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 C3 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., 2008a), 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
such as economically important cultivars. Several C3 grass species able to maintain high root viability and new root production under stressful conditions, and to identify heat-regulated proteins associated with thermotolerance in roots of cool-season grasses.


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 C3 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 C3 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., 2006a, 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 replicates), 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. (2008b). 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)].
(TCA) and 0.07% 2-mercaptoethanol in acetonitrile] 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 μg of proteins. The voltage settings for IEF were 500 V for 1 h, 1000 V for 1 h, and 8000 V to a total of 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, Piscataway, NJ, USA) with bovine serum albumin (BSA) as a standard.

**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% trifluoroacetic acid (TFA) followed by C18 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. 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 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.
Proteomic responses to heat stress in grass roots

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         | 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 |
|            |                                                                     |          |               |     |            |      |      |      |
|            |                                                                     |          |               |     | 2 d        |      |      |      |
|            |                                                                     |          |               |     | 10 d       |      |      |      |

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 92 Q8W0Q7 351 9 N*** P*** N**, P***
3 Methionine synthase protein (EC 2.1.1.14) [Sorghum bicolor] 5.93/83 788 211 15 P** P** N**, P***
4 Cytosolic glutamine synthetase (EC 6.3.1.2) [Populus alba/Populus tremula] 6.61/84 429 gi37956277 209 5 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***
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*** N**, P*** N**, P***

Category 02 Energy
8 Cytoplasmic aconitase 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) [Solomon tuberosum] 8.01/52 999 gi1488652 268 5 N*** N***
10 Malate dehydrogenase (EC 1.1.1.37) [Oryza sativa] 7.42/36 609 Q94J22 132 5 N** N**
11 Sucrose synthase (EC 2.4.1.13) (fragment) [Hordeum vulgare] 5.94/92 211 S29242 354 23 P*** P*** P***
12 Pyrophosphate-dependent phosphofructokinase alpha subunit (EC 2.7.1.90) [Citrus paradise Grapefruit] 6.71/67 373 Q9ZST2 162 11 P*** P***
13 Pyruvate kinase (EC 2.7.1.40) [Glycine max] 7.50/55 302 T07787 176 5 P** P***
14 Pyruvate kinase (EC 2.7.1.40) [Solomon tuberosum] 6.64/55 170 P22200 238 7 P** P***
15 Fructose-bisphosphate (FBP) aldolase (EC 4.1.2.13) [Oryza sativa] 6.55/58 719 Q40676 692 11 P** P**
16 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (EC 1.2.1.12) cytosolic (fragment) [Hordeum vulgare] 6.20/33 235 A24159 885 8 P** P**
17 Non-symbiotic (non-legume) haemoglobin [Gossypium hirsutum] 8.97/18 442 gi3913789 203 4 P*** P***
18 Phosphoglucomate 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 dehydrogenase (ubiquione) (EC 1.6.99.3) flavoprotein 1 precursor [Solomon tuberosum] 8.45/53 618 S52261 117 8 P** N**, P***

Category 04 Protein destination and storage
23 Mitochondrial processing peptidase (MPP) (EC 3.4.24.64) alpha-chain [Dactylis glomerata] 6.53/53 377 Q5FN39 250 5 N** N** N**
24 Putative disulphide-isomerase (EC 5.3.4.1) [Oryza sativa] 5.01/56 854 Q53LQ0 257 4 P**
25 26S protease regulatory subunit 7 [Oryza sativa] 6.03/47 682 Q9FXT9 176 9 N**

Category 05 Transporters
26 H+-transporting two-sector ATPase (EC 3.6.3.14) alpha chain mitochondrion [Trichium aestivum] 5.70/55 306 Q36567 604 14 N*** N*** P*** N***, P***

Category 06 Cell structure
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 07 Signal transduction
29 GTP-binding protein [Oryza sativa] 8.39/60 830 Q8W315 260 12 N*** N*** N***, P***
30 GTP-binding protein beta chain homologue curled-leaved [Catharanthus roseus] 7.02/36 006 T16970 251 4 N** N** 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 Q8RV16 164 2 N** N** N**

Category 11 Disease/defense
33 Probable peroxidase (EC 1.11.1.3) precursor anionic [Zea mays] 5.41/37 774 T04360 68 4 P** N*, P*** N*
34 Probable peroxidase (EC 1.11.1.3) precursor anionic [Zea mays] 5.41/37 774 T04360 69 3 N** N**
35 Probable peroxidase (EC 1.11.1.3) precursor anionic [Zea mays] 5.41/37 774 T04360 69 3 N** N**
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* compared to *A. thaliana*.

### 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 |
| 36   | Phenylalanine ammonia-lyase (PAL) (fragment) ([*Hordeum vulgare*]) |          |               |      |     |        | N**   | N*** | N**   | N*** |
| 37   | [Arabidopsis thaliana]                                              |          |               |      |     |        | N***  | N**  | N***  | N**  |
| 38   | dDTP-glucose 4–6-dehydratases-like protein ([*Arabidopsis thaliana*]) |          |               |      |     |        | P**   | P*** | N***  | P*** |
| 39   | Adenosine deaminase ([*Triticum aestivum*])                         |          |               |      |     |        | N**   | P**  | N**   | P**  |
| 40   | 5-Adenosylmethionine synthase ([*Arabidopsis thaliana*])            |          |               |      |     |        | N***  | N*** | N***  | N*** |
| 41   | SAMs ([*Dendrobium crumenatum*])                                   |          |               |      |     |        | N***  | N*** | N***  | N*** |
| 42   | AB019533 [Oryza sativa]                                             |          |               |      |     |        | N**   | P*** | N**   | P*** |
| 43   | AY135561 [Arabidopsis thaliana]                                     |          |               |      |     |        | N***  | N**  | N***  | N**  |
| 44   | No confident ID                                                    |          |               |      |     |        | N***  | N*** | N***  | N*** |
| 45   | No confident ID                                                    |          |               |      |     |        | N***  | N*** | N***  | N*** |
| 46   | No confident ID                                                    |          |               |      |     |        | N***  | N*** | N***  | N*** |
| 47   | No confident ID                                                    |          |               |      |     |        | N***  | N*** | N***  | N*** |
| 48   | Phosphoserine aminotransferase ([*Oryza sativa*])                  |          |               |      |     |        | N**   | P*** | N**   | P*** |
| 49   | Phosphoserine aminotransferase ([*Oryza sativa*])                  |          |               |      |     |        | N**   | P*** | N**   | P*** |
| 50   | Mitochondrial aldehyde dehydrogenase ([*Oryza sativa*])            |          |               |      |     |        | N***  | N*** | N***  | N*** |
| 51   | Plasticid ATP sulphurylase ([*Oryza sativa*])                      |          |               |      |     |        | N**   | N**  | N**   | N**  |
| 52   | Sucrose synthase ([*Triticum aestivum*])                            |          |               |      |     |        | N**   | N*   | N**   | N*   |
| 53   | GAPDH (phosphorylation) ([*Triticum aestivum*])                    |          |               |      |     |        | N***  | N**  | N***  | N**  |
| 54   | GAPDH (phosphorylating) ([*Triticum aestivum*])                    |          |               |      |     |        | P**   | N*** | P**   | N*** |
| 55   | Cytoplasmic FBP aldolase ([*Oryza sativa*])                        |          |               |      |     |        | N***  | N*** | N***  | N*** |
| 56   | Cytoplasmic FBP aldolase ([*Oryza sativa*])                        |          |               |      |     |        | N***  | N*** | N***  | N*** |
| 57   | Putative asparagine-tRNA ligase ([*Oryza sativa*])                 |          |               |      |     |        | N**   | N**  | N**   | N**  |
| 58   | Cyclophilin A-2 ([*Hordeum vulgare*])                               |          |               |      |     |        | N**   | N*   | N**   | N*   |
| 59   | Glutathione S-transferase GST 34 ([*Zea mays*])                    |          |               |      |     |        | N***  | N*** | N***  | N*** |
| 60   | GTP-binding nuclear protein Ran2 ([*Arabidopsis thaliana*])         |          |               |      |     |        | N***  | N*** | N***  | N*** |
| 61   | Glutathione S-transferase GST 34 ([*Zea mays*])                    |          |               |      |     |        | N**   | P**  | N**   | P**  |
| 62   | Glutathione S-transferase GST 34 ([*Zea mays*])                    |          |               |      |     |        | N***  | N*** | N***  | N*** |
| 63   | Glutathione S-transferase GST 34 ([*Zea mays*])                    |          |               |      |     |        | N***  | N*** | N***  | N*** |
| 64   | Superoxide dismutase ([*Zea mays*])                                |          |               |      |     |        | N***  | N*** | N***  | N*** |
| 65   | Glutathione S-transferase GST 34 ([*Zea mays*])                    |          |               |      |     |        | N***  | N*** | N***  | N*** |
| 66   | Glutathione S-transferase GST 34 ([*Zea mays*])                    |          |               |      |     |        | N***  | N*** | N***  | N*** |
| 67   | Glutathione S-transferase GST 34 ([*Zea mays*])                    |          |               |      |     |        | N***  | N*** | N***  | N*** |
| 68   | UDP-glucose 6-dehydrogenase ([*Glycine max*])                      |          |               |      |     |        | N**   | N**  | N**   | N**  |
| 69   | r40c1 protein ([*Oryza sativa*])                                   |          |               |      |     |        | N**   | N**  | N**   | N**  |
| 70   | Os03g0737000 ([*Oryza sativa*])                                     |          |               |      |     |        | N**   | N**  | N**   | N**  |

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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., 2006a, 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.

**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., 2008a). 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
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 contribute to root adaptation to heat stress by lowering respiratory energy consumption (Rachmilevitch et al., 2006). 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 O2 or low temperature, and the increase in the activity of SS was suggested to contribute to low O2 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

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 | Transmitters | 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   |

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PPIase accelerates the folding of proteins. It catalyses the affect one or both of the proteins contained in this spot. 

contained two proteins (phosphogluconate dehydrogenase A. stolonifera 58 [peptidyl-prolyl cis–trans isomerase (PPIase)] were 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 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 (phosphoglucurate 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 (Florn et al., 2006), protein transport across mitochondria (Chan et al., 2006), regulation of meristem cellular organization (Guyomarch 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 H₂O₂ detoxification, peroxidases are also implicated in various physiological processes such as auxin catabolism, lignification, 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 H₂O₂-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 (Kawahara 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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