Proteomics evidence of quality stresses caused by changing environment

Branlard G[1], Bancel E[1], Majoul T[2], Martre P[1]

1-INRA UMR 1095 Génétique Diversité et Ecophysiologie des Céréales (GDEC)-Université Blaise Pascal, 234 av. du Brézet, 63100 Clermont Ferrand, France.

2- ISSTE - Institut Supérieur des Sciences et Technologies de l'Environnement, Technopole de Borj Cédria - CP 1003 hammam-lif. Tunisie.

INTRODUCTION

Tremendous advances have been achieved in genomic sequencing and serial analysis of gene expression at the mRNA level. At a particular time point, genome itself is static and cannot provide the biological characteristics of an organism. Genomic sequences do not provide any direct information on protein levels and their post translational modifications, which result in the absence of correlation between mRNA and protein abundance (Gygi et al., 1999). It is the proteins which are directly responsible for the functions and phenotypes of cells; that is the reason why scientists working on any one of the hundred of sequenced species are now using proteomics approach to understand the gene functions. The term proteome covers all proteins present in a sample at a given point in time and under given conditions (Wilkins et al., 1995). Proteomics in biological science and particularly in plant biology comprises three scopes or disciplines: the functional proteomics with specific directed approach, the expression proteomics (using quantitative study of protein expression between samples) and the structural proteomics where complex of proteins or proteins present in cell organelles are studied. Literature survey shows that plant proteomics, although far less abundant than animal proteomics, reveals its explosive growth after the nineties. Among the factors involved in this success the following are important: (1) advances in two-dimensional gel electrophoresis (2-DGE) enabling thousands of proteins to be simultaneously separated, stained, scanned and compared using computer analysis, (2) Edman sequencing which was time consuming was replaced by mass spectrometry (MS) (Mann, et al., 2001) with continuous improvement in mass accuracy and sensitivity for protein identification , (3) the growth of proteomics which is the direct result of genomics, also benefit from genome sequencing in using dedicated software's and bioinformatics tools for proteome mining. Today proteomics and particularly plant proteomics is the exponentially growing field of the functional genomics era, helping to understand gene function in its environment. (Rossignol 2001, Agrawal and Rakwal 2006, Thiellement et al., 2006). The identification of all the proteins expressed in a given tissue, cell, or in sub-cellular components of a living organism at a given time, the study of their genetic determination (gene location and sequence), the study of their possible polymorphism through post-translational modifications, and the identification of their function in physiological metabolism are the major tasks of the proteomics approach.

Several reviews have already been proposed on plant proteomics (see for example Thiellement et al., 2006) consequently the present report will only focus on wheat grain proteomics. In addition the lecture delivered will review some major advances in plant proteomics through protein preparation, 2-DGE improvements, image analysis, quantitative evaluation and statistical comparisons and bio-informatics analysis. Proteomics is not a new approach particularly for wheat storage proteins (WSPs) which have received a great deal of attention in the last two decades. The early studies on genetic determination, allelic diversity, protein sequences, and functional properties were in fact the main achievements of proteomics analysis long before this approach became a powerful tool.

Using 2-DGE, mass spectrometry and database interrogation several studies of the wheat grain were focused on the impacts of aneuploidy mainly on WSPs (Islam et al., 2002, Dumur et al., 2004). Removing only one chromosome revealed an over expression of the WSPs encoded by the active loci located on the remaining unique chromosome. Whereas removing the chromosome pair not only resulted in absence of the proteins encoded on the eliminated chromosome pair but strongly reduced or over expressed many other WSPs suggesting complex regulation mechanisms involved in these major wheat endosperm proteins. This complex regulation effect was confirmed in analysing wheat RILs having or not the 1BL/1RS translocation (Gobaa et al., 2007, 2008) where the elimination of the *Glu-B3* encoded low molecular weight glutenin subunits was compensated by the increase in the amount of some other polymerized proteins. Deferential proteomics was mainly used to understand the protein responses of wheat subjected to heat stress during grain formation and components accumulation (Skylas et al., 2002, Majoul et al., 2003, 2004, Dupont et al., 2006). In addition to the heat shock proteins HSPs which are directly associated to heat treatment, numerous proteins involved in carbohydrate metabolism were also up- or down- regulated. Many researchers in different countries have reported that high temperatures during grain filling severely affect ratios between protein fractions (Triboï and Triboï-Blondel 2001), dough properties and quality characteristics (Ciaffi et al., 1996, Perrotta et al., 1998, Gibson et al., 1998, Peterson et al., 1992, Stone et al., 1994, Wardlaw and Wrigley, 1994). Heat stress during the accumulation of grain components is known to have a strong influence on wheat grain physiology, leading to reduced grain size and increased protein concentration and modification of the rheological properties

(Blumenthal et al., 1993; Triboi et al., 2003) Using proteomics new insights on grain responses to environmental variations affecting wheat quality can be revealed. Several studies carried out at INRA on heat stress and heat treatment will be briefly shown in the present report.

MATERIAL AND METHODS

Plant material

Three experiments were carried out with three cultivars of hexaploid bread wheat to analyse endosperm responses to heat treatments and heat shocks. In these experiments, wheat crops where shown at a density of 500 seed m^{-2} in 2 m^2 containers (0.5 m deep) and were grown outside from emergence till anthesis. In order to monitor and control the air temperature, water supply, gas exchange, the containers were transferred under sunlit growth chambers 4-5 days after anthesis (daa). The crops were fully irrigated and fertilised, and pests where chemical controlled. The treatments and samplings are outlined below.

Experiment 1 was carried out with the cultivar Thésée. Crops were subjected to two day/night thermal regimes from 4-5 daa to grain ripeness: 18°C/10°C (control, C) and 34°C/10°C (stressed, S). Grains were sampled in the two treatments at early (315°Cdays) and late (480°Cdays) stages and at the end (700°Cdays) of the grain-filling period, and their composition in storage proteins (Majoul et al., 2003) and albumins-globulins (AG; Majoul et al., 2004) were analysed using proteomics tools.

Experiment 2 was carried out with the cultivar Récital. Crops were exposed to 4-hour periods at mid-day at 38°C for four consecutive days between 300°Cdays and 400°Cdays – i.e. at mid-grain filling. The day/night thermal regimes: was 18°C/10°C for the control as well as for the treated one out of these four days. Total proteins and AG were analysed by proteomics at four stages: just before (S1) and just after the heat chock period (S2), one week after (S3), and at physiological maturity (S4).

Experiment 3 was carried out with the cultivar Arche. Crops were subjected to two day/night thermal regimes from 4-5 daa to grain ripeness: 23°C/11°C (C) and 28°C/15°C (T). Grains were collected from both treatments at regular thermal time intervals from 163°Cdays (early grain filling) to 781°Cdays (physiological maturity).

Methods

The following were the main steps if the proteomic analysis:

1 Protein extraction. Total proteins were extracted from 40 mg of whole grain flour with a 500µl of solubilisation buffer, containing 7M urea, 4% CHAPS, 2M thiourea, 2% (v/v) ampholytes 3-10 and 6-95, 2% DTT and 0.4% protease inhibitor cocktail as described by Majoul et al., 2003. Extraction of AG from mature grain was performed in a sodium phosphate buffer as previously described (Branlard and Bancel, 2006). The proteins extracted were then precipitated with glacial acetone and dried before storing at -80°C, or used for isofocussing.

2- 2-DGE. Although 2-DGE is not perfect and must be performed technically correct, it is currently the most efficient method for protein separation (Rabilloud, 2002). In our case, Immobiline pH gradient gel electrophoresis (IPGE) was used as the first dimension and SDS poly-acrylamide gel electrophoresis as the second dimension. The IPG gradients were on 13cm strip pH 3-10 L, for experiments 1 and 2 and on 18 cm pH 3-11NL for experiment 3. Three to six replicates were performed to identify the common spots that characterise each proteic sample. Coomassie blue G250 (CB) was used before gel scanning in transmission mode. The protein content being increased under high temperature (Daniel and Triboï, 2000), the same amount of proteins was loaded on 2-DGE, enabling to detect differential expression due to the temperature treatment.

3- Image analysis. Melanie-3 software (GeneBio, Geneva, Switzerland) or 2D Platinum (GE Healthcare) was used to compare images and spots. The SAS GLM procedure (SAS, 1985) was used to test spot volume and percentage of spot volume (%SV) between images of consecutive sampling in developing grain.

4- Mass spectrometry (MS). Spots of interest were excised and subjected to trypsic digestion as described by Gobaa et al., (2007). Two spectrometers were used, (1) matrix-assisted laser desorption – the time of flight (MALDI-TOF) spectrometer giving peptide mass fingerprints and/or (2) Electrospray ion trap MS/MS spectrometer giving partial sequences of peptides.

5- Protein identification. The monoisotopic peptide masses resulting from the trypsic digest were compared with peptide masses in databases such as SwissProt, (http://www.expasy.org/sprot/), NCBI (http://www.ncbi.nlm.nih.gov/) or EST databases using Mascot and Profound software (http://www.matrixscience.com) and http://prowl.rockefeller.edu). Matches to protein sequences from the Viridiplantae taxon were considered acceptable if at least four peptide masses from the peptide mass fingerprint (PMF) matched, and a Z score of 1.60 or higher was obtained using ProFound, or a significant score was obtained using MASCOT based on the program's algorithm.

Mass data collected during the LC-MS/MS analysis were processed with Sequest software. The proteins were identified by searching for the peptide masses and the MS/MS sequences in the SwissProt (http://www.expasy.org/sprot/) and NCBI (http://www.ncbi.nlm.nih.gov/) non-redundant sequence databanks. Proteins were considered to have been identified if at least two non-redundant peptides matched a single reference in the databases.

RESULTS and DISCUSSION

In each of the three experiments reported here, the crops were grown under the same conditions until the temperature treatment was applied. Therefore,

differential proteomics may provide reliable information on heat effect during grain filling, independently of any competition factors that might arise if the ears number per plant or grain number per ear would have been different between the sampled plots.

1-On heat shock proteins: Experiment 1 showed that the kinetics of accumulation of both total proteins and AG are significantly different when wheat crops are grown at 18°C/10°C compared with 34°C/10°C. At physiological maturity, 60 proteins were up-regulated and 20 were down-regulated at 34°C/10°C compared with 18°C/10°C. Forty one heat-induced and nine heat-suppressed proteins were identified. HSPs of the 90 kDa family, which were constitutively present at 18°C/10°C, were increased by 5- to 9-folds at 34°C/10°C compared with 18°C/10°C. A 70 kDa HSP was also increased 2.6-folds in response to elevated temperature. The small HSPs, of the 20kDa family, were increased 2- to 3-folds at 34°C/10°C compared with 18°C/10°C. The 90 kDa HSP has been reported to act as chaperon proteins with ATPase activity and to interact mainly with proteins involved in transcription regulation and signal transduction pathways (Zhao et al., 2001). The small HSPs have a molecular chaperone function preventing thermal aggregation of proteins and, under normal growing conditions, they generally increase as seed dehydrates.

In experiment 2 after the 4 days of heat shock on cv Récital, 109 AG spots had changed: including 29 new spots, 37 up- and 43 down- regulated spots. At physiological maturity (i.e: 25 days after the end of the heat shock treatment) 21 AG still had significantly different %SV compared with control grains.. Several HSPs were significantly increased in response to heat shock at S2 and also at S3 but with no significant difference at maturity as shown in Figure 1 for a 70kDa HSP. The HSP70 bind ATP and they are involved in the folding and oligomerization of newly synthesized polypeptides. The HSP70 are among all the HSPs, undoubtedly those having the strongest influence on kinetics of storage protein accumulation. It has been suggested that HSPs, in response to heat stress during grain filling, modify the folding and aggregation of gluten protein, thereby weakening the dough properties (Blumenthal et al., 1998).

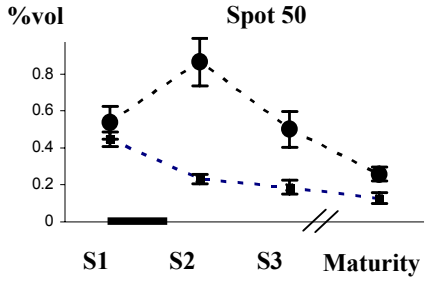

Figure 1: Evolution of the %SV for a HSP70 spot for grain of cv. Récital for control (■) and in response to four-consecutive days of heat shock stress (●).The thick bar on the X-axis indicates the four-consecutive days during which the heat shock was applied.

2-On storage proteins

High temperatures have been shown to increase the synthesis of gliadins, but to decrease the synthesis of glutenins (Blumenthal et al., 1993, Daniel and Triboï 2000). In experiment 1, 34°C vs 18°C day temperature decreased the amount of storage proteins. Surprisingly, since constant total protein amount were loaded on the 2-DGE, no differences were detected for the HMW-GS between the two temperature treatments, whereas many gliadins and only few LMW-GS were over-expressed at 34°C/10°C compared with 18°C/10°C. At maturity, several α-gliadins were increased 2- to 27-folds at 34°C/10°C compared with 18°C/10°C (Majoul et al., 2003). Blumenthal et al., (1990) have suggested that the activation of gliadin synthesis is due to the presence of heat-stress responsive elements in the upstream regions of some gliadin genes. In experiment 2, statistical comparison revealed that not all gliadins were over expressed. The proportion of individual storage protein was analysed before and after the heat-shock treatment and at physiological maturity. Only few gliadins, mostly α-gliadins, were over-expressed at just after and one week after the end of heat-shock treatment; none was significantly over-expressed at physiological maturity. Only one HMW-GS, encoded at *Glu-A1*, was significantly over expressed after the heat-shock treatment and stayed at higher %vol for the heat-chock treatment compared with the control till physiological maturity (Figure 2).

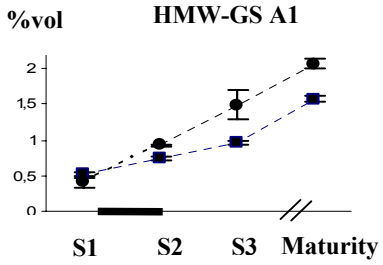

Figure 2: Evolution of the %SV for the HMW-glutenins subunit 1, in grain formation of cv Récital for control ■ and in response to 4 days of heat shock stress ●.

At physiological maturity, no difference in %vol for the HMW-GS were observed between the heat-shock and control treatments. At S2, S3, and at maturity, the gliadin to glutenin ratio was not significantly different between the two treatments. Interestingly among the numerous AGs that were significantly down-regulated after 4 days of heat shock, two protein disulfide isomerases (PDI) were down regulated. (Figure 3), suggesting that variations of PDIs are not related to the variations of the gliadin to glutenin ratio observed at S2.

In experiment 3, the mild temperature increase throughout grain filling (28°C/15°C vs. 23°C/11°C) had

little influence on the presence or absence of storage proteins. However, considerable decrease of the total

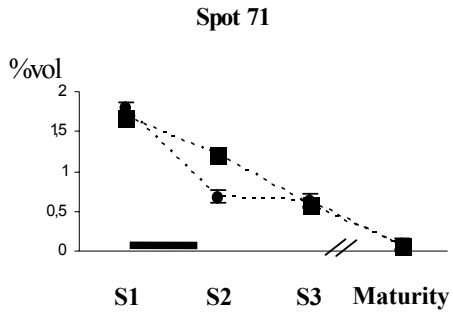

Figure 3: Evolution of the %SV for spot 71 identified as PDI, in grain formation of cv Récital for control ■ and in response to 4 days ▬ of heat shock stress ●.

amount of storage proteins per grain was observed. At 28°C/15°C, the total amount of gliadins and glutenins in mature grains was only 65.7% and 65.5%, respectively, of that observed at 23°C/11°C. Image analysis of the six replicates performed for each of the seven stages during grain development enabled to accurately follow the accumulation of 78 storage proteins. The kinetics of storage proteins accumulation was analysed on individual spots as well as total gliadins and glutenins. Except at physiological maturity, the gliadins to glutenins ratio did not differ significantly at 28°C/15°C vs. 23°C/11°C. In contrast, there was a significant difference in the HMW-GS to LMW-GS ratio between C and T at four out of seven stages but no difference was found at full maturity. Individual storage proteins did not display identical kinetics in C and T. Two ω-gliadins were 200% over-expressed, whereas one γ-gliadin and one LMW-GS were 50% under-expressed at three consecutive stages in 28°C/15°C compared with 23°C/11°C.

All together the three experiments indicate that regulation of the amount of glutenins and gliadins is not related to specific HSPs or PDIs. The extent of the influence of these enzymes on the synthesis of specific glutenin or gliadin need to be analysed both with transcriptomics and proteomics tools. Recent analyses of lines having or not the 1BL/1RS translocation suggest the existence of a mechanism of regulation for reaching a polymerized level of proteins in wheat grain (Gobaa et al., 2007, 2008).

3-On proteins associated to carbohydrates.

Starch is the main carbohydrate of wheat grain and its quantitative variation is strongly associated to grain weight and yield. In addition to genotype, environmental factors may affect grain weight and yield. In particular, heat stress during grain filling has been shown to reduce the grain weight and the yield of wheat (Jenner 1994), partly because of reduced rate of starch synthesis under high temperature conditions (Bullar and Jenner, 1985).

In the experiment 1, the 16°C increase of the day-time temperature throughout grain filling reduced by 50% the amount of the glucose-1-phosphate adenyltransferase precursor (Majoul et al., 2003) and of the glucose-1-phosphate adenyltransferase (Majoul et al., 2004). The G-1-P adenyltransferase is the first committed enzyme of the starch biosynthesis pathway. Its reduced level of expression under high temperature may explain the observed reduction of grain size and starch synthesis under such conditions. Two spots identified as Granule Binding Starch Synthase (GBSS) precursor and GBSS, the key enzyme of amylose synthesis (Morell et al., 2001), were increased 5- and 3-folds, respectively, in response to the 16°C increase in day-time temperature. At maturity, a β-Amylase was increased 3-folds in S sample. This finding suggests that starch hydrolysis occurred in response to heat stress probably to fuel the living grain with energy. In addition two glucose-6-phosphate isomerases, which are involved in glycogenesis and gluconeogenesis, were increased 2.7-folds in S sample.

The 4 days of heat shock (Experiment 2) induced the over expression of a sucrose synthase (Figure 4). This protein was 5 fold increased at S2 and decreased thereafter during maturation. This enzyme that ensures sucrose cleavage, provides uridine diphosphate (UDP-glucose) glucose and fructose which are important carbohydrates for various metabolic pathways. Macleod and Duffus (1988) showed that sucrose synthase activity was significantly reduced at 30°C in developing barley endosperm. In the case of short heat shock, other major enzymes of starch metabolism, still remain to be detected since all the varying spots have not been identified.

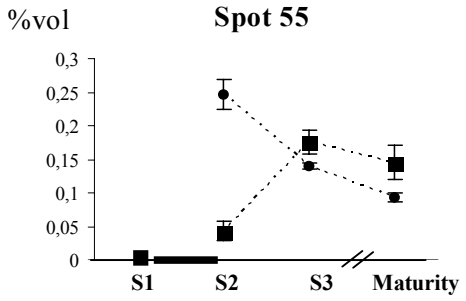

Figure 4: Evolution of the %SV for spot 55 identified as sucrose synthase 2, in grain formation of cv Récital for control ■ and in response to 4 days ▬ of heat shock stress ●.

CONCLUSION

Many proteins previously reported by other teams (Skylas et al., 2000, Vensel et al., 2005, Dupont et al., 2006) were also identified in the experiments reported here. Together, these experiments have increased our understanding of what is currently happening in wheat in response to the influence of heat in many countries. Genes encoding some of these proteins could be specifically analysed for future wheat genotyping with the aim of ensuring greater stability in grain composition in response to heat treatment. Further analyses performed on contrasted genotypes and using transcriptomics together with proteomics tools will progressively deliver the key enzymes that breeders will

need to focus on for a better adaptation of wheat to changing environment.

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