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Proteomic analysis of small heat shock protein isoforms in barley shoots

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Abstract

The analysis of stress-responsiveness in plants is an important route to the discovery of genes conferring stress tolerance and their use in breeding programs. High temperature is one of the environmental stress factors that can affect the growth and quality characteristics of barley (*Hordeum vulgare*). In this study a proteomic analysis (2D-PAGE, MS) was used to detect the effects of heat shock on the protein pattern of an abiotic stress-tolerant (Mandolina) and an abiotic stress-susceptible (Jubilant) barley cultivar. Evaluation of two-dimensional gels revealed several proteins to be differentially expressed as a result of heat stress in both cultivars. The protein spots of interest were, after an in-gel tryptic digestion, further investigated by mass spectrometry. For the analysis of the peptide mixture, we both used a matrix-assisted laser desorption/ionization (MALDI) tandem time of flight mass spectrometer (TOF/TOF) and an automated nano-HPLC system coupled to an electrospray ionization-quadrupole linear ion trap (Q-TRAP) instrument. The hyphenation of the latter techniques proved to be a powerful technique as shown by the identification of six isoforms of a 16.9 kDa sHSP in one single spot. We observed that S-adenosylmethionine synthetase (SAM-S) was differentially expressed between the two cultivars. Recent results refer to the role of SAM-S as being involved in abiotic stress tolerance. Furthermore, comparison of the heat shock treated samples also revealed several small heat shock proteins (sHSP), of which distinct isoforms could be characterised.

Keywords: *Hordeum vulgare*; Barley; Heat shock proteins; MALDI TOF/TOF; Q-TRAP LC-MS/MS system

1. Introduction

Any living organism has to cope with conditions of stress. Specifically for plants, the possibilities to escape from stress are limited because plants are immobile (Kuiper, 1998). In general, typical response to environmental stress conditions is established by the induction of a set of stress proteins that protects the organism from cellular damage. The yield and quality of cereals are severely affected by heat stress in many countries (Treglia et al., 1999). Barley (*Hordeum vulgare*) cultivars are among the most important crops in the world, being used in the malting industry and as feeding-stuffs. Therefore, understanding the effect of high temperature on barley is an important issue for the improvement of the quality of these crops in temperate and warm countries.

In plants, a heat shock response is a ubiquitous phenomenon resulting in altered gene expression and protein translation. Most of the proteins produced are heat shock proteins (HSPs) (Howarth, 1991). The HSPs have either high (80–100 kDa, HMW HSPs), intermediate (68–73 kDa, IMW-HSPs), or small molecular masses (15–20 kDa, sHSPs). The latter are classified as gene products of six gene families based on DNA sequence similarity, immunological cross-reactivity and intracellular localization (Vierling, 1991; Waters et al., 1996; Sun et al., 2002).

The evolutionary conservation of HSPs has suggested that these proteins are involved in fundamental cellular

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functions. HSPs are associated with protein folding, protein translocation across membranes, assembly of oligomeric proteins, modulation of receptor activities, mRNA protection, prevention of enzyme – especially photosynthesizing – denaturation and their stress-induced aggregation, and with post-stress ubiquitin and chaperonin-aided repair. Based on these functions, HSPs have been termed "molecular chaperones" (Georgopoulos and Welch, 1993; Leone et al., 2000). Apart from being synthesized as heat shock protein, HSPs are also accumulated in plants in response to a large number of other stress factors such as arsenite, ethanol, heavy metals, water stress, light, hormones, abscisic acid, wounding, excess NaCl, chilling, and anoxic conditions (Prasad and Rengel, 1998; Anderson et al., 1994; Lee et al., 1996; Sabehat et al., 1998).

Proteomics is a very elegant approach for the understanding of cellular processes. This tool provides more fundamental insights into organism development and homeostatic control than provided by the genome sequence (Shihua et al., 2003). In a standard approach, two techniques, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry, are combined. Electrophoresis is still the preferred separation technique of many researchers in a global, comparative analysis of proteins. In fact, the 2D-PAGE technique was established early on for barley (Görg et al., 1988; Hurkman and Tanaka, 1988; Flengsrud and Kobro, 1989). In a differential analysis set-up, this method provides powerful insights into the stress-responsiveness of genes (Zivy and de Vienne, 2000).

Mass spectrometry, implementing fingerprinting and partial sequencing is the method of choice for the identification of differentially displayed proteins. The mass spectrometric identification of proteins strongly relies on the presence of sequence information in the databases. This is clearly a limitation for barley, for which the genome has not yet been sequenced. However, a large, publicly available expressed sequence tag (EST) database, which comprises 2028018 sequences to date, together with the genome sequence information of closely related organisms, such as rice (*Oryza sativa*), mainly circumvents this drawback.

Several plant species, including barley, have already been investigated by a proteomic approach (Skylas et al., 2002; Prime et al., 2000; Haebel and Kehr, 2001). In barley proteome studies, several authors mainly focussed either on a more descriptive overview of occurring proteins (Kristoffersen and Flengsrud, 2000; Finnie and Svensson, 2003) or they investigated the changes in protein synthesis during seed development (Finnie et al., 2002). In other plant species, like wheat (*Triticum aestivum*), proteomics has already been applied to study the effects of heat shock at the protein expression level during grain filling (Skylas et al., 2002). These authors made a comparison between two cultivars (heat-susceptible and heat-tolerant), the analysis revealing differential expression of several sHSPs.

In the present study, we investigated the influence of short-term (2 h) heat stress on the protein expression levels of barley. We used a heat-tolerant and a heat-susceptible cultivar and attempted to analyse the differentially displayed proteins after shock treatment of both cultivars, in order to identify proteins responsible for heat tolerance. Such proteins are thought to be potential markers for heat tolerance of barley cultivars in breeding programs.

2. Results and discussion

2.1. 2D-gel electrophoresis and image analysis

The composition of the soluble protein fraction of an abiotic stress-tolerant (Mandolina) and an abiotic stress-susceptible (Jubilant) cultivar, grown under different temperature conditions, was compared. Four barley batches of 25 seedlings of the Mandolina genotype were grown five days at 24 °C, with a heat shock (40 °C) of 2 h allowed to act upon two of those samples. Concomitantly, a similar experiment was set up in which the abiotic stress-susceptible genotype was used. A representative CBB G-250 stained 2-D gel pattern of the soluble protein fraction of the control state and of heat shock shoots from both cultivars is presented in Fig. 1. Approximately 400–500 proteins were detected on each gel. Two replicate groups were created for the Mandolina cultivar (24 and 40 °C) and two were created for the Jubilant cultivar (24 and 40 °C). The reproducibility of the gel patterns was high as reflected by the figures for the scatter plot tool (>80%) and the mean coefficient of variations (CV < 35%). In a first analysis, the spot lists of the two genotypes at both temperatures were compared. Comparison of the proteomes at 24 °C revealed one differential protein spot present in the Mandolina cultivar, while comparison of the proteomes at 40 °C revealed two proteins unique to the Jubilant cultivar. Because we were mainly interested in the influence of a short term heat shock on barley and due to the small differences between the two genotypes, we continued with two replicate groups. We created one replicate group for the 2D-images at 24 °C and one group for the 2D-images at 40 °C. This approach slightly improved the mean coefficient of variations. The spot lists of the two replicate groups were compared. A comparison of the relative integrated densities averaged from three or four replicate gels was performed, and only proteins with at least a two-fold increase in density ratio and with a statistical relevance ($p < 0.05$) were submitted to further mass spectrometric analysis. The applied short term heat shock to both cultivars affected mainly the protein profile in the 15–30 kDa range and in the pH region of

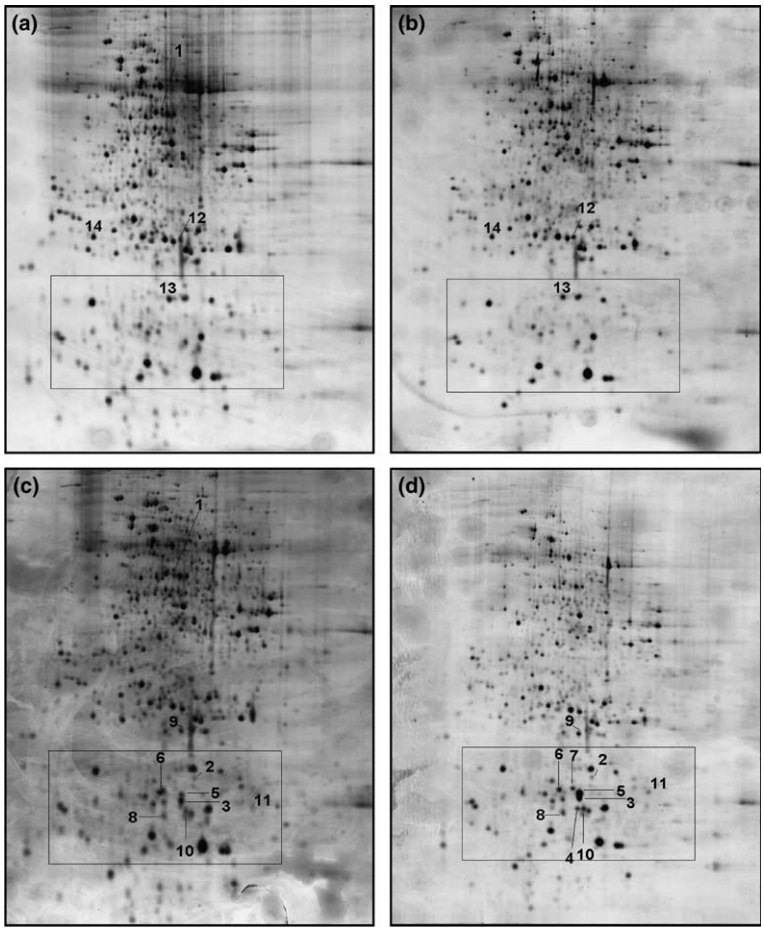

Fig. 1. Coomassie G-250 stained 2D-PAGE gels of the soluble protein fractions of (a) the Mandolina cultivar, 24 °C; (b) the Jubilant cultivar, 24 °C; (c) the Mandolina cultivar, 40 °C and (d) the Jubilant cultivar, 40 °C.

4.0–8.5 (Fig. 2). The spots were numbered, indicated in Figs. 1 and 2 and listed in Table 1.

2.2. Mass spectrometry

Essential in a proteomic approach is the availability of sequence information from the organism.

Due to the lack of the genome sequence, we were forced to generate as much information as possible through our mass spectrometric analyses, the use of data from peptide mass fingerprinting not being sufficient. We therefore used two different, state-of-the-art mass spectrometers, a matrix-assisted laser desorption/ionization (MALDI) TOF/TOF instrument (4700 Proteomics Analyzer, Applied Biosystems, Framingham, CA, USA) and a Q-TRAP LC-MS/MS system (Applied Biosystems, Framingham, CA, USA). Both instruments allow to obtain amino acid sequence information from peptides using their MS/MS capabilities. In a first analysis step, the MALDI TOF/TOF spectrometer was used to investigate the peptide mixture, because a minimum of sample handling is inherent to this fast and sensitive ionisation method. Furthermore, this instrument can be used in both the MS and MS/MS mode, due to the availability of a gas cell between the two Time of Flight tubes. In Fig. 3(a) we show a MS spectrum of a tryptic digest mixture obtained in MS mode, while Fig. 3(b) gives a representative MS/MS spectrum.

Due to sample impurities, we were not able to unambiguously identify several protein spots by this analysis. A second hyphenated strategy, an automated nano-HPLC coupled to an ESI-Q-TRAP mass spectrometer, was therefore assayed, a method which allows to clean up the peptide mixture thoroughly. In Fig. 4(a), the TIC chromatogram of the tryptic digest of spot 8, after LC separation, is shown. In Fig. 4(b) and (c), MS/MS spectra of two of the tryptic peptides from the same spot (retention times: 28.94 and 28.23 min) are shown.

2.3. Proteins identified by mass spectrometry

As mentioned under 2.1, we observed increased synthesis of the spot 1 protein in the barley genotype Mandolina compared to Jubilant. The O.D. values of this protein spot at 24 and 40 °C were comparable. By means of the LC-MS/MS set-up, several peptide

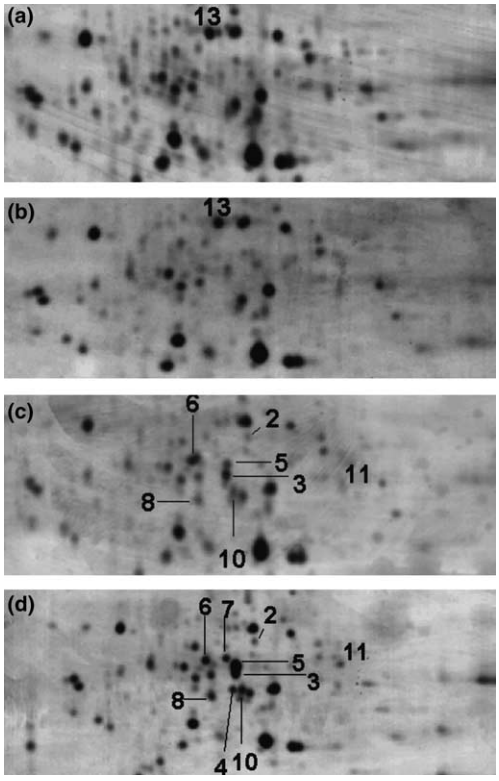

Fig. 2. Detailed picture of the individual boxes in Fig. 1.

sequences could be deduced (see Table 1) which identified the protein as *S*-adenosylmethionine synthetase (SAM-S) on the basis of homology with a protein (93% identity and 95% similarity at the amino acid level) from rice (*Oryza sativa*). The enzyme catalyses the conversion of ATP and L-methionine into *S*-adenosyl-L-methionine (SAM or AdoMet). Within the cell, SAM-S is the major methyl group donor for numerous transmethylation reactions (Boerjan et al., 1994). SAM-S has been proposed to play a role during drought-stress-induced betaine biosynthesis (Hanson et al., 1995). The high activity of the enzyme, combined with a high mRNA abundance during drought conditioning and after rewetting of the drought-conditioned seedlings, suggests that SAM-S may participate in reactions that enhance the ability of the seedling to survive prolonged drought stress (Mayne et al., 1996). Therefore, it is very likely that a reduced amount of SAM-S in the Jubilant cultivar contributes to heat suffering.

Except for protein spot 2, the other proteins showing an increased abundance after heat shock treatment are sHSPs (Table 1). They can be subdivided in three categories: proteins that are homologous to the 16.9 kDa sHSP from wheat, proteins that are homologous to the 17.8 kDa sHSP from the same organism and those that are homologous to a low molecular weight HSP from rice. In protein spot 3 we found six isoforms of the 16.9 kDa HSP, differing by only a few amino acids. Those six proteins were in the current set-up not separated on the basis of their charge and molecular weight. On the other hand, protein spot 4 is also an isoform of this protein that is nicely separated from protein spot 3 (Fig. 2). Interestingly, this protein is only present in the Jubilant cultivar. A specific correlation of the presence of this isoforms to heat susceptibility must be further investigated. In a similar study in mature wheat grain (Skylas et al., 2002), one of those isoforms showed an increase in protein abundance in a heat tolerant cultivar. The authors determined this protein as a possible marker protein for heat-tolerance. As revealed in the present work, the presence of multiple closely related isoforms shows the need for more refined analysis of the specific species that are present in the different cultivars, before they can be used as heat-tolerance markers.

Protein spots 5, 6 and 7 were identified and are homologous (>65% identity) to a low molecular weight HSP from rice. These three isoforms are nicely separated on the 2D gel (Fig. 2). It was possible to differentiate spot 7 from the spots 5 and 6, but the latter two were indistinguishable. Protein spot 7 is again unique in the stress-susceptible cultivar (cf. spot 4).

We also observed a decrease in abundance for three other proteins, listed in Table 1. In protein spot 12 we identified, besides other proteins, a 23 kDa oxygen-evolving protein of photosystem II (98% identity). The exposure of photosynthetic organisms to temperatures above the normal physiological range often results in an irreversible inactivation of photosynthesis. Photosystem II (PSII) is one of the most susceptible protein complexes to heat among various components of the photosynthetic apparatus. Previous studies have indicated that heat-related inhibition of PSII is often attributed to damage of the thylakoid-membrane proteins (Berry and Björkman, 1980). Preczewski et al. (2000) showed that the chloroplast small heat-shock protein referred to as 'chip Hsp24' protects photosystem II against heat stress, and that the phenotypic variation in production of chip Hsp24 is positively related to PS II thermotolerance.

Protein spot 13 was identified as being translation initiation factor 5A (eIF-5A). The function of this protein is to promote the formation of the first peptide bond during protein synthesis. Takeuchi et al. (2002) have suggested that loss of the active form of eIF-5A upon 45 °C heat stress is an important factor in the irreversible process of heat stress-induced death of a human pancreatic cancer cell line, but similar behaviour in plants has not been demonstrated before.

3. Conclusion

Our proteomic approach, high-resolution 2-D gel electrophoresis combined with mass spectrometry, proved to be a successful strategy in the study of heat

Table 1
Identification of the differentially displayed proteins

Spot no.[a]	Accession no.[b]	Protein ID[c]	Organism	Peptide mass[d]	Sequence[e]	Comments	Method[f]
Protein with increased abundance in the Mandolina cultivar compared to the Jubilant cultivar							
1	11895380	*S*-adenosyl methionine synthetase	*Oryza sativa*	1469.73	TNMVMVFGEITTK		LC-MS/MS
				2042.98	QVTVEYHNDNGAMVPIR		
Proteins showing an increased abundance after a short term heat shock							
2	18234603	Unknown protein	*Arabidopsis thaliana*	1410.88	ILIPTLSVLSLSR		LC-MS/MS
3	18213982	Small heat shock protein 16.9B	*Triticum aestivum*	974.52	FRLPEDAK		LC-MS/MS
				1026.60	AEVKKPEVK		
				1396.79	AGLENGVLTVTVPK		
				1616.76	MDWKETPEAHVFK		
				1931.97	SIVPAISGGNSETAAFANAR		
	21940839	Small heat shock protein 16.9B	*T. aestivum*	1918.98	SIIPAISGNSETATFANAR		LC-MS/MS
	24238923	Small heat shock protein 16.9B	*T. aestivum*	1056.62	TEVKKPEVK		LC-MS/MS
	21964213	Small heat shock protein 16.9B	*T. aestivum*	1961.98	SIVPAISGSNSETAAFANAR		LC-MS/MS
	18212283	Small heat shock protein 16.9B	*T. aestivum*	1908.93	SIVPAFSGNSETAAFANAR		LC-MS/MS
	21195581	Small heat shock protein 16.9B	*T. aestivum*	1888.96	SIIPAISGNSETAAFANAR		LC-MS/MS
4	21968553	Small heat shock protein 16.9B	*T. aestivum*	1979.97	SIFPAISGGNSETAAFANAR		LC-MS/MS
				2225.04	SNVLDPFADLWADPFDTFR		
				1824.04	AGLENGVLTVTVPKAEVK		
				1967.09	VDEVKAGLENGVLTVTVPK		
5	13091926	Heat shock protein, low molecular weight	*O. sativa*	1153.54	TSSDTAAFAGAR		MALDI TOF/TOF
				1598.81	IDWKETPEAHVFK		
6	13091926	Heat shock protein, low molecular weight	*O. sativa*	1153.54	TSSDTAAFAGAR		MALDI TOF/TOF
				1198.54	TDTWHRVER		
7	16292234	Heat shock protein, low molecular weight	*O. sativa*	1296.56	TTDSETAAFAGAR		MALDI TOF/TOF
				1584.79	IDWKETPDAHVFK		
8	16321745	Small heat shock protein 17.8	*T. aestivum*	1307.60	FVLPENADMEK	Met ox	LC-MS/MS
				989.49	AMAATPADVK	Met ox	
				1291.61	FVLPENADMEK		
				1027.61	VLVISGERR		
				1818.01	DGVLTVTVEKLPPPEPK		
				2151.06	ELPGAYAFVVDMPGLGSGDIK	Met ox	
				3122.53	AMAATPADVKELPGAYAFVVD-MGLGSGDIK	2 × Met ox	
	21967462	Small heat shock protein 17.8	*T. aestivum*	2193.07	ELPDAFAFVVDMPGLGSGDIK	Met ox	LC-MS/MS
				3148.54	AMAAPADVKELPDAFAFVVDMP-GLGSGDIK	Met ox	

(*continued on next page*)

Table 1 (*continued*)

Spot no.[a]	Accession no.[b]	Protein ID[c]	Organism	Peptide mass[d]	Sequence[e]	Comments	Method[f]
				3164.54	AMAAPADVKELPDAFAFVVDMPGL-GSGDIK	2 × Met ox	
	16321080	Small heat shock protein 17.8	*T. aestivum*	1277.66	FVLPDNADMEK		LC-MS/MS
				1293.59	FVLPDNADMEK	Met ox	
9	NOT IDENTIFIED						
10	NOT IDENTIFIED						
11	NOT IDENTIFIED						
Proteins showing decreased abundance, after a short-term heat shock							
12	21146652	23 kDa oxygen evolving protein of photosystem II	*T. aestivum*	1242.66	QYYSITVLTR		LC MS/MS
				1561.74	KNTDFVAYSGEGFK		
				2083.02	TADGDEGGKHQLITATVADGK		
				3048.51	KTITDYGSPEEFLSQVGFLLGQQ-SYGGK		
	24963702	Triosephosphate isomerase	*T. aestivum*	1288.63	TNVSPEVAESTR		LC MS/MS
				1311.65	IIYGGSVTGASCK	Carbami-domethyl	
	16308176	Adenosine diphosphate glucose pyrophosphatase	*Hordeum vulgare*	1262.69	VTFLDDAQVKK		LC MS/MS
				2504.07	LTQDFCVADLSCSDTPAGYPCK	3 × Carb-amidom-ethyl	
	12036592	Dehydroascorbate reductase	*T. aestivum*	1201.68	IFSTFVTFLK		LC MS/MS
				1826.84	AAVGHPDTLGDCPFSQR	Carbami-domethyl	
13	18228088	Translation initiation factor 5A	*O. sativa*	1087.58	TFPQQAGAIR		LC-MS/MS
				1310.75	LPTDDVLLGQIK		
				1419.70	CHFVAIDIFNGK	Carbami-domethyl	
				2216.06	KLEDIVPSSHNCDVPHVDR	Carbami-domethyl	
				2769.34	TGFADGKDLILSVMSAMGEEQI-CAVK	Carbami-domethyl	
14	9743953	Hypothetical protein	*H. vulgare*	1059.56	SVGGITGDNLK		LC MS/MS
				1210.53	WDEGYDVTAR		
				1318.74	LKAGYVAANWVK		
				1778.87	DTNGIASTSGSTIELSAR		
				2466.15	QVLSDASSFTWGIFNQPDPSDR		

[a] Numbers refer to the spot numbers given in Fig. 1.
[b] Nucleotide entry code of the National Center for Biotechnology Information (NCBI): http://ncbi.nlm.nih.gov.
[c] Annotation of the protein sequence performed using the BLAST program (NCBI).
[d] Theoretical molecular mass of the peptide.
[e] Peptide sequence corresponding to the appropriate peptide mass. Parts of the sequence, determined by mass spectrometry, could indisputably confirm the peptide.
[f] Protein spots analyzed and identified using either MALDI-TOF/TOF MS or nano-HPLC Q-TRAP MS. For detailed information, see Section 4.5.

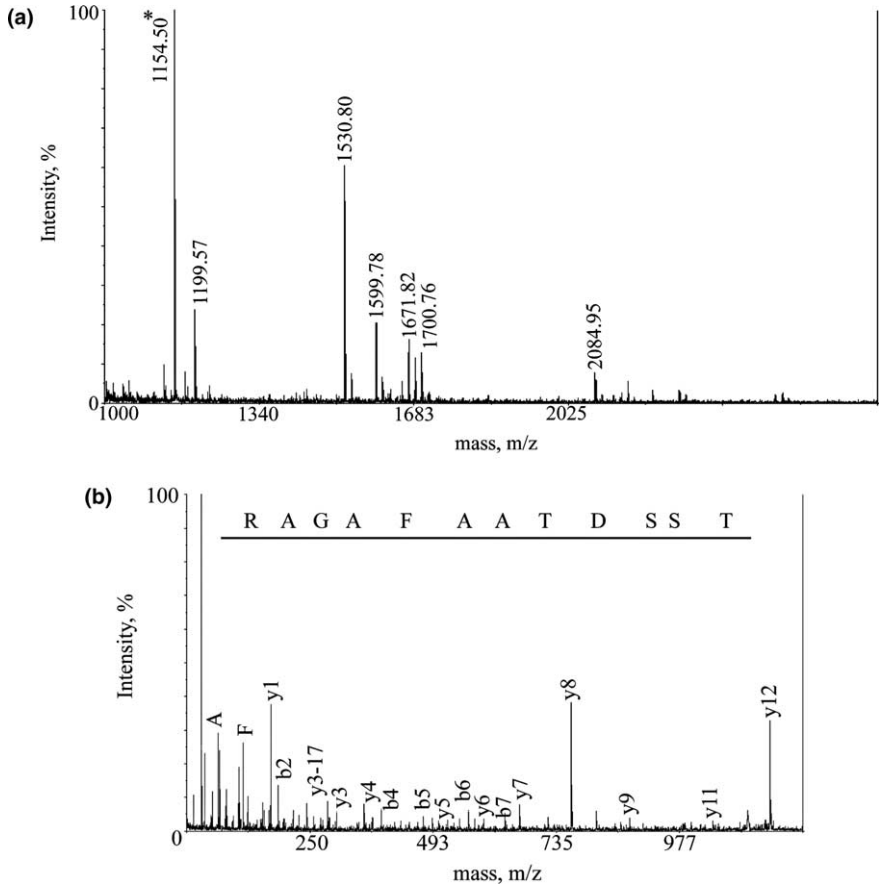

Fig. 3. (a) MS spectrum obtained from the tryptic digest of spot 6. Peptide mass fingerprint analysis could not identify this protein. (b) A characteristic MS/MS spectrum of the peak labelled (*) in (a). Using this amino acid sequence information, we could identify the protein as a sHSP homologue of rice.

shock associated proteins in different barley cultivars. Although the genome sequence is not yet available, we were able to identify most of the proteins displaying differential protein synthesis. This is largely due to the improvements, sensitivity and MS/MS capabilities in the mass spectrometric analyses, enabling to perform 'de novo sequencing' and, consequently, identify more protein spots.

The protein SAM-S displayed an increased protein synthesis in the stress-tolerant barley cultivar. SAM-S is known to participate in surviving prolonged drought stress. Loss of resistance of the Jubilant cultivar against abiotic stress factors could be partially due to the absence of this protein.

In the differential analysis of the heat-treated Jubilant and Mandolina barley shoots, we detected increased protein abundance for several sHSPs. One of them is highly homologous to the 16.9 kDa HSP from wheat. In a different study, this protein was referred to as a potential marker for heat-tolerance in wheat grains. We showed the presence of several isoforms of this protein.

We also found two proteins (protein spots 4 and 7, see Figs. 1 and 2) unique to the stress-susceptible cultivar; an in-depth analysis of those proteins and of their isoforms will be necessary in order to better understand their role in relation to heat-tolerance and susceptibility. In future, we will therefore investigate the effect of a prolonged heat shock period on the proteome of both cultivars.

4. Experimental

4.1. Plant growth

Barley (*Hordeum vulgare*) genotypes (Jubilant, abiotic stress-susceptible, spring type and Mandolina, abiotic stress-tolerant, spring type) were sterilized with 3% H_2O_2 for 30 min and thereafter soaked in MilliQ water, with frequent changes, for 2 h. Grains were germinated under sterile conditions on wet filter paper, in Petri dishes (25 grains/dish), in the dark in a thermostat (24 °C) for 5 days. After 5 days of growth half of the barley seedlings were subjected to a heat shock treatment at 40 °C for 2 h, and protein extraction was immediately performed.

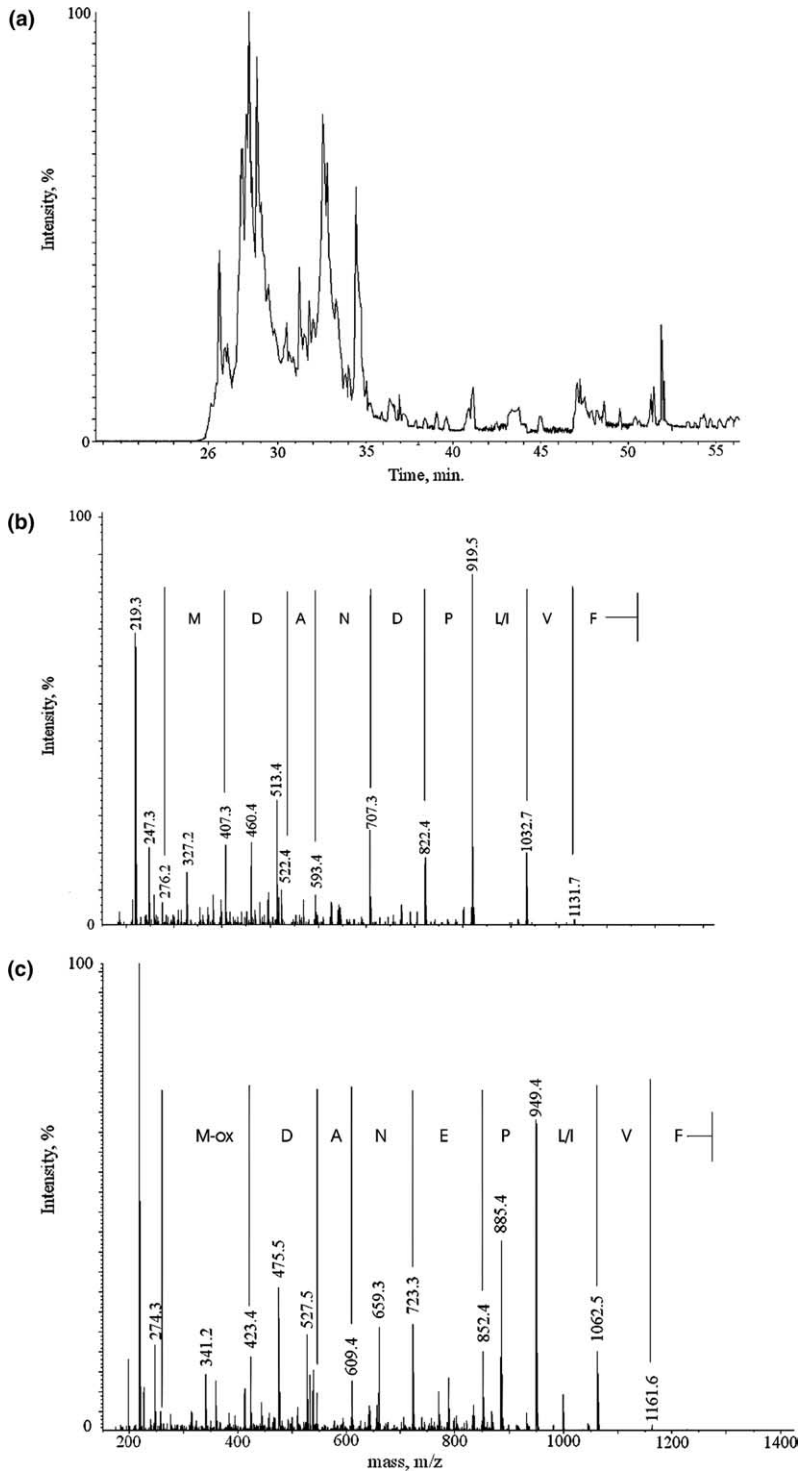

Fig. 4. (a) TIC chromatogram, after the LC separation, of spot 8. (b) A typical MS/MS spectrum of peak 1277.66 Da, obtained via the LC-MS/MS system. (c) MS/MS spectrum of another peptide, 1307.6 Da, in the same peptide mixture. The deduced amino acid sequence from the two spectra indicates the presence of different isoforms.

4.2. Protein extraction of the barley shoots

Barley shoots were crushed in liquid nitrogen and then homogenized with 10% TCA and 0.07% β-mercaptoethanol in cold acetone. Proteins were allowed to precipitate 1 h at −20 °C. After centrifugation (10 min at 16,000*g*, 4 °C), the pellets were washed twice with cold acetone containing 0.07% β-mercaptoethanol (1 h

at −20 °C). The supernatants were discarded and the pellets were vacuum-dried and dissolved in an extraction buffer (25μl per mg of dry matter) for 30 min on a shaker at 37 °C. This buffer consisted of 8 M urea, 40 mM Tris-base, 4% CHAPS and 1 mM PMSF. After centrifugation (10 min at 16,000*g*, 4 °C), the supernatant containing the soluble protein fraction was recovered and the protein concentration was determined using the Bradford Kit Assay (Bio-Rad, Hercules, Ca, USA). The samples were stored at −80 °C until use.

4.3. 2D-gel electrophoresis

Approximately 550 μg of protein was loaded on 18 cm immobilized pH gradient (IPG) strips, pH range 3–10 l (Bio-Rad) via the passive in-gel rehydration protocol. The isoelectric focusing (IEF) was performed on a Multiphor II system (Amersham Biosciences, Uppsala, Sweden) running a standard program provided by the manufacturer. The temperature was kept at 18 °C. After completion of the IEF program, the strips were equilibrated for 10 min in a 50 mM Tris–HCl solution (pH 8.8) containing 6 M Urea, 30% glycerol, 2% sodium dodecylsulfate (SDS) and 1% dithiothreitol (DTT). Thereafter, the solution was replaced by the same Tris–HCl solution except that DDT was exchanged for 2.5% iodoacetamide. The strips were then placed on home-casted SDS–PAGE gels (12.5% T and 2.6% C). Electrophoresis was carried out in a Protean Plus Dodeca Cell system (Bio-Rad) at 10 mA/gel for 15 min, followed by a ±10 h run at 200 V, until the bromophenol blue front reached the bottom of the gel. Staining was performed using Coomassie Brillant Blue G-250 according to Anderson et al. (1989). The gel images were digitized with a 12-bit GS-710 calibrated densitometer (Bio-Rad) and analyzed with the PDQuest 7.1 software (Bio-Rad). After spot detection, the 2-D maps were automatically aligned followed by manual spot editing to increase the correlation between the different 2-D maps. Statistical analysis of the relative abundance of each matched protein spot was accomplished by using a two-tailed *t*-test. Only quantitative differences with a *p*-value of at least <0.05 were considered.

4.4. In-gel digestion

Protein digestion was performed according to Rosenfeld et al. (1992), with minor modifications. In short, the spots were excised from the gel and washed twice with 150 μl of 200 mM ammonium bicarbonate in 50% acetonitrile/water (20 min at 30 °C). After drying at room temperature, for 10 min, the tubes were chilled on ice. Afterwards, 8 μl of digestion buffer was added (50 mM ammonium bicarbonate, pH 7.8), containing 0.002 μg/μl trypsin. The samples were kept on ice (45 min), and 20 μl of digestion buffer was added. After overnight incubation (37 °C), the supernatant was recovered and the remaining peptides were extracted from the gel piece (60% acetonitrile/0.1% formic acid in water). For mass spectrometric analysis, the samples were dried and then dissolved in 12 μl of 0.1% formic acid.

4.5. Protein identification and mass spectrometric analyses

The genome of *Hordeum vulgare* has not been fully sequenced, but several EST databases can be accessed on the World Wide Web. Such a suitable *Hordeum vulgare* EST database (www.ncbi.nlm.nih.gov) was downloaded and formatted to make it accessible via the database searching program MASCOT (www.matrixscience.com). If mass spectral data fitted a translated EST sequence, this sequence was loaded into a BLAST query for annotation.

Tryptic peptide mixtures were measured on a MALDI TOF/TOF mass spectrometer (Applied Biosystems, Framingham, CA, USA). In the MS mode, the generated ions are accelerated at the source (20 kV) and are separated in the second TOF tube. In the MS/MS mode, the parent ion is focused into the gas cell where the peptide is fragmented using collision induced dissociation (CID). The fragments formed are reaccelerated (15 kV) and the m/z-values are then determined in the second TOF tube. The voltage applied to the source was 8 kV and to the gas cell 7 kV; air (1×10^{-6} Torr) was introduced in the cell as fragmenting gas.

The peptide mixture (1 μl) was co-crystallized with an equal volume of matrix solution (100 mM α-cyano-4-hydroxycinnamic acid dissolved in 50% v/v acetonitrile/0.1% TFA in water) and applied to the target plate. Prior to analysis, the instrument was externally calibrated with a standard peptide mixture, as outlined by the manufacturer. When the peptide mass fingerprinting was not conclusive, several peptides were subjected to further MS/MS analysis. Mass spectral data were then submitted for database searching to our local MASCOT server for protein identification. If the identification was uncertain, the peptide mixture was separated by nano-HPLC and detected on-line by an ESI-Q-TRAP mass spectrometer (Applied Biosystems). The experimental set-up of the separation system has been described elsewhere (Devreese et al., 2002). Briefly, the samples were loaded onto the nano-column (PEPMAP, 150 mm × 75 μm I.D) using an in-line pre-concentration step on a micro pre-column cartridge (2 mm × 800 μm I.D.). Afterwards, peptides were separated using a linear gradient from 5% acetonitrile/0.1% formic acid in water to 80% acetonitrile/0.1% formic acid in water (50 min). The eluted peptides were 'on-line' detected by the Q-TRAP mass spectrometer. In this method, an automated MS to MS/MS switching protocol was used. First, an enhanced MS scan as survey scan (m/z 400–1500) was performed,

followed by an enhanced resolution scan of the two most intense ions. The last scan allowed to determine the charge state of the ions. If their charge state was two or three, an enhanced product ion scan (MS/MS) of these ions was performed. The total cycle time of this set-up was approximately 4.5 s.

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