *A. scabrum* ‘FTAS’ and ‘NTAS’, being the most tolerant, followed by *A. stolonifera* genotypes ‘Declaration’, ‘Penn A-4’, ‘Independence’, and ‘Shark’, and with ‘L-93’ and ‘Pennlinks’ having intermediate tolerance, and ‘Kingpin’, ‘Backspin’, ‘Century’, and ‘Putter’ being the most sensitive. The two most heat-tolerant ecotypes of thermal *A. scabrum* (‘FTAS’ and ‘NTAS’) and two tolerant genotypes of *A. stolonifera* (‘Declaration’ and ‘Penn A-4’) exhibited the strongest *AsEXP1* expression, the heat-sensitive *A. stolonifera* genotypes had no expression, and the genotypes with moderate heat tolerance exhibited an intermediate level of *AsEXP1* expression. Furthermore, the correlation analysis between the level of *AsEXP1* expression and physiological data (EL and $F_{m}/F_{n}$) confirmed the positive relationship of *AsEXP1* expression and physiological responses to heat stress in *Agrostis* species. The present results indicated that *AsEXP1* expression level was highly correlated with the level of whole-plant heat tolerance in both *Agrostis* species, which was associated with the maintenance of higher leaf cell membrane stability and photochemical efficiency. It is interesting to note that the correlation coefficient between photochemical efficiency or cell membrane stability and the *AsEXP1* expression level were greater than between the level of gene expression and leaf expansion. These results suggest a novel function of *AsEXP1* in regulating physiological processes, in addition to the known function controlling cell wall loosening or extensibility.

In summary, the present study is the first to identify an expansin gene, *AsEXP1*, associated with heat tolerance in cool-season perennial grass species. This gene was highly up-regulated in shoots exposed to heat stress and was induced within only 1 h of heat stress. *AsEXP1* may be a useful molecular marker in evaluating the ability of cultivars to withstand heat stress and could be used for selecting heat-tolerant grass germplasms. However, the modes of action of expansins in regulating heat tolerance deserve further investigation. Further understanding of the molecular genetic mechanisms of heat tolerance associated with expansins may lead to the development of novel strategies for improving grass productivity in high temperature environments.

Acknowledgements

The authors wish to thank the United States Golf Association for funding support of this research.

References


