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RESEARCH PAPER

Root proteomic responses to heat stress in two *Agrostis* grass species contrasting in heat tolerance

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Abstract

Protein metabolism plays an important role in plant adaptation to heat stress. This study was designed to identify heat-responsive proteins in roots associated with thermotolerance for two C_3 grass species contrasting in heat tolerance, thermal *Agrostis scabra* and heat-sensitive *Agrostis stolonifera* L. Plants were exposed to 20 °C (control), 30 C (moderate heat stress), or 40 °C (severe heat stress) in growth chambers. Roots were harvested at 2 d and 10 d after temperature treatment. Proteins were extracted and separated by two-dimensional polyacrylamide gel electrophoresis. Seventy protein spots were regulated by heat stress in at least one species. Under both moderate and severe heat stress, more proteins were down-regulated than were up-regulated, and thermal *A. scabra* roots had more up-regulated proteins than *A. stolonifera* roots. The sequences of 66 differentially expressed protein spots were identified using mass spectrometry. The results suggested that the up-regulation of sucrose synthase, glutathione *S*-transferase, superoxide dismutase, and heat shock protein Sti (stress-inducible protein) may contribute to the superior root thermotolerance of *A. scabra*. In addition, phosphoproteomic analysis indicated that two isoforms of fructose-biphosphate aldolase were highly phosphorylated under heat stress, and thermal *A. scabra* had greater phosphorylation than *A. stolonifera*, suggesting that the aldolase phosphorylation might be involved in root thermotolerance.

Key words: Grass, heat tolerance, phosphoproteomics, protein, proteomics, thermotolerance.

Introduction

An increase in temperature associated with global warming is a growing concern, as it limits plant growth and productivity, especially for temperate species. Physiological mechanisms of heat tolerance have been examined extensively in various plant species, but the molecular basis of heat tolerance is not well understood (Wahid *et al*., 2007). Plant adaptation to environmental stresses is dependent upon the activation of cascades of molecular networks involved in stress perception, signal transduction, and the expression of stress-related proteins. Knowledge of heat-responsive proteins is critical for further understanding of the molecular mechanisms of stress tolerance.

Proteomics offers a powerful approach to discover the proteins and pathways that are crucial for stress responsiveness and tolerance. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) in combination with mass spectrometry (MS) allows rapid and reliable protein identification and can provide information about abundance and post-translation modification (PTM). In recent years, proteomic-based technologies have been successfully applied to the systematic study of the proteomic responses in many plant species to a wide range of abiotic stresses, including heat (Ferreira *et al*., 2006; Lee *et al*., 2007), drought (Pinheiro *et al*., 2005), cold (Yan *et al*., 2006), oxidative stress (Wang *et al*., 2004), anoxia (Chang *et al*., 2000), salt (Yan *et al*., 2005), ultraviolet-B (Xu *et al*., 2008*a*), and metal stress (Labra *et al*., 2006). Lee *et al*. (2007) found that heat shock proteins (HSPs) and antioxidant enzymes were up-regulated under heat stress in rice (*Oryza sativa*) leaves, and also the enzymes related to metabolic pathway were differentially accumulated. Ferreira *et al*. (2006) reported that in *Populus euphratica* a moderate heat response involves changes in proteins related to lipid biogenesis, cytoskeleton structure, sulphate assimilation, thiamine and

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hydrophobic amino acid biosynthesis, and nuclear transport. Protein phosphorylation is one of the most prominent PTMs by which cells transduce signals (Kalume *et al*., 2003; Bentem and Hirt, 2007). It has been inferred that 5% of the *Arabidopsis thaliana* genome encodes kinases and phosphatases, representing >1000 enzymes controlling the phosphorylation status of thousands of proteins (Arabidopsis Genome Initiative, 2000; Kerk *et al*., 2002). Previous studies have suggested a critical role for protein phosphorylation in plant stress responses (Mizoguchi *et al*., 1996; Xiong and Yang, 2003).

In recent years, knowledge of the mechanisms underlying plant responses to heat stress has grown (Wahid *et al*., 2007). However, most research focuses on stress adaptation mechanisms of the aboveground organs, whereas mechanisms of root tolerance to heat stress are much less investigated. Various studies have demonstrated that roots are more sensitive to heat stress, and suggest that high soil temperature is more detrimental than high air temperature for whole-plant growth (Xu and Huang, 2001; Liu and Huang, 2005). Roots may express different proteomes from leaves, grains, or fruits due to their different sensitivity to heat stress and unique functions. Proteomic profiling associated with root thermotolerance will enable molecular dissection of heat tolerance mechanisms. One approach to understanding the mechanisms of plant tolerance to stresses is to examine plants adapted to extremely stressful environments, since these plants may retain regulatory mechanisms enabling their survival. The dissection of such mechanisms may reveal a set of genes and proteins that may contribute to genetic improvement for stress tolerance in other plants, such as economically important cultivars. Several C_3 grass species have been identified growing in geothermally heated areas in Yellowstone National Park (YNP) (Stout and Al-Niemi, 2002; Tercek *et al*., 2003). Thermal *Agrostis scabra* ('thermal' rough bentgrass) is one of the predominant grass species in thermal areas. This geothermal grass species can survive and even grow at temperatures up to 45–50 °C in soils that are permeated by steam (Tercek *et al*., 2003). In contrast, the growth temperature for common C_3 grass species is between 10 and 18 °C for roots and between 15 and 24 °C for shoots, and physiological injury and death occur in roots of temperate grass species when soil temperatures reach 23 °C (Pote *et al*., 2006). Previous studies found that thermal *A. scabra* was able to maintain high root viability and new root production under high temperatures (35–40 °C) whereas severe root death occurred for *A. stolonifera* (Pote *et al*., 2006; Rachmilevitch *et al*., 2006*a*, *b*). The fact that thermal *A. scabra* is able to survive extreme temperatures marks it out as an important plant species to study the mechanisms responsible for survival after heat stress. Investigation into differentially accumulated proteins in the roots of heat-tolerant plants in comparison with heat-sensitive plants may identify specific proteins related to root thermotolerance, which could be used to develop molecular markers to select heat-tolerant germplasm or to create tolerant grasses through genetic manipulation.

The objectives of this study were to compare protein/phosphoprotein profiles of roots between thermal *A. scabra* and heat-sensitive *A. stolonifera*, under heat stress conditions, and to identify heat-regulated proteins associated with thermotolerance in roots of cool-season grasses.

Materials and methods

Plant materials and treatments

Thermal *A. scabra* plants were generated from seeds collected from a geothermal site in YNP, Wyoming, USA. *Agrostis stolonifera* L. (cv. Penncross) plants were collected from field plots from the turfgrass research farm at Rutgers University (New Brunswick, NJ, USA). Both species were propagated vegetatively in a greenhouse. Clonal plants of approximately 60 d old were then transplanted into plastic pots (20 cm deep and 15 cm in diameter) filled with washed, fine sand. Plants were maintained in a greenhouse for 28 d and then moved to a growth chamber set at 20/15 °C (day/night temperature), 75% relative humidity, 600 mmol m^{-2} s^{-1} of photosynthetically active radiation, and a 12 h photoperiod. Plants were allowed to acclimate to the growth chamber conditions for 7 d before being exposed to three air temperature regimes: 20 °C (control), 30 °C (moderate heat stress), and 40 °C (severe heat stress). The soil temperatures were 20.1, 29.5, and 39.3 °C (average of four replicaes), respectively, under control, moderate heat stress, and severe heat stress conditions. Each treatment was repeated three times in three different chambers to minimize chamber effects. During plant establishment and temperature treatment, plants were watered every day until water drained from the bottom of each pot in order to ensure full hydration of plants and avoid the occurrence of water deficit, and fertilized once a week with full-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950).

Evaluation of root thermotolerance

Root viability was determined to evaluate root thermotolerance. At 10 d of temperature treatments, roots were washed free of soil. About 0.4 g (fresh weight) of roots (whole roots with base and tips) was collected for the measurement of root viability using a modified 2,3,5-triphenyltetrazolium chloride (TTC) reduction technique (Knievel, 1973). Roots were incubated in the dark for 24 h in 0.6% TTC at 37 °C, then rinsed with deionized water and placed in 95% ethanol at 60 °C for formazan extraction. The absorbance of the incubation solution was measured at 490 nm with a spectrophotometer (Model U-1100, Hitachi, Tokyo, Japan). Four independent samples were determined for each treatment. Live roots were mixed with different proportions of autoclave-killed roots to construct a standard curve. Root viability was expressed as the percentage of live root biomass to total root biomass.

Protein extraction

Roots were harvested at 2 d and 10 d of temperature treatment, immediately frozen in liquid nitrogen, and then stored at –80 °C prior to analysis. Four independent samples were harvested from each treatment. Root protein extraction followed the procedure described by Xu *et al*. (2008*b*). A 1 g aliquot of root sample was ground to powder with liquid nitrogen, homogenized, and incubated with 10 ml of precipitation solution [10% trichloroacetic acid

(TCA) and 0.07% 2-mercaptoethanol in acetone] for 2 h at –20 °C. The precipitated proteins were pelleted and washed with ice-cold acetone containing 0.07% 2-mercaptoethanol until the supernatant was colourless. The pellet was vacuum-dried, resuspended in resolubilization solution [8 M urea, 2 M thiourea, 2% CHAPS, 1% dithiothreitol (DTT), 1% pharmalyte], and sonicated to extract proteins. Insoluble tissue was removed by centrifugation at 21 000 *g* for 20 min. Protein concentration was determined according to Bradford (1976) using a commercial dye reagent (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin (BSA) as a standard.

Two-dimensional PAGE

An IPGPhor apparatus (GE Healthcare, Piscataway, NJ, USA) was used for isoelectric focusing (IEF) with immobilized pH gradient (IPG) strips (pH 3.0–10.0, linear gradient, 13 cm). The IPG strips were rehydrated for 12 h at 20 °C with 250 µl of rehydration buffer [8 M urea, 2 M thiourea, 2% (w/v) CHAPS, 1% (v/v) IPG buffer, 1% DTT, and 0.002% bromophenol blue] containing 300 mg of proteins. The voltage settings for IEF were 500 V for 1 h, 1000 V for 1 h, and 8000 V to a total 56.50 kVh. Following IEF, the protein in the strips was denatured with equilibration buffer (50 mM Tris–HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 1% DTT) and then incubated with the same buffer containing 2.5% iodoacetamide instead of DTT for 20 min. The second dimension electrophoresis was performed on a 12.5% gel using a Hoefer SE 600 Ruby electrophoresis unit (GE Healthcare). For phosphoprotein detection, 2-D gels were stained with a modified protocol using Pro-Q Diamond Phosphoprotein Stain (Pro-Q DPS; Molecular Probes) (Agrawal and Thelen, 2005). Following scanning of Pro-Q DPS-stained gels, gels were stained with colloidal Coomassie brilliant blue (CBB) G-250 to detect total proteins (Newsholme *et al*., 2000).

Gel images were analysed with Progenesis (version 4.01) (nonlinear) software. Image analysis included the following procedures: spot detection, spot measurement, background subtraction, and spot matching. Only spots that were detected on all the four replicate gels were analysed further. To correct the variability due to staining, the spot volumes were normalized as a percentage of the total volume of all spots on the gel. Data were subjected to analysis of variance (ANOVA) to test for the effects of species, heat, and their interactions. Means were separated by least significance difference test (P <0.05).

Protein identification

The gel spots were excised and washed with 30% acetonitrile (ACN) in 50 mM ammonium bicarbonate prior to DTT reduction and iodoacetamide alkylation. Trypsin was used for digestion at 37 °C overnight. The resulting peptides were extracted with 30 ml of 1% trifluoracetic acid (TFA) followed by C_{18} Ziptip desalting. For the MS analysis, the peptides were mixed with 7 mg ml^{-1} α-cyano-4-hydroxy-cinnamic acid matrix in a 1:1 ratio and spotted onto a matrix-assisted laser desorption/ionization (MALDI) plate. The peptides were analysed on a 4800 MALDI TOF/TOF analyser (Applied Biosystem, Framingham, MA, USA). Mass spectra (m/z 880–3200) were acquired in positive ion reflector mode. The 25 most intense ions were selected for subsequent MS/MS sequencing analysis in 1 kV mode. Protein identification was performed by searching the combined MS and MS/MS spectra against the green plant NCBI database using a local MASCOT search engine (V.1.9) on a GPS (V. 3.5, ABI) server. Proteins containing at least two peptides with confidence interval (CI) values no less than 95% were considered as being identified.

Experimental design and statistical analysis

The experimental design was a split-plot design with temperature as the main plot and grass species as the subplot, and each treatment had four replicates. Root viability was subjected to ANOVA to test for the effects of heat and species. Treatment means were separated by the least significant difference test at a P-value of <0.05.

Results

Changes in root viability in response to heat stress

Root viability of thermal *A. scabra* did not change as temperature increased from 20 °C to 30 °C, but decreased at 40 °C (Table 1). A significant decline in root viability was observed at both 30 °C and 40 °C, compared with the control at 20 °C for *A. stolonifera*. The root viability of the two species did not differ at 20 °C, but thermal *A. scabra* had significantly higher root viability than *A. stolonifera* at 30 °C and 40 °C.

Proteomic responses to heat stress between grass species

The 2-D polyacrylamide gels were reproducible and exhibited clearly separated protein spots. Root protein profiles of the two grass species exposed to 20 °C were similar, except that *A. stolonifera* had higher intensities of spots 52, 53, and 33, and lower intensities of spots 34, 35, and 36, than *A. scabra*. However, the response patterns of proteins to heat stress varied between the two species. A representative gel image stained by CBB is presented in Fig. 1. Protein spots that were significantly affected by heat stress at one or both sampling times in at least one species were analysed further. A total of 70 protein spots exhibited differential accumulation under heat stress, and four regions of differentially expressed proteins are presented in Fig. 2.

Among the 70 protein spots, one spot (spot 52) exhibited increases in intensity or up-regulation in thermal *A. scabra*, but decreases in the intensity or down-regulation in *A. stolonifera* at moderate or severe heat

Table 1. *Root viability of thermal* A. scabra *and* A. stolonifera *as affected by heat stress (30 °C and 40 °C) at 10 d of treatment*

Data are the means of four replicates. Means followed by the same letters were not statistically different based on the least significance test at P=0.05. Uppercase letters are for comparison between two grass species at a given temperature treatment. Lowercase letters are for comparisons between temperature treatments for a given grass species.

Species	Root viability (% live roots)		
	20 °C	30 °C	40 °C
A. scabra	83.8 Aa	79.5 Aa	55.4 Ab
A. stolonifera	85.9 Aa	65.2 Bb	33.8 Bc

stress. The intensity of 47 spots (spots 1–47; Fig. 1; Table 2) decreased and that of 22 spots (spots 48–51 and 53–70; Fig. 2; Table 2) increased under moderate or severe heat stress in at least one species. More protein spots exhibited down-regulation than those showing up-regulation under heat stress. In the group of up-regulated spots, 13 spots (spots 48, 53, 54, 56, 58–60, 62, 63, 65–67, and 70) were increased in both species and nine spots (spots 49–51, 55, 57, 61, 64, 68, and 69) were increased only in thermal *A. scabra* (Table 2; Fig. 3). Thermal *A. scabra* had more up-regulated protein spots than *A. stolonifera* under moderate and severe heat stress. Among the 47 down-regulated spots, 25 spots (spots 1–5, 7, 8, 15, 18, 21, 22, 25–27, 29, 32, 33, 36–42, and 44) were decreased in both species, nine spots (spots 6, 11–14, 16, 17, 24, and 45) were decreased only in *A. stolonifera*, and 13 spots (spots 9, 10, 19, 20, 23, 28, 30, 31, 34, 35, 43, 46, and 47) were decreased only in *A. scabra* (Table 2; Fig. 3). Eleven protein spots (spots 9, 10, 12, 13, 17, 24, 30, 34, 35, 46, and 68) were responsive only to short-term heat stress (2 d), while 18 (spots 6, 8, 16, 18–20, 43, 45, 47, 48–51, 57, 59, 67, and 70) were responsive only to long-term heat stress (10 d). The remaining 41 protein spots were responsive to both short-term and long-term heat stress (Table 2; Fig. 3). Most spots were responsive to heat stress in both species, nine spots (spots 6, 11–14, 16, 17, 24, and 45) only in *A. stolonifera*, and 23 (spots 9, 10, 19, 20, 23, 28, 30, 31, 34, 35, 43, 46, 47, 49–52, 55, 57, 61, 64, 68, and 69) only in *A. scabra* (Table 2). Most of the differentially accumulated protein spots were regulated by

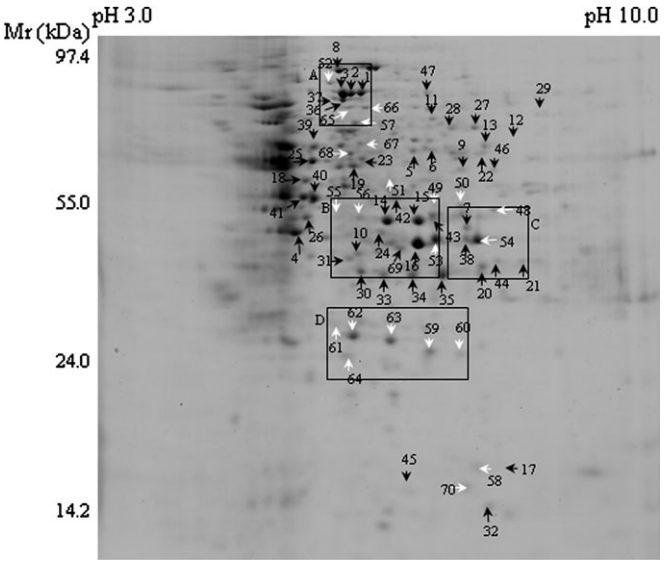

Fig. 1. Coomassie-stained 2-D polyacrylamide gel of separated proteins from *A. scabra* roots grown at 20 °C. Proteins were separated in the first dimension on an IPG strip (pH 3.0–10.0) and in the second dimension on a 12.5% polyacrylamide gel. The numbered spots were affected by heat stress.

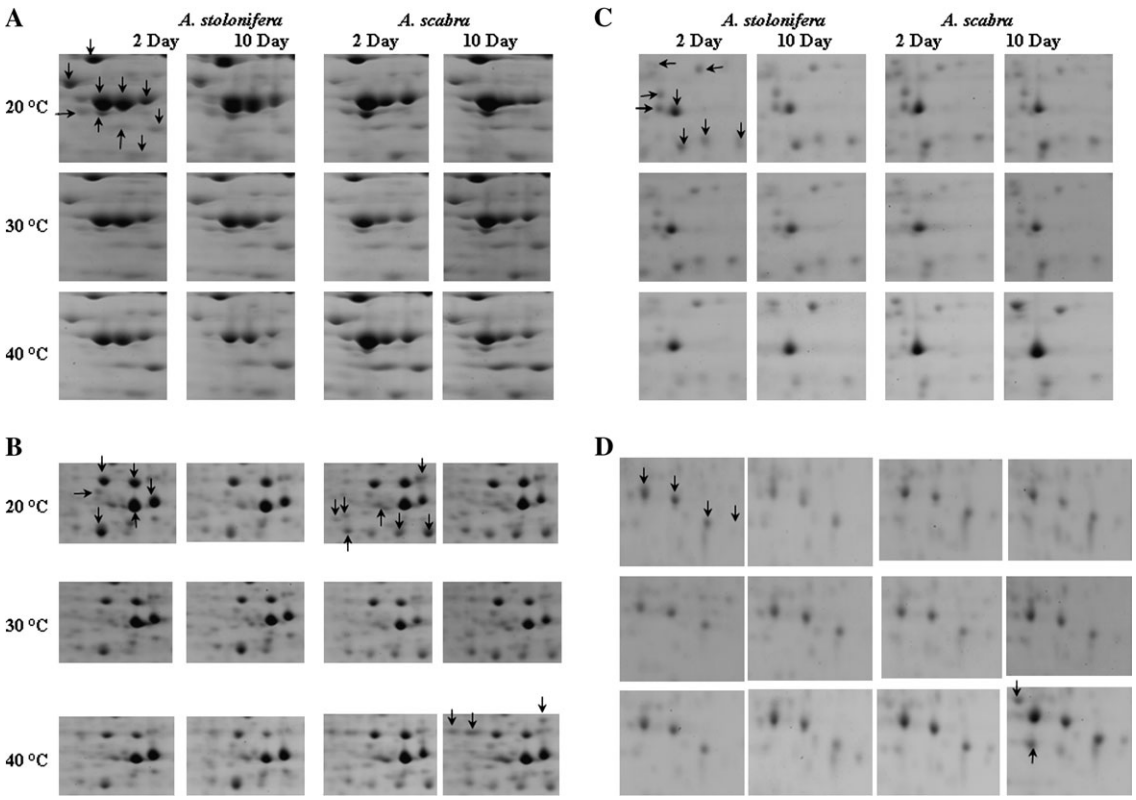

Fig. 2. Selected differentially expressed protein spots in two species growing under different temperatures.

Table 2. *Differentially expressed proteins identified by mass spectrometry between thermal* A. scabra *(ecotype 'NTAS', N) and* A. stolonifera *(cultivar 'Penncross', P) under heat stress (30 °C and 40 °C) compared with those at normal temperature (20 °C)*

ID, spot ID (corresponding to Fig. 1); H pI/MW, hypothetical isoelectrical point/molecular weight; PS, protein score; PM, the number of unique peptides matched.*0.05>P⩾0.01; **0.01>P⩾0.001; ***0.001>P.

ID	Protein identification [source]	H. pI/MW	Accession no.	PS	PM	Heat stress treatment			
						2 d		10 d	
						30 °C	40 °C	30 °C	40 °C
Protein spots decreased by heat stress									
Category 01 Metabolism									
1	Methionine synthase protein (EC 2.1.1.14) [*Catharanthus roseus*]	6.10/84 857	S57636	190	14	N***	N*, P*	P***	N**, P***
2	Methionine synthase protein (EC 2.1.1.14) [*Sorghum bicolor*]	5.93/83 788	Q8W0Q7	351	9	N***	P**	P***	N**, P***
3	Methionine synthase protein (EC 2.1.1.14) [*Sorghum bicolor*]	5.93/83 788	Q8W0Q7	211	15	P**	P**	N**, P**	N***, P***
4	Cytosolic glutamine synthetase (EC 6.3.1.2) [*Populus alba×Populus tremula*]	6.61/18 429	gil37956277	209	5		N**, P**	N**, P**	N**, P**
5	Serine hydroxymethyltransferase (SHMT) (EC 2.1.2.1) [*Arabidopsis thaliana*]	7.12/51 797	Q9FPJ3	214	5	N**, P**	N**, P**	N**, P**	N**, P**
6	SHMT (EC 2.1.2.1) [*Arabidopsis thaliana*]	7.12/51 797	Q9FPJ3	196	6			P**	P**
7	Nucleotide-sugar dehydratase [*Arabidopsis thaliana*]	8.58/38 621	F84688	504	10	N***, P**	N***, P***	P***	N***, P***
Category 02 Energy									
8	Cytoplasmic aconitate hydratase (EC 4.2.1.3) [*Arabidopsis thaliana*]	6.72/10 8201	B84471	186	8			N**, P***	N**, P**
9	Fumarase (EC 4.2.1.2) [*Solanum tuberosum*]	8.01/52 999	gil1488652	268	5	N***	N***		
10	Malate dehydrogenase (EC 1.1.1.37) [*Oryza sativa*]	8.74/35 460	Q94JA2	132	5		N**		
52	Sucrose synthase (EC 2.4.1.13) Ss1 [*Hordeum vulgare*]	5.94/92 211	S29242	354	23	P***	P***	P**	P***
11	Pyrophosphate-dependent phosphofructokinase alpha subunit (EC 2.7.1.90) [*Citrus paradise Grapefru*]	6.71/67 373	Q9ZST2	162	11		P***		P***
12	Pyruvate kinase (EC 2.7.1.40) [*Glycine max*]	7.50/55 302	T07787	176	5	P**	P***		
13	Pyruvate kinase (EC 2.7.1.40) cytosolic [*Solanum tuberosum*]	6.64/55 170	P22200	238	7	P**			
14	Fructose-bisphosphate (FBP) aldolase (EC 4.1.2.13) [*Oryza sativa*]	6.55/38 719	Q40676	692	11	P**	P***	P**	
15	FBP aldolase (EC 4.1.2.13) [*Oryza sativa*]	6.55/38 719	Q40676	545	10		P***	N**, P***	N***, P***
16	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (EC 1.2.1.12) cytosolic (fragment) [*Hordeum vulgare*]	6.20/33 235	A24159	885	8				P**
17	Non-symbiotic (non-legume) haemoglobin [*Gossypium hirsutum*]	8.97/18 442	gil3913789	203	4	P***	P***		
18	Phosphogluconate dehydrogenase (decarboxylating) (EC 1.1.1.44) cytosolic [*Zea mays*]	5.92/53 055	T01658	744	13			N**, P**	N**, P**
19	Phosphogluconate dehydrogenase (decarboxylating) (EC 1.1.1.44) (fragment) [*Zea mays*]	10.00/8528	T01660	186	2				N**
20	Ferredoxin-NADP reductase (EC 1.18.1.2) precursor [*Zea mays*]	8.37/36 375	S53305	210	13			N*	N***
21	Ferredoxin-NADP reductase (EC 1.18.1.2) precursor root (fragment) [*Zea mays*]	8.37/36 375	S53305	432	12		P***		N**
22	$NADH_2$ dehydrogenase (ubiquinone) (EC 1.6.99.3) flavoprotein 1 precursor [*Solanum tuberosum*]	8.45/53 618	S52261	117	8		P**		N**, P**
Category 06 Protein destination and storage									
23	Mitochondrial processing peptidase (MPP) (EC 3.4.24.64) alpha-chain [*Dactylis glomerata*]	6.53/53 377	Q9FNU9	250	5	N**	N**	N**	N**
24	Putative disulphide-isomerase (EC 5.3.4.1) [*Oryza sativa*]	5.01/56 854	Q53LQ0	257	4		P**		
19	26S protease regulatory subunit 7 [*Oryza sativa*]	6.03/47 682	Q9FXT9	176	9				N**
Category 07 Transporters									
25	H^+-transporting two-sector ATPase (EC 3.6.3.14) alpha chain mitochondrion [*Triticum aestivum*]	5.70/55 306	Q36567	604	14		N***	N***, P***	N***, P***
Category 09 Cell structure									
26	Reversibly glycosylated polypeptide [*Triticum aestivum*]	5.82/41 498	gil4158232	517	12		N**, P**	N**, P**	N**, P**
27	Putative oxidase [*Oryza sativa*]	8.93/74 298	Q9ZQP2	147	2		N***, P**		N***, P**
28	Putative oxidase [*Oryza sativa*]	8.93/74 298	Q9ZQP2	133	2		N***		N**
Category 10 Signal transduction									
29	GTP-binding protein [*Oryza sativa*]	8.39/68 030	Q8W315	260	12	N***	N***, P***	N**	N***, P**
30	GTP-binding protein beta chain homologue curled-leaved [*Nicotiana tabacum*]	7.02/36 006	T16970	251	4		N*		
31	GTP-binding protein beta chain *Nicotiana tabacum*]	7.02/36 006	T16970	94	6		N**	N**	
32	Nucleoside diphosphate kinase (EC 2.7.4.6) [*Pinus pinaster*]	8.38/26 144	Q8RVI6	164	2	N**	N**	N**	N***, P***
Category 11 Disease/defence									
33	Probable peroxidase (EC 1.11.1.-) 1 precursor anionic [*Zea mays*]	5.41/37 774	T04360	68	4	P***	N*, P***		N*
34	Probable peroxidase (EC 1.11.1.-) 1 precursor anionic [*Zea mays*]	5.41/37 774	T04360	69	3		N**		
35	Probable peroxidase (EC 1.11.1.-) 1 precursor anionic [*Zea mays*]	5.41/37 774	T04360	69	3		N***		

Table 2. *Continued*

ID	Protein identification [source]	H. pI/MW	Accession no.	PS	PM	Heat stress treatment			
						2 d		10 d	
						30 °C	40 °C	30 °C	40 °C
	Category 20 Secondary metabolism								
36	Phenylalanine ammonia-lyase (PAL) (fragment) (EC 4.3.1.5) [*Hordeum vulgare*]	5.73/54 073	T05968	427	11	N**	N***	N**, P*	N***, P*
37	PAL (EC 4.3.1.5) [*Hordeum vulgare*]	5.73/54 073	T05968	285	6	N***	P**	P***	N**, P***
38	dDTP-glucose 4–6-dehydratases-like protein [*Arabidopsis thaliana*]	7.09/38 389	T45701	297	7	P**	P***		N***, P***
39	Adenosylhomocysteinase (EC 3.3.1.1) [*Triticum aestivum*]	5.65/53 436	T06764	560	11		N**, P**	N**, P**	N**, P**
40	*S*-Adenosylmethionine synthase (SAMS) (EC 2.5.1.6) [*Arabidopsis thaliana*]	5.51/42 795	Q9LUT2	437	8		N***, P***	N***, P***	N***, P***
41	SAMS (EC 2.5.1.6) [*Dendrobium crumenatum*]	5.42/43 209	Q944U4	906	12		N***, P***	N***, P***	N***, P***
	Category 12 Unclear classification								
42	AB019533 NID [*Oryza sativa*]	6.68/41 341	BAA77337	445	9	N**, P***	N**	N**, P***	N**, P**
43	AY135561 NID [*Arabidopsis thaliana*]	8.02/43 358	AAN15218	191	6			N***	N**
44	No confident ID						N***, P***		N***, P***
45	No confident ID								P**
46	No confident ID					N***	N***		
47	No confident ID							N**	N**
	Protein spots increased by heat stress								
	Category 01 Metabolism								
48	Phosphoserine aminotransferase (EC 2.6.1.52) [*Oryza sativa*]	8.53/44 931	Q8LMR0	243	7				N**, P***
49	Phosphoserine aminotransferase (EC 2.6.1.52) [*Oryza sativa*]	8.53/44 931	Q8LMR0	380	7				N***
50	Phosphoserine aminotransferase (EC 2.6.1.52) [*Oryza sativa*]	8.53/44 931	Q8LMR0	420	8				N***
51	Plastidic ATP sulphurylase (APS) (EC 2.7.7.4) [*Oryza sativa*]	9.00/52 354	Q9ZWM0	320	13				N**
	Category 02 Energy								
52	Sucrose synthase (EC 2.4.1.13) Ss1 [*Hordeum vulgare*]	5.94/92 211	S29242	354	23		N**	N*	
53	GAPDH (phosphorylation) (EC 1.2.1.12) [*Hordeum vulgare*]	6.20/33 235	P08477	850	8		N***	N**	N***, P**
54	GAPDH (phosphorylating) (EC 1.2.1.12) [*Hordeum vulgare*]	6.20/33 235	P08477	880	11		P**		N***, P***
55	Cytoplasmic FBP aldolase (EC 4.1.2.13) [*Oryza sativa*]	6.55/38 719	Q40676	237	10		N***	N***	N***
56	Cytoplasmic FBP aldolase. (EC 4.1.2.13) [*Oryza sativa*]	6.55/38 719	Q40676	217	10		N***		N***, P***
67	Mitochondrial aldehyde dehydrogenase (EC 1.2.1.3) [*Secale cereale*]	6.58/59 323	Q8LST6	190	6			N*	N*, P**
	Category 05 Protein biosynthesis								
57	Putative asparagine-tRNA ligase (EC 6.1.1.22) [*Oryza sativa*]	5.68/62 588	Q93WM3	281	10				N**
	Category 06 Protein destination and storage								
58	Cyclophilin A-2 (EC 5.2.1.8) (peptidyl-prolyl *cis–trans* isomerase) [*Triticum aestivum*]	8.52/18 379	Q93XQ6	108	3		P**	P***	N***, P***
65	Stress-induced protein (Os02g0644100) [*Oryza sativa*]	6.03/64 914	gil115447567	369	16		N***, P***	P**	N**, P***
66	Sti (stress-inducible protein) [*Glycine max*]	5.81/63 585	Q43468	178	4		N***, P***	N**	N***, P***
	Category 08 Intracellular traffic								
59	Ran (Small GTP-binding protein) (Ran2) [*Oryza sativa*]	6.66/25 038	Q9XJ45	601	10				N**, P**
60	GTP-binding nuclear protein Ran2 [*Arabidopsis thaliana*]	6.38/25 062	P41917	189	6		P***		N***, P***
	Category 11 Disease/defence								
61	Glutathione *S*-transferase GST 34 (EC 2.5.1.18) [*Zea may*]	5.63/24 573	Q9FQA5	80	4		N***	N***	N***
62	GST (EC 2.5.1.18) [*Triticum aestivum*]	5.79/23 338	Q9SP56	238	5		N***		N***, P***
63	GST (EC 2.5.1.18) [*Triticum aestivum*]	5.79/23 338	Q9SP56	176	4	P**		N**, P***	N***, P***
64	Superoxide dismutase (EC 1.15.1.1) (Mn) 3.2 precursor [*Zea mays*]	6.71/25 238	B48684	282	5				N***
	Category 20 Secondary metabolism								
68	UDP-glucose 6-dehydrogenase (EC 1.1.1.22) [*Glycine max*]	5.74/52 941	T08818	290	12		N**		
	Category 12 Unclear classification								
69	r40c1 protein [*Oryza sativa*]	6.30/38 822	Q40705	162	7		N*		N**
70	Os03g0737000 [*Oryza sativa*]	9.18/22 307	gil115455195	293	6				N*, P*

both moderate and severe heat stress. Twenty-seven spots were only affected by severe heat stress, while one spot (spot 13, down-regulated only in *A. stolonifera*) was only affected by moderate heat stress (Table 2).

Root phosphoproteomic responses to heat stress were also investigated; a representative image is presented in Fig. 4A. The phosphorylation level of two proteins increased under heat stress, to a greater extent in *A. scabra*

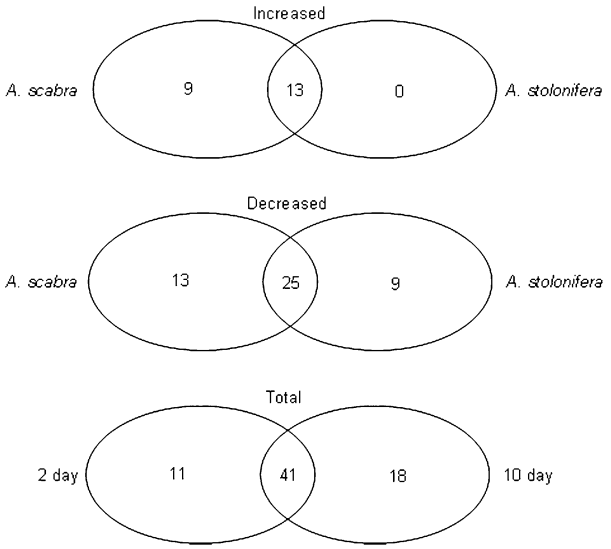

Fig. 3. Venn diagram illustrating the expression patterns of heat-responsive proteins in the roots of *Agrostis* grass.

than in *A. stolonifera*. The magnified regions of these two spots from different treatments are presented in Fig. 4B. The comparison of two images from the same gel by different staining methods showed that these two spots corresponded to spots 55 and 56 in the image of CBB-stained gels. These two protein spots exhibited a higher intensity by the Pro-Q DPS staining method than by the CBB staining method.

The 70 differentially accumulated protein spots were digested with trypsin, subjected to MALDI TOF/TOF MS, and 66 protein spots were identified. The results are listed in Table 2. Most spots contained only one protein, while one spot contained two proteins (spot 19: phosphogluconate dehydrogenase and 26S protease regulatory subunit 7). The identified proteins were classified according to the functional categories described by Bevan *et al.* (1998): they belonged to the categories of metabolism, energy, protein destination/storage, protein synthesis, transporters, intracellular traffic, disease/defence, and secondary metabolism (Tables 2 and 3).

Discussion

Higher root viability in thermal *A. scabra* under heat stress suggests that *A. scabra* had superior thermotolerance to *A. stolonifera*. This result is in agreement with results from previous studies (Rachmilevitch *et al.*, 2006*a*, *b*). Superior root thermotolerance in thermal *A. scabra* could be associated with the expression of certain heat-responsive proteins. In fact, the proteomic response to heat stress varied between the two species, and the differentially accumulated proteins have diverse functions, as shown in Table 3 and discussed below.

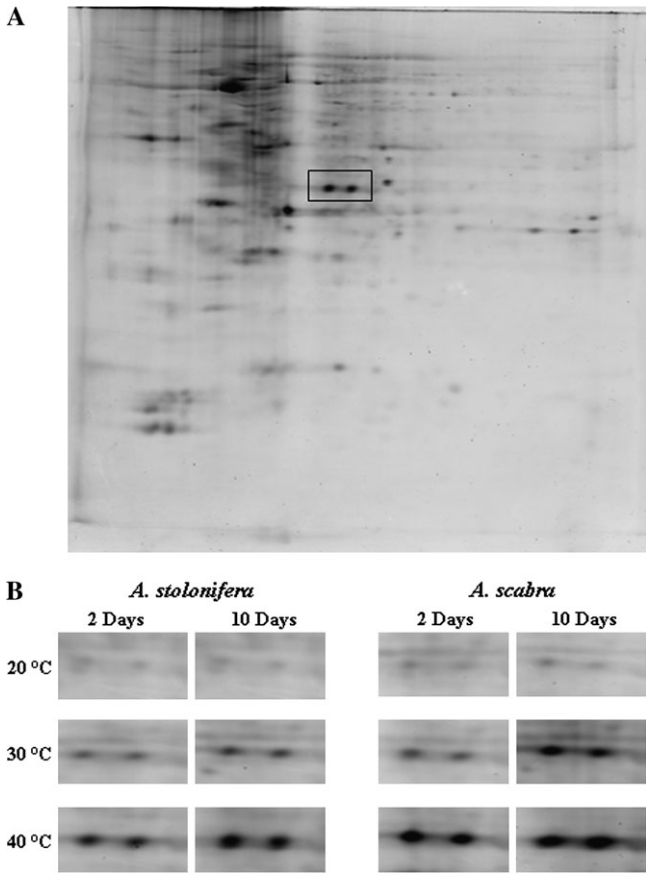

Fig. 4. Pro-Q DPS-stained 2-D polyacrylamide gel of separated proteins from *A. scabra* roots growing under 40 °C at 10 d of treatment (A), and magnified regions of differentially phosphorylated protein spots in two species growing under different temperatures (B).

Metabolism category

This category included 11 protein spots regulated by heat stress in at least one species. The down-regulated proteins are cytosolic glutamine synthetase (GS; spot 4), methionine synthase (spots 1–3), serine hydroxymethyltransferase (SHMT; spots 5 and 6), and nucleotide-sugar dehydratase (spot 7). All seven spots were decreased in both species, except spot 6 which decreased only in *A. stolonifera*. GS catalyses the assimilation of ammonium to glutamine using glutamic acid as its substrate (Chen and Silflow, 1996). Reduction of GS under stress conditions has been reported, and this may be a protective mechanism because nitric oxide, an intermediate of nitrogen assimilation, is an active radical (Wang *et al.*, 2004; Yan *et al.*, 2005; Xu *et al.*, 2008*a*). However, Sahu *et al.* (2001) reported that GS activities increased and decreased under salt stress in tolerant and sensitive rice leaves, respectively. Plomion *et al.* (2006) also found that GS protein was increased by drought in leaves of poplar (*Populus alba* L.). El-Khatib *et al.* (2004) reported that overexpression of cytosolic GS in poplar enhanced photorespiration during drought and could contribute to

Table 3. *Functional distribution of protein spots responsive to heat stress*

Proteins were grouped according to the functional categories described by Bevan *et al*. (1998). Protein spot 19, containing two proteins, and spot 52, which was decreased in *A. stolonifera* and increased in *A. scabra*, were each counted twice.

Heat effect	Total number of protein spots											
	Metabolism	Energy	Protein biosynthesis	Protein destination and storage	Transporters	Intracellular traffic	Cell structure	Signal transduction	Disease defence	Secondary metabolism	Unclear classification	Total
Decrease	7	16	0	3	1	0	3	4	3	6	6	49
Increase	4	6	1	3	0	2	0	0	4	1	2	23

the protection of photosynthesis. Methionine synthase catalyses the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine, resulting in the formation of methionine. SHMT catalyses interconversion of serine and glycine. The down-regulation of these proteins suggests that heat stress suppressed amino acid synthesis, including methionine, serine, and glycine in roots of the two cool-season grass species. One spot (spot 48) of phosphoserine aminotransferase was up-regulated in both species. Phosphoserine aminotransferase is the enzyme catalysing the second step in serine biosynthesis.

There are variations in the response of phosphoserine aminotransferase (spots 49 and 50) and plastidic ATP sulphurylase (APS; spot 51) to heat stress between the two species. APS catalyses the first step in sulphur assimilation. The up-regulation of APS at the transcription and protein levels under metal stress was reported (Roth *et al*., 2006; Weber *et al*., 2006). Enhanced sulphur assimilation may increase glutathione generation for active oxygen species scavenging. APS and two spots (spots 49 and 50) of phosphoserine aminotransferase were up-regulated only in roots of thermal *A. scabra* following 10 d of moderate or severe heat stress, suggesting the importance of serine and sulphur metabolism in root thermotolerance, particularly during prolonged periods of stress.

Energy category

In this category, 21 protein spots were altered by heat stress. Among the 21 proteins, four spots [spots 53 and 54, glyceraldehyde-3-phosphate dehydrogenase (GAPDH); spots 55 and 56, fructose-bisphosphate (FBP) aldolase] were up-regulated while 16 spots were down-regulated (Table 2). Thirteen protein spots involved in carbon degradation and the electron transport chain in mitochondria were down-regulated by heat stress, including aconitate hydratase (spot 8), fumarase (spot 9), malate dehydrogenase (spot 10), sucrose synthase (spot 52), pyrophosphate-dependent phosphofructokinase (spot 11), pyruvate kinase (spots 12 and 13), FBP aldolase (spots 14 and 15), GAPDH (spot 16), phosphogluconate dehydrogenase (spots 18 and 19), and $NADH_2$ dehydrogenase (spot 22). The down-regulation of these proteins involved in respiration may contribute to root adaptation to heat stress by lowering respiratory energy consumption (Rachmilevitch *et al*., 2006*a*, *b*). In addition, these results suggest the sensitivity of root respiration to heat stress.

Agrostis scabra and *A. stolonifera* had different response patterns of sucrose synthase (SS) and GAPDH to heat stress. The SS was down-regulated in *A. stolonifera* while it was up-regulated in thermal *A. scabra* by heat stress. SS catalyses both synthesis and degradation of sucrose (Geigenberger and Stitt, 1993), but the degradation process dominates *in vivo*. SS in the cytosol is thought to supply UDP-glucose and fructose produced by sucrose cleavage for glycolysis, and possibly starch synthesis. The expression of SS was enhanced under low O_2 or low temperature, and the increase in the activity of SS was suggested to contribute to low O_2 or low temperature tolerance (Crespi *et al*., 1991; Harada and Ishizawa, 2003; Harada *et al*., 2005). The increased accumulation of SS in thermal *A. scabra* may contribute to superior root thermotolerance by regulating sucrose metabolism. In this study, GAPDH was present in three spots (spots 16, 53, and 54). The intensity of spot 16 decreased only in *A. stolonifera* while that of another two spots (spots 53 and 54) increased in both grass species. All three spots are abundant proteins. Interestingly, thermal *A. scabra* had a higher level of spot 54 and

a lower level of spot 53 than *A. stolonifera*, which may be due to the different cellular locations and functions of these isoforms. In addition to catalysing a reaction in glycolysis, GAPDH has been shown to exhibit protein kinase activity (Duclos-Vallee *et al*., 1998), bind RNA (Nagy and Rigby, 1995), suppress the production of active oxygen species (Baek *et al*., 2008), and enhance ribozyme (Sioud and Jespersen, 1996) and phosphotransferase activities (Engel *et al*., 1998). In leaves of *P. euphratica*, GAPDH increased under heat stress (Ferreira *et al*., 2006). Although many studies indicated that GAPDH was up-regulated under different stress conditions (Yang *et al*., 1993; Chang *et al*., 2000), little is known about how GAPDH in involved in the defence mechanism against heat stress. Elucidation of the multifaceted properties of this protein during heat stress would help to understand how this protein regulates thermotolerance.

The levels of FBP aldolase phosphorylation under heat stress were also different between the two species. FBP aldolase catalyses a glycolysis reaction in which FBP is broken down into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Higher plants contain two isoforms, one in the cytosol and the other in the chloroplasts (Lebherz *et al*., 1984). Riccardi *et al*. (1998) reported that FBP aldolase was increased by water deficit in maize (*Zea mays*) leaves. In the present study, four protein spots were identified as FBP aldolase. Two abundant spots (spots 14 and 15) exhibited down-regulation while two weak spots (spots 55 and 56) showed up-regulation under heat stress. Interestingly, aldolase in these two weak spots was greatly phosphorylated in both species by heat stress. Also, the phosphorylation occurred early during 2 d of heat stress and thermal *A. scabra* had a greater level of phosphorylation than *A. stolonifera*. The phosphorylation of these two FBP aldolase isoforms might be related to the defence mechanism against heat stress. However, little is known about the function of FBP aldolase in plant response to stresses. It would be interesting to identify the kinase that phosphorylates FBP aldolase, and find out how aldolase and carbon metabolism are regulated in plants by FBP aldolase phosphorylation.

Protein destination and storage category

This category had six protein spots regulated by heat stress. Spots 19 (26S protease regulatory subunit 7) and 23 [mitochondrial processing peptidase (MPP)] were down-regulated only in thermal *A. scabra*, and spot 24 (disulphide-isomerase) was down-regulated only in *A. stolonifera*, while spots 65 and 66 (HSP Sti) and spot 58 [peptidyl-prolyl *cis–trans* isomerase (PPIase)] were up-regulated in both grass species. However, spot 19 contained two proteins (phosphogluconate dehydrogenase and 26S protease regulatory subunit 7). Heat stress may affect one or both of the proteins contained in this spot. PPIase accelerates the folding of proteins. It catalyses the *cis–trans* isomerization of proline imidic peptide bonds in oligopeptides. Little is known about the function of PPIases under heat stress conditions.

Interestingly, MPP was down-regulated only in thermal *A. scabra*, and disulphide-isomerase was down-regulated only in *A. stolonifera*. Most mitochondrial proteins encoded in the nucleus are synthesized as precursor proteins with extension peptides and are targeted to the mitochondria. After import of the precursors into the mitochondria, the extension peptides are cleaved off by MPP. This protein was also decreased by drought in roots of poplar (Plomion *et al*., 2006). In this study, MPP was decreased only in thermal *A. scabra* under both moderate and severe heat stress. How changes in the expression of MPP under heat stress are involved in root thermotolerance requires further investigation. Disulphide-isomerase catalyses the rearrangement of -S–S- bonds in proteins and participates in the folding of proteins containing disulphide bonds. The down-regulation of this protein only in *A. stolonifera* indicates that heat damage in roots may be related to the disruption of protein folding associated with the degradation of disulphide-isomerase. HSP Sti, also known as stress-inducible protein Sti, contains two heat shock chaperonin-binding motif (STI1), three tetratricopeptide repeat (TPR), and two Sti1 domains. It is believed that the function of TPR-containing proteins is mediated through protein–protein interaction to modulate diverse cellular processes, including Hsp90 signalling and interaction (Flom *et al*., 2006), protein transport across mitochondria (Chan *et al*., 2006), regulation of meristem cellular organization (Guyomarc'h *et al*., 2004), and gibberellin signalling (Izhaki *et al*., 2001). This protein was up-regulated in response to salt stress (Dooki *et al*., 2006). In this study, it was also up-regulated by heat stress, and thermal *A. scabra* had a higher level of this protein than heat-sensitive *A. stolonifera*, suggesting its positive relationship with root thermotolerance.

Stress defence category

Seven protein spots in this category were altered by heat stress (Table 3). Spots 34 and 35 were down-regulated only in thermal *A. scabra* and spot 33 was down-regulated in both species; all three spots were identified as peroxidase. Spots 61 [glutathione *S*-transferase (GST)] and 64 [superoxide dismutase (SOD)] were increased only in thermal *A. scabra*, and spots 62 and 63 (GST) were up-regulated in both species. GST is an abundant protein and has functions in conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles. Recent studies have also suggested GSTs as components of ultraviolet-inducible cell signal pathways and as potential regulators of apoptosis (Dixon *et al*., 2002). The plant-specific phi class might counteract the consequences of generation of reactive oxygen species during photosynthesis (Edwards *et al*., 2000). The

increased expression of GSTs has been identified in several proteomics or transcription analyses of plants that were exposed to different stresses (Dixon *et al*., 2002; Roth *et al*., 2006; Gazanchian *et al*., 2007; Yang *et al*., 2007), although Plomion *et al*. (2006) reported that it was reduced by drought in poplar roots. Hajheidari *et al*. (2007) reported that drought stress increased GST in a tolerant cultivar of sugar beet (*Beta vulgaris* L.) while it decreased it in a sensitive cultivar.

In this study, one spot (spot 61) of GST was induced by heat stress only in thermal *A. scabra*, and another two spots (spots 62 and 63) had higher intensity in thermal *A. scabra* than in *A. stolonifera* under heat stress. The higher GST level in *A. scabra* may lead to lower production of active oxygen species, resulting in superior root thermotolerance. Also, the two species had variation in the levels of SOD and peroxidase. SOD acts as the first line of defence converting superoxide to the less toxic hydrogen peroxide molecule. In the present study, thermal *A. scabra* had a higher level of SOD than *A. stolonifera* under heat stress. In addition to H_2O_2 detoxification, peroxidases are also implicated in various physiological processes such as auxin catabolism, liginfication, suberization, stress response, and senescence (Hiraga *et al*., 2001; Passardi *et al*., 2005). Three differentially accumulated spots (spots 33–35) were identified as peroxidase and all were decreased by heat stress. Interestingly, *A. stolonifera* only had spots 33 and 34, and the intensity of spot 33 was higher, while the intensity of spot 34 was lower in *A. stolonifera* than in thermal *A. scabra* under both control and stress conditions, indicating that the peroxidase isoforms presented in spots 34 and 35 might be important for heat tolerance. The higher level of SOD and some isoforms of peroxidase in roots of thermal *A. scabra* may contribute to the superior thermotolerance by suppressing the production of active oxygen species.

Secondary metabolism category

In this category, seven protein spots were affected by heat stress, of which one exhibited up-regulation (spot 68, UDP-glucose 6-dehydrogenase) only in thermal *A. scabra* and six [spots 36 and 37, phenylalanine ammonia-lyase (PAL); spot 38, dDTP-glucose 4–6-dehydratases-like protein; spot 39, adenosylhomocysteinase; spots 40 and 41, *S*-adenosylmethionine synthase (SAMS)] showed down-regulation in both species. PAL is a key enzyme in plant secondary metabolism, catalysing the first reaction in the biosynthesis from L-phenylalanine to a wide variety of natural products based on the phenylpropane skeleton. SAMS catalyses the production of *S*-adenosyl-L-methionine (SAM) from L-methionine and ATP. SAM serves as a methyl group donor in numerous transmethylation reactions and is the precursor for the biosynthesis of polyamines and ethylene among other metabolites. Several authors have shown that the SAMS gene and/or enzyme activity are stimulated under different stress conditions, suggesting the induction of lignification during stress (Chang *et al*., 1995; Yan *et al*., 2006). However, other studies indicated that the protein and transcript levels of SAMS were decreased under salt and mental stress (Jiang *et al*., 2007; Yang *et al*., 2007). The roles of dDTP-glucose 4–6-dehydratases-like protein, adenosylhomocysteinase, and UDP-glucose 6-dehydrogenase in plant tolerance of heat stress are unclear.

Other proteins

Nucleoside diphosphate kinase (NDPK) is believed to use ATP to maintain cellular levels of CTP, GTP, and UTP. It is also associated with H_2O_2-mediated mitogen-activated protein kinase (MAPK) signalling (Moon *et al*., 2003). The up-regulation of NDPK has been reported in response to drought (Salekdeh *et al*., 2002; Hajheidari *et al*., 2005), cold (Imin *et al*., 2004), heat, and salt stress (Dooki *et al*., 2006; Lee *et al*., 2007). However, in this study it was down-regulated by heat stress. Ran is an evolutionarily conserved eukaryotic GTPase, which is likely to be involved in protein import into the nucleus and RNA export from the nucleus, in chromatin condensation, and in cell cycle control (Kahana and Cleveland, 1999; Yang, 2002). However, little is known about the function of Ran in plant response to stresses. It was found that its abundance was increased under salt and heat stress (Ferreira *et al*., 2006; Jiang *et al*., 2007). In this study it was also increased by heat stress, and thermal *A. scabra* had a higher level than *A. stolonifera*, suggesting that Ran could play roles in nucleocytoplasmic interactions under heat stress.

In summary, different proteomic profiles were detected between thermal *A. scabra* and heat-sensitive *A. stolonifera* under heat stress, and more proteins were up-regulated in *A. scabra* than in *A. stolonifera*. The higher levels of SS, GST, SOD, Sti, and some peroxidase isoforms in thermal *A. scabra* could be related to its superior root thermotolerance relative to *A. stolonifera*. In addition, phosphorylation of FBP aldolase isoforms may also contribute to better root thermotolerance in *A. scabra*. Genes encoding these differentially regulated proteins between the two grass species may be further investigated using molecular approaches, which may provide the molecular basis of root thermotolerance in cool-season grass species.

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References

Agrawal GK, Thelen JJ. 2005. Development of a simplified economical polyacrylamide gel staining protocol for phosphoproteins. *Proteomics* **5,** 4684–4688.

Arabidopsis Genome Initiative. 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408,** 796–815.

Baek D, Jin Y, Jeong JC, *et al*. 2008. Suppression of reactive oxygen species by glyceraldehyde-3-phosphate dehydrogenase. *Phytochemistry* **69,** 333–338.

Bentem SF, Hirt H. 2007. Using phosphoproteomics to reveal signalling dynamics in plants. *Trends in Plant Science* **12,** 404–411.

Bevan M, Bancroft I, Bent E, *et al*. 1998. Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*. *Nature* **391,** 485–488.

Bradford MM. 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry* **72,** 248–54.

Chan NC, Likić VA, Waller RF, Mulhern TD, Lithgow T. 2006. The C-terminal TPR domain of Tom70 defines a family of mitochondrial protein import receptors found only in animals and fungi. *Journal of Molecular Biology* **358,** 1010–1022.

Chang S, Puryear JD, Dias MADL, Funkhouser EA, Newton RJ, Cairney J. 1995. Gene expression under water deficit in loblolly pine (*Pinus taeda* L.): isolation and characterization of cDNA clones. *Physiologia Plantarum* **95,** 1–10.

Chang WWP, Huang L, Shen M, Webster C, Burlingame AL, Roberts JKM. 2000. Patterns of protein synthesis and tolerance of anoxia in root tips of maize seedlings acclimated to a low-oxygen environment, and identification of proteins by mass spectrometry. *Plant Physiology* **122,** 295–317.

Chen Q, Silflow CD. 1996. Isolation and characterization of glutamine synthetase genes in *Chlamydomonas reinhardtii*. *Plant Physiology* **112,** 987–996.

Crespi MD, Zabaleta EJ, Pontis HG, Salerno GL. 1991. Sucrose synthase expression during cold acclimation in wheat. *Plant Physiology* **96,** 887–891.

Dixon DP, Lapthorn A, Edwards R. 2002. Plant glutathione transferases. *Genome Biology* **3,** REVIEWS3004.

Dooki AD, Mayer-Posner FJ, Askari1 H, Zaiee A, Salekdeh GH. 2006. Proteomic responses of rice young panicles to salinity. *Proteomics* **6,** 6498–6507.

Duclos-Vallee JC, Capel F, Mabit H, Petit MA. 1998. Phosphorylation of the hepatitis B virus core protein by glyceraldehyde-3-phosphate dehydrogenase protein kinase activity. *Journal of General Virology* **79,** 1665–1670.

Edwards R, Dixon D, Walbot V. 2000. Plant glutathione *S*-transferases: enzymes with multiple functions in sickness and in health. *Trends in Plant Science* **5,** 193–198.

El-Khatib RT, Hamerlynck EP, Gallardo F, Kirby EG. 2004. Transgenic poplar characterized by ectopic expression of a pine cytosolic glutamine synthetase gene exhibits enhanced tolerance to water stress. *Tree Physiology* **24,** 729–736.

Engel M, Seifert M, Theisinger B, Seyfert U, Welter C. 1998. Glyceraldehyde-3-phosphate dehydrogenase and Nm23-H1/nucleoside diphosphate kinase A: two old enzymes combine for the novel Nm23 protein phosphotransferase function. *Journal of Biological Chemistry* **273,** 20058–20065.

Ferreira S, Hjernø K, Larsen M, Wingsle G, Larsen P, Fey S, Roepstorff P, Salomé Pais M. 2006. Proteome profiling of *Populus euphratica* Oliv. upon heat stress. *Annals of Botany* **98,** 361–377.

Flom G, Weekes J, Williams JJ, Johnson JL. 2006. Effect of mutation of the tetratricopeptide repeat and asparatate–proline domains of Sti1 on Hsp90 signaling and interaction in *Saccharomyces cerevisiae*. *Genetics* **172,** 41–51.

Gazanchian A, Hajheidari M, Sima NK, Salekdeh GH. 2007. Proteome response of *Elymus elongatum* to severe water stress and recovery. *Journal of Experimental Botany* **58,** 291–300.

Geigenberger P, Stitt M. 1993. Sucrose synthase catalyses a readily reversible reaction *in vivo* in developing potato tubers and other plant tissues. *Planta* **189,** 329–339.

Guyomarc'h S, Vernoux T, Traas J, Zhou D, Delarue M. 2004. *MGOUN3*, an *Arabidopsis* gene with tetratrico-peptide-repeat-related motifs, regulates meristem cellular organization. *Journal of Experimental Botany* **55,** 673–684.

Hajheidari M, Salekdeh GH, Heidari M, Abdollahian-Noghabi M, Sadeghian SY. 2005. Proteome analysis of sugar beet leaves under drought stress. *Proteomics* **5,** 950–960.

Hajheidari M, Eivazi A, Buchanan BB, Wong JH, Majidi I, Salekdeh GH. 2007. Proteomics uncovers a role for redox in drought tolerance in wheat. *Proteome Research* **6,** 1451–1460.

Harada T, Ishizawa K. 2003. Starch degradation and sucrose metabolism during anaerobic growth of pondweed (*Potamogeton distinctus* A. Benn.) turions. *Plant and Soil* **253,** 125–135.

Harada T, Satoh S, Yoshioka T, Ishizawa K. 2005. Expression of sucrose synthase genes involved in enhanced elongation of pondweed (*Potamogeton distinctus*) turions under anoxia. *Annals of Botany* **96,** 683–692.

Hiraga S, Sasaki K, Ito H, Ohashi Y, Matsui H. 2001. A large family of class III plant peroxidases. *Plant and Cell Physiology* **42,** 462–468.

Hoagland DR, Arnon DI. 1950. The water-culture method for growing plants without soil. *California Agricultural Experiment Station Circular* 247.

Imin N, Kerim T, Weinman JJ, Rolfe BG. 2004. Effect of early cold stress on the maturation of rice anthers. *Proteomics* **4,** 1873–1882.

Izhaki A, Swain SM, Tseng T, Borochov A, Olszewski NE, Weiss D. 2001. The role of SPY and its TPR domain in the regulation of gibberellin action throughout the life cycle of *Petunia hybrida* plants. *The Plant Journal* **28,** 181–190.

Jiang Y, Yang B, Harris NS, Deyholos MK. 2007. Comparative proteomic analysis of NaCl stress-responsive proteins in *Arabidopsis* roots. *Journal of Experimental Botany* **58,** 3591–3607.

Kahana JA, Cleveland DW. 1999. Beyond nuclear transport. RAN-GTP as a determinant of spindle assembly. *Journal of Cell Biology* **146,** 1205–1210.

Kalume DE, Molina H, Pandey A. 2003. Tackling the phosphoproteome: tools and strategies. *Current Opinion in Chemical Biology* **7,** 64–69.

Kerk K, Bulgrien J, Smith DW, Barsam B, Veretnik S, Gribskov M. 2002. The complement of protein phosphatase catalytic subunits encoded in the genome of *Arabidopsis*. *Plant Physiology* **129,** 908–925.

Knievel DP. 1973. Procedures for estimating ratio of live or dry matter in root samples. *Crop Science* **13,** 124–126.

Labra M, Gianazza E, Waitt R, Eberini I, Sozzi A, Grassi F, Agradi E. 2006. *Zea mays* L. protein changes in response to potassium dichromate treatments. *Chemosphere* **62,** 1234–1244.

Lebherz HG, Leadbetter MM, Bradshaw RA. 1984. Isolation and characterization of the cytosolic and chloroplast form of spinach leaf fructose diphosphate aldolase. *Journal of Biological Chemistry* **259,** 1011–1017.

Lee DG, Ahsan N, Lee S, Kang KY, Bahk JD, Lee I, Lee B. 2007. A proteomic approach in analyzing heat-responsive proteins in rice leaves. *Proteomics* **7,** 3369–3383.

Liu X, Huang B. 2005. Root physiological factors involved in creeping bentgrass response to high soil temperatures. *Environmental and Experimental Botany* **53,** 233–245.

Mizoguchi T, Irie K, Hirayama T, Hayashida N, Yamaguchi-Shinozaki K, Matsumoto K, Shinozaki K. 1996. A gene

encoding a mitogen-activated protein kinase kinase kinase is induced simultaneously with genes for a mitogen-activated protein kinase and an S6 ribosomal protein kinase by touch, cold, and water stress in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences, USA* **93,** 765–769.

Moon H, Lee B, Choi G, *et al*. 2003. NDP kinase interacts with two oxidative stress-activated MAPKs to regulate cellular redox state and enhances multiple stress tolerance in transgenic plants. *Proceedings of the National Academy of Sciences, USA* **100,** 358–363.

Nagy E, Rigby WFC. 1995. Glyceraldehyde-3-phosphate dehydrogenase selectively binds Au-rich RNA in the NAD^+-binding region (Rossmann fold). *Journal of Biological Chemistry* **270,** 2755–2769.

Newsholme SJ, Maleeft BF, Steiner S, Anderson NL, Schwartz LW. 2000. Two-dimensional electrophoresis of liver proteins: characterization of a drug-induced hepatomegaly in rats. *Electrophoresis* **21,** 2122–2128.

Passardi F, Cosio C, Penel C, Dunand C. 2005. Peroxidases have more functions than a Swiss army knife. *Plant Cell Reporter* **24,** 255–265.

Pinheiro C, Kehr J, Ricardo CP. 2005. Effects of water stress on lupin stem protein analysed by two-dimensional gel electrophoresis. *Planta* **221,** 716–728.

Plomion C, Lalanne C, Claverol S, Meddour H, Kohler A, Bogeat-Triboulot M. 2006. Mapping the proteome of poplar and application to the discovery of drought-stress responsive proteins. *Proteomics* **6,** 6509–6527.

Pote J, Wang Z, Huang B. 2006. Timing and temperature of physiological decline for creeping bentgrass. *Journal of the American Society of Horticultural Science* **131,** 608–615.

Rachmilevitch S, Huang B, Lambers H. 2006*a*. Assimilation and allocation of carbon and nitrogen of thermal and nonthermal *Agrostis* species in response to high soil temperature. *New Phytologist* **170,** 479–490.

Rachmilevitch S, Lambers H, Huang B. 2006*b*. Root respiratory characteristics associated with plant adaptation to high soil temperature for geothermal and turf-type *Agrostis* species. *Journal of Experimental Botany* **57,** 623–631.

Riccardi F, Gazeau P, Vienne D, Zivy M. 1998. Protein changes in response to progressive water deficit in maize. Quantitative variation and polypeptide identification. *Plant Physiology* **117,** 1253–1263.

Roth U, Roepenack-Lahaye E, Clemens S. 2006. Proteome changes in *Arabidopsis thaliana* roots upon exposure to Cd^{2+}. *Journal of Experimental Botany* **57,** 4003–4013.

Sahu AC, Sahoo SK, Sahoo N. 2001. NaCl-stress induced alteration in glutamine synthetase activity in excised senescing leaves of a salt-sensitive and a salt-tolerant rice cultivar in light and darkness. *Plant Growth Regulation* **34,** 287–292.

Salekdeh GH, Siopongco J, Wade LJ, Ghareyazie B, Bennett J. 2002. Proteomic analysis of rice leaves during drought stress and recovery. *Proteomics* **2,** 1131–1145.

Sioud M, Jespersen L. 1996. Enhancement of hammerhead ribozyme catalysis by glyceraldehyde-3-phosphate dehydrogenase. *Journal of Molecular Biology* **257,** 775–789.

Stout RG, Al-Niemi TS. 2002. Heat tolerant flowering plants of active geothermal areas in Yellowstone National Park. *Annals of Botany* **90,** 259–267.

Tercek MT, Hauber D, Darwin SP. 2003. Genetic and historical relationships among geothermally adapted *Agrostis* (Bentgrass) of North America and Kamchatka: evidence for a previously unrecognized thermally adapted taxon. *American Journal of Botany* **90,** 1306–1312.

Wahid A, Gelani S, Ashraf M, Foolad MR. 2007. Heat tolerance in plants: an overview. *Environmental and Experimental Botany* **61,** 199–223.

Wang SB, Chen F, Sommerfeld M. 2004. Proteomic analysis of molecular response to oxidative stress by the green alga *Haematococcus pluvialis* (Chlorophyceae). *Planta* **220,** 17–29.

Weber M, Trampczynska A, Clemens S. 2006. Comparative transcriptome analysis of toxic metal responses in *Arabidopsis thaliana* and the Cd^{2+}-hypertolerant facultative metallophyte *Arabidopsis halleri*. *Plant, Cell and Environment* **29,** 950–963.

Xiong L, Yang Y. 2003. Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. *The Plant Cell* **15,** 745–59.

Xu Q, Huang B. 2001. Lowering soil temperatures improves creeping bentgrass growth under heat stress. *Crop Science* **41,** 1878–1883.

Xu C, Sullivan JH, Garrett WM, Caperna TJ, Natarajan S. 2008*a*. Impact of solar ultraviolet-B on the proteome in soybean lines differing in flavonoid contents. *Phytochemistry* **69,** 38–48.

Xu C, Xu Y, Huang B. 2008*b*. Protein extraction for 2-dimensional gel electrophoresis of proteomic profiling in turfgrass. Crop Science in press.

Yan S, Tang Z, Su W, Sun W. 2005. Proteomic analysis of salt stress-responsive proteins in rice root. *Proteomics* **5,** 235–244.

Yan S, Zhang Q, Tang Z, Su W, Sun W. 2006. Comparative proteomic analysis provides new insights into chilling stress responses in rice. *Molecular Cellular Proteomics* **5,** 484–496.

Yang Y, Kwon HB, Peng HP, Shih MC. 1993. Stress responses and metabolic regulation of glyceraldehyde-3-phosphate dehydrogenase genes in Arabidopsis. *Plant Physiology* **101,** 209–216.

Yang Z. 2002. Small GTPases: versatile signaling switches in plants. *The Plant Cell* **14,** S375–S388.

Yang Q, Wang Y, Zhang J, Shi W, Qian C, Peng X. 2007. Identification of aluminum-responsive proteins in rice roots by a proteomic approach: cysteine synthase as a key player in Al response. *Proteomics* **7,** 737–749.