Plant Physiology and Biochemistry 135 (2019) 253–262

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Comparative proteomic analysis of durum wheat shoots from modern and ancient cultivars

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ARTICLE INFO

Keywords:
Durum wheat
Germination
Proteomics
Local varieties

ABSTRACT

Durum wheat is widespread cultivated in the Mediterranean basin, where it is used to produce high-quality semolina for pasta. Although over the years local and ancient wheat cultivars have been replaced by new ones, better suited to intensive cultivation, the increasing demand of consumers for nutritional and sensory qualities, as well as their attention to sustainable agronomic practices, renewed the interest toward traditional varieties. In order to fully exploit their agronomical and nutritional potential, a systematic analysis of molecular traits would be desirable. Nowadays, this examination is greatly facilitated by the current availability of high-throughput genomic and proteomic methods, which are integrated with classical measurements on plant physiology. To this purpose, we performed a comparative study on germination performances, hormone level variations, and differential protein representations of three-days germinated shoots of two traditional wheat cultivars from Southern Italy, namely Senatore Cappelli and Saragolla, and the commercial elite variety Svevo. Two-dimensional electrophoresis- and nanoLC-ESI-LIT-MS/MS-based proteomic analysis revealed 45 differentially represented spots, which were associated with 32 non-redundant protein species grouping into storage, stress/defense and metabolism/energy production functional categories. Major differences in the traditional varieties concerned over-representation of glutenins, gamma-gliadin and some enzymes of glycolysis and TCA cycle, as well as a down-representation of proteins involved in the response to stress conditions. These features were here discussed in relation to the hormone profile and the known agronomic features of traditional varieties, as compared to the commercial one.

1. Introduction

In the Mediterranean area, durum wheat (*Triticum turgidum* **ssp** *durum*) is the most widespread crop and it is of high economic relevance, since it is used for the production of semolina, breakfast products and other food derivatives. In Italy, about 70% of production is carried out in Southern regions where it is used mostly to produce pasta, a worldwide renewed Italian food. In Southern Italy, different traditional durum wheat cultivars, which provide high quality semolina but do not adapt to parameters of intensive cultivation, have been progressively abandoned in favor of few genetically uniform, high-yield varieties. This reduction of biodiversity may because of reduced productivity under adverse environmental conditions or of enhanced susceptibility to pathogen attack and diseases, as well as of decreased grain quality in terms of protein and secondary metabolite content (Martini et al., 2015). The latter issue is in contrast with the increasing quality demands of the pasta industry to meet consumers expectations. Nowadays, landraces and ancient cultivars are increasingly attracting the attention of consumers, due to their peculiar organoleptic and nutritional features (Hidalgo and Brandolini, 2013), as well as to the possibility to be grown under environmental-friendly conditions, such as organic farming (Fagnano et al., 2012). Therefore, traditional

Abbreviations: ABA, abscisic acid; ENO2, enolase 2; FBA, fructose-bisphosphate aldolase; IAA, indolacetic acid; GA3, gibberellin A3; GA4, gibberellin A4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GAPN, NADP-dependent non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase; HMW glutenin, high molecular weight glutenin; HSPs, Heat shock proteins; LEAP, late embryogenesis abundant protein; MDH, cytoplasmic malate dehydrogenase; NDK, nucleoside diphosphate kinase; PDI, protein disulfide isomerase; PPI, peptidyl-prolyl *cis-trans* isomerase; PER1, 1-cys peroxiredoxin; TPIS, cytosolic triosephosphate isomerase

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https://doi.org/10.1016/j.plaphy.2018.12.010
Received 6 July 2018; Received in revised form 10 December 2018; Accepted 16 December 2018
Available online 18 December 2018
0981-9428/

cultivars and local varieties may represent a pool of genetic resources both for the selection of useful agronomical traits, as well as to increase economic revenues in agriculture. In order to fully exploit their genetic potential in breeding programmes, or to determine best agricultural practises to improve yields, a systematic evaluation of relevant traits is necessary, which at present is greatly facilitated by recent advances in wheat genome sequencing (*http://www.wheatgenome.org*) and by high-throughput genomic and proteomic analyses. The latter investigations have mostly been performed on different cultivars of bread wheat (*T. aestivum* L.), particularly concerning the quantitative changes of proteins associated with different grain developmental (Arena et al., 2017; Ma et al., 2014; Mazzeo et al., 2017; Yang et al., 2016) or germination (Dong et al., 2015) stages. In these contexts, comparative experiments were also accomplished to gain information on the different response of specific cultivars or treatments toward environmental challenges (Fercha et al., 2013; Ge et al., 2012).

Ancient varieties of durum wheat have received recent attention due to their functional and toxicological characteristics (Cooper, 2015; Valli et al., 2018). Following this rediscover process, comparative experiments with modern scientific approaches are encouraged to evaluate physiological and agronomic properties of these wheat varieties. In this context, we decided to perform a differential analysis of two ancient wheat cultivars of Southern Italy, namely Senatore Cappelli and Saragolla, which were compared to the commercial elite variety Svevo used for food industry applications. These traditional cultivars have excellent qualitative traits; for example, Senatore Cappelli, cultivated under low-input conditions, presents the lowest starch content and the highest protein and gluten content as compared to modern varieties (Dinelli et al., 2013). On the other hand, Saragolla shows the best performances among five durum wheat cultivars in terms of yield amount and quality of pasta, thus resulting the more adapt to organic farming (Fagnano et al., 2012). In this study, a comparison of the germination performances, hormone level variations, and protein representation changes in three-days germinated shoots was accomplished and corresponding results are described.

2. Materials and methods

2.1. Seed germination

Seeds of the tetraploid *Triticum turgidum* ssp *durum* cultivars Svevo (Agrisemi Minicozzi, Benevento, Italy), Senatore Cappelli (Società Italiana Sementi, Bologna, Italy) and Saragolla (Syngenta, Milano, Italy) were sterilized in 1% w/v sodium hypochlorite for 20 min, and then rinsed with distilled water to remove the excess of chemicals. Seeds were germinated in Petri plates placed in a controlled environment chamber (model ISCO, series MICRA), at 30 °C, for 24, 48, 72, 96, 120, 144 and 168 h. Three biological replicates of 100 seeds per plate were used.

2.2. Hormone extraction and analysis

IAA, gibberellin A3 (GA3), gibberellin A4 (GA4) and ABA content within wheat cultivars was measured by using a chromatographic procedure. Two grams of frozen samples were reduced to powder with methanol (2.5 ml/g of fresh tissue) in a mortar with pestle. 1-Naphthaleneacetic acid (Sigma) was added to each sample as standard (10 nmol/g of fresh tissue). Each extract was centrifuged (16,000 x *g*), for 10 min, at 4 °C. The supernatant was concentrated under vacuum and a volume of pure water was added to each sample, and extracted with an equal volume of ethyl acetate. Aqueous and organic phases were separated by centrifugation (16,000 x *g*), for 2 min. The lower aqueous phase was transferred to a new tube, the pH of the solution was adjusted below 3 to keep all the hormones in a protonated form. The upper organic phase was recovered, completely dried under vacuum and then dissolved in 30 µl of methanol before further analysis by reversed phase-HPLC. The latter was performed on a LC-20 Prominence HPLC system (Shimadzu, Japan) equipped with a LC-20AT quaternary gradient pump, a SPD-M20A photo diode array detector (PDAD) and a SIL-20 AH autosampler (20 µl injection vol). Plant hormones were separated on a Gemini-NX C18 column (250 × 4.5 mm, 5 µm) (Phenomenex, Torrance, CA), which was assembled with a Security Guard® (Phenomenex) pre-column and eluted with a gradient of acetonitrile (ACN) containing 0.1% v/v trifluoroacetic acid (TFA) in aqueous 0.1% v/v TFA, at 45 °C; ACN ramped from 15 to 30% in 5 min, from 30 to 50% in 5 min, from 50 to 80% in 2 min, at a flow rate of 1.5 ml/min. Separated compounds were identified through their retention times, UV spectra and literature data by comparison with IAA (12886, Sigma, St Louis, MO), GA3 (G7645, Sigma), GA4 (G7276, Sigma) and ABA (A1049, Sigma) standards. These standard compounds were also used to build up calibration curves (in the range 5–200 µg/ml) at specific wavelengths (λIAA = 254 nm; λABA = 254 nm; λGAs = 205 nm). For quantitative analysis, two different extract amounts from unknown samples were injected in triplicate. Reported values represent the concentration (expressed as µg of hormone per gram of fresh tissues). Gibberellin concentration was reported as the sum of GA3 and GA4 content.

2.3. 2D-electrophoretic analysis of protein extracts

T. durum cultivar grains, collected 3 days after the start of germination, were finely powdered in liquid N_2. Powder samples were aliquoted (about 1 g) and suspended in 3 ml of acetone containing 10% v/v trichloroacetic acid and 0.07% β-mercaptoethanol. After vortexing, samples were placed at −80 °C for 15 min, centrifuged (11,000 x *g*) for 10 min, and discarded of the supernatant; pellet samples were multiply washed with acetone and then dried at 25 °C. Pellet samples were treated with 5 ml of 700 mM sucrose, 500 mM Tris-HCl, pH 7.5, 50 mM EDTA, 100 mM KCl, 0.07% w/v β-mercaptoethanol, 1 mM PMSF, 2% SDS, 1 mM HCl, 1 mM protease inhibitor cocktail, and an equal volume of phenol. After 20 min, samples were centrifuged to obtain the separation of aqueous and phenolic phases. The phenolic phases were recovered, added with 4 vol of 0.1 M ammonium acetate in methanol, and stored at −20 °C, overnight. Samples were centrifuged (11,000 x *g*), for 10 min, to induce protein precipitation and the corresponding supernatants were discarded.

Protein pellets were washed with ice-cold methanol (once) and ice-cold acetone (three times), dried and solved in IEF buffer (8 M urea, 2% w/v CHAPS, 0.30% DTT and 2% w/v carrier ampholytes pH 3–10) (Bio-Rad, Hercules, CA, USA). Protein concentration was quantified using the BioRad protein assay, using BSA as standard compound. IPG strips (17 cm, pH 3–10 NL, BioRad ReadyStrip, BioRad) were rehydrated overnight with 300 µl of IEF buffer containing 350 µg of total proteins. Proteins were focused using a Protean IEF Cell (BioRad) at 12 °C, applying 250 V (90 min), 500 V (90 min), 1000 V (180 min) and 8000 V (until run ending), for a total of 55 KVh. After focusing, proteins were reduced by incubating the IPG strips with 6 M urea, 30% w/v glycerol, 2% w/v SDS and a dash of bromophenol blue containing 1% w/v dithiothreitol for 20 min; proteins within IPG strips were alkylated with the same buffer containing 1% w/v iodoacetamide instead of the reducing agent, for 20 min. Second dimension electrophoresis was carried out on 12% T polyacrylamide gels with a Protean apparatus (BioRad) in 25 mM Tris-HCl (pH 8.3), 1.92 M glycine and 1% w/v SDS, with 200 V, constant applied for 6/8 h. Two-dimensional electrophoretic gels were stained with colloidal Coomassie G-250. Biological samples were run in technical triplicate.

2-D electrophoretic gels were scanned using a GS-800 calibrated densitometer (Bio-Rad). Images analysis was performed using the PD Quest software (Bio-Rad). Spot detection and matching between gels were performed automatically, followed by manual verification. Protein spots were annotated only if detectable in all gels. After normalization of the spot densities against the whole-gel densities, the

percentage volume of each spot was averaged for three different replicates of each biological sample, and Student's t-test with ($P < 0.05$) was used to identify significant changes in protein amounts between samples.

2.4. Spot digestion, mass spectrometric analysis and protein identification

Spots from 2-DE were excised from the gel and shattered. Proteins were *in-gel* reduced with dithiothreitol, *S*-alkylated with iodoacetamide, and then digested with trypsin. Resulting peptide mixtures were subjected to a desalting/concentration step on μZip-TipC_{18} devices (Millipore Corp., Bedford, MA, USA) before MS analysis. Recovered peptides were then analyzed for protein identification by nanoLC-ESI-LIT-MS/MS, using an LTQ XL mass spectrometer (Thermo Fisher Scientific, USA) equipped with a Proxeon nanospray source connected to an Easy-nanoLC (Proxeon, Denmark). Peptides were resolved on an Easy C_{18} column (100 mm × 0.075 mm, 3 μm) (Proxeon) (Paiardini et al., 2014). Mobile phases were 0.1% *v*/v formic acid (FA) (solvent A) and 0.1% v/v FA in ACN (solvent B), running at a total flow rate of 300 nl/min. Linear gradient was initiated 20 min after sample loading; solvent B ramped from 5 to 35% over 45 min, from 35 to 60% over 10 min, and from 60 to 95% over 20 min. Spectra were acquired in the range *m/z* 400–2000. Each peptide mixture was analyzed under CID-MS/MS data-dependent product ion scanning procedure, enabling dynamic exclusion (repeat count 1 and exclusion duration 60 s), over the three most abundant ions. Mass isolation window and collision energy were set to *m/z* 3 and 35%, respectively. Raw nanoLC-ESI-LIT-MS/MS data were searched with v.2.2.06 MASCOT software (Matrix Science, UK) against an updated (07/2017), non-redundant UniProtKB database (taxonomy *T. aestivum*) to identify protein(s) present within each gel spot. Database searching was performed by using Cys carbamidomethylation and Met oxidation as fixed and variable protein modifications, respectively, a mass tolerance value of 1.8 Da for precursor ion and 0.8 Da for MS/MS fragments, trypsin as proteolytic enzyme, and a missed cleavage maximum value of 2. Other MASCOT parameters were kept as default. Protein candidates assigned on the basis of at least two sequenced peptides with an individual peptide expectation value < 0.05 (corresponding to a confidence level for peptide identification > 95%) were considered confidently identified. Definitive peptide assignment was always associated with manual spectra visualization and verification.

2.5. Data analysis

Results of germination assay and HPLC-based hormone analysis are expressed as means ± SD and represents three independent experiments. Comparisons of means to identify significant differences was performed by Anova followed by the Student-Newman-Keulus test, with the minimum level of significance being $P < 0.05$. Statistical significance of differences in protein spot densities from densitometric analysis of 2D-electrophoresis gels was assessed automatically by the PD Quest software (Bio-Rad) using the Student's *t*-test ($P < 0.05$).

3. Results and discussion

3.1. Germination assay

Seeds of Svevo, Senatore Cappelli and Saragolla wheat varieties were subjected to germination tests after 24, 48, 72, 96,120, 144 and 168 h. As shown in Fig. 1, the ancient cultivar Saragolla exhibited the best performances, both in terms of germination percentage as well as of precocity. In fact, Saragolla germination percentage values were the highest among the three cultivars at every time of sampling tested. Moreover, Saragolla variety showed an early (from 48 to 72 h) sharp increase in germination percentage (from 30 to 50%), which was unparalleled by the other cultivars. Senatore Cappelli exhibited germination characteristics similar to those of Saragolla, although less pronounced and lacking the early increase phase, which finally reached a maximum yield of germination at 168 h, close to that of Saragolla. On the other hand, Svevo cultivar showed the lowest performances both in terms of germination percentages as well as of precociousness. Overall, reported data indicate that the two ancient varieties, and in particular Saragolla, have a better germination potential than the elite counterpart Svevo.

3.2. Hormone content

Plant hormones balance is of pivotal importance in germination. In particular, it is well-characterized at the molecular level the stimulatory role of gibberellins in cereal grains as opposed to the inhibitory one of abscisic acid (Camoni et al., 2018). In order to correlate the hormone content of the three wheat varieties with their germination potential, seeds were collected after 3 days of germination, and assayed for abscisic acid (ABA), indoleacetic acid (IAA) and gibberellin (GA) content. Results reported in Fig. 2 show that GA content in the Saragolla cultivar was much higher (50 μg/g fresh tissue) than that of Senatore Cappelli (25 μg/g fresh tissue) and Svevo (16 μg/g fresh tissue) varieties. In particular, Svevo cultivar showed the lowest content of GA, an amount that was about one third of that of Saragolla. On the other hand, ABA and IAA concentrations were much reduced as compared to those of GA, and their differences among varieties were statistically not significant. Hence, reported data are in accordance with the notion that GA levels in germinating cereal seeds strongly increase, whereas ABA levels drop. Indeed, GA levels among the different cultivars fairly correlated with wheat variety germination performances.

3.3. Proteomic analysis

With the aim to describe differences in the protein repertoire among Svevo, Senatore Cappelli and Saragolla, a proteomic approach centered on 2-DE electrophoresis-based quantification of resolved proteins and mass spectrometry identification of differentially represented species was undertaken. This analysis was focused on 3-days germinating grains (Fig. 3), because at this time differences in germination percentages and GA levels among cultivars were at maximum. An effective separation of extracted proteins was obtained by 2-DE, applying a non-linear 3–10 pH gradient (in the first dimension) and using a 12% T polyacrylamide gel (in the second dimension), which solved components in the range 10–150 kDa. The master gel and representative gel counterparts of the three cultivars are shown in Fig. 4, whereas triplicates of the gels are shown in supplementary material (Fig. S1). Average proteomic maps showed 534, 550, and 549 spots in Svevo, Saragolla, and Senatore Cappelli gels, respectively. Software-assisted statistical analysis of colloidal Coomassie-stained gels revealed 45 protein spots as differentially represented between various samples ($P < 0.05$). These spots were excised from the gel, trypsinolyzed and subjected to nanoLC–ESI–LIT–MS/MS experiments for protein assignment. Fourty-four positive identification results were obtained. Two spots were associated with multiple proteins; due to their concordant quantitative trend with identical proteins from other spots, they were included in further bioinformatics analysis. Globally, identified spots were associated with 32 non-redundant protein entries. The list of the identified proteins, together with their quantitative variations in the different wheat cultivars is reported in Table 1. Some proteins occurred as multiple spots, whose structural differences were not further characterized. Functional categorization according to Gene Ontology annotation and literature data allowed to group identified proteins into three broad functional categories, namely protein storage, stress/defense and metabolism/energy production. Different proteins of uncharacterized function or not grouping in the above categories were also identified. These groups are discussed in the dedicated sections reported below.

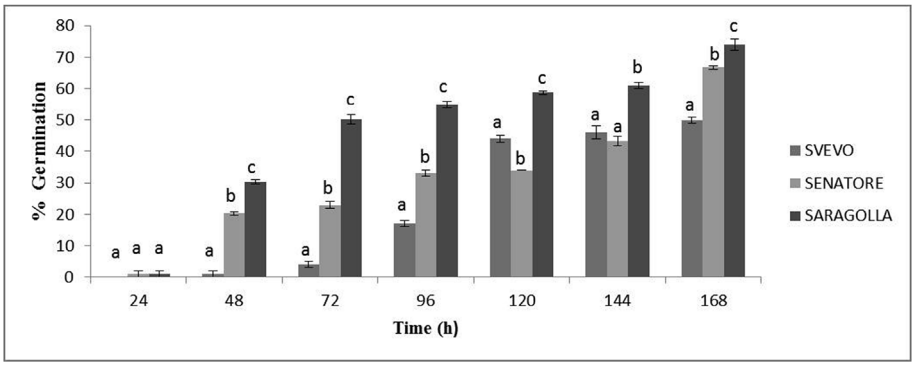


Fig. 1. Germination trend of wheat seeds of Svevo, Senatore Cappelli and Saragolla cultivars. Seeds were sterilized and germinated in Petri plates, and placed in a controlled environment chamber, at 30 °C, for 24, 48, 72, 96, 120, 144 and 168 h. Three biological replicates of 100 seeds per plate were used. Histograms indicate the germination percentage for each sample; vertical bars represent the standard deviation of mean of three replicates; bars labeled with dissimilar letters are significantly different ($P < 0.05$).

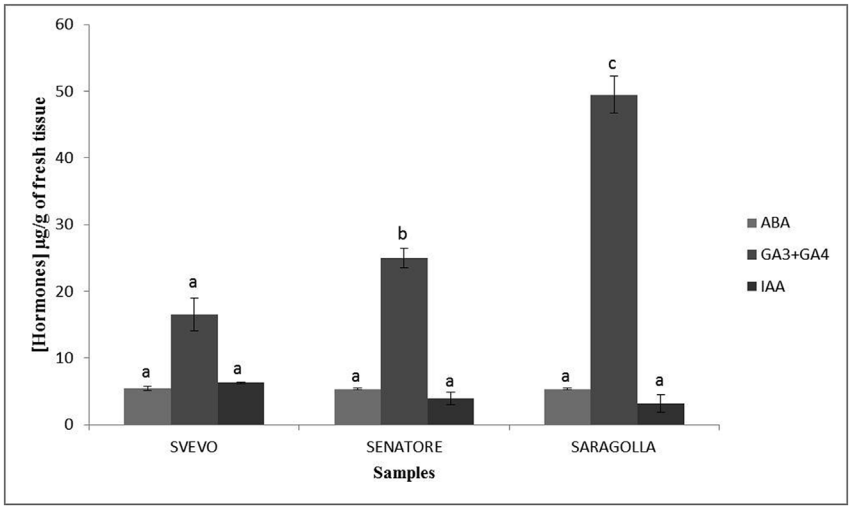


Fig. 2. Endogenous hormones concentration in durum wheat grains of Svevo, Senatore Cappelli and Saragolla cultivars, collected three days after germination. Three-days germinated seeds of different cultivars were extracted with methanol and analyzed by reversed–phase HPLC, as reported in Materials and methods. Reported values of abscisic acid (ABA) gibberellins (GA3 + GA4) and indolacetic acid (IAA) are the mean of hormone content per weight of fresh tissues (µg g^{-1}). Vertical bars represent the standard deviation of mean of three replicates; bars labeled with dissimilar letters are significantly different ($P < 0.05$).

3.4. Storage

Storage proteins in wheat caryopses include water/salt soluble albumins and globulins (non-prolamins) and insoluble gluten proteins, which are a mixture of monomeric gliadins and polymeric glutenins (prolamins). Gliadins and glutenins represent the major storage fraction (about 80% of the endosperm proteins) and determine the rheological and viscoelastic properties of wheat food derivatives, whereas albumins and globulins play important roles during grain development, metabolism and response to stresses (Singh et al., 1990). In this work, 8 storage proteins were ascertained as differentially represented among the three cultivars. On the overall, they accounted for 23%, 38.5% and 53.8% of total differential proteins in Svevo, Senatore Cappelli and Saragolla, respectively, supporting previous findings on an augmented protein content in the latter (De Santis et al., 2017). In particular, among the globulin family, 12 S seed storage globulin 1 (spot 11), 63 kDa globulin-like protein (spots 3 and 4), globulin 1 S allele (spot 6) and globulin 1 (spot 18) were identified. Their differential representation showed a heterogeneous trend among cultivars, with globulin 1 and 12 S seed storage globulin 1 over-represented in the Saragolla cultivar, and globulin 1 S allele in Senatore Cappelli cultivar. On the other hand, 63 kDa globulin-like protein showed an heterogeneous trend, with a form (spot 3) more abundant in the Svevo variety and another one (spot 4) in Senatore Cappelli counterpart. It has been reported that this cupin-domain-containing allergen can be glycosylated and phosphorylated. Accordingly, its occurrence as a large train of spots showing variable internal representation suggests the occurrence of variable post-translational modification changes between cultivars.

Fig. 3. Wheat seedlings of Svevo, Senatore Cappelli and Saragolla cultivars 72 h after germination. Seeds were sterilized and germinated in Petri plates, and placed in a controlled environment chamber, at 30 °C, for 72 h.

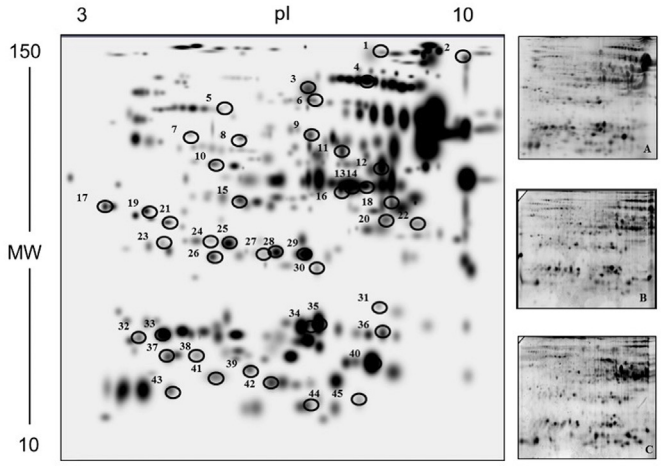


Fig. 4. Two-dimensional electrophoretic reference map of three-days germinated wheat seedlings and representative gels of Svevo (A), Senatore Cappelli (B) and Saragolla (C) varieties. Proteins were resolved on pH 3–10 NL IPG strips (first dimension), 12% T SDS-PAGE (second dimension) and visualized by colloidal Coomassie blue staining. Spot numbering refers to Table 1, where protein identification results are reported.

Indeed, it has been reported that different key proteins, including globulins, become phosphorylated during wheat germination (Dong et al., 2015); this molecular event may promote storage protein degradation.

As far as the gliadin/glutenin class, HMW glutenin x-type subunit Bx7 (spots 1 and 2), and γ-gliadin (spot 30) resulted over-represented in the local wheat varieties. Similarly to what observed for the 63 kDa globulin, HMW glutenins appeared as two spots of different pI values, probably reflecting a different degree of post-translational modification. Avenin-like a1 and b1 isoforms (spot 15, 13 and 14, respectively) were also identified among gluten proteins. Saragolla cultivar showed a reduced amount of both proteins as compared to the other two varieties. Avenin is a major allergen in oat and the presence of avenin-like proteins in wheat may contribute to the widespread gluten sensitivity and celiac disease syndrome.

On the overall, analysis of differentially synthesized storage proteins pointed out that local cultivars, and particularly Saragolla cultivar, present a higher representation of specific HMW glutenin and gamma-gliadin forms than Svevo. Since the quality of wheat flour-derived food products is largely determined by HMW glutenins and gliadins (Cornish et al., 2006), our data confirm the possibility to successfully use these varieties to prepare very high-quality dough for food derivatives.

3.5. Stress and defense

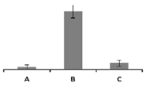
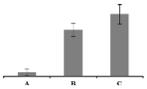
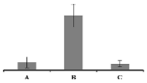
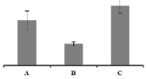
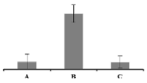
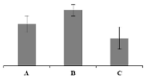
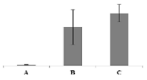
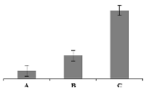
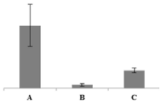
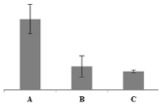
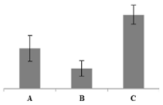
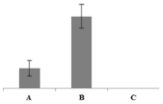
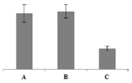
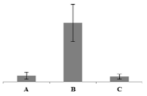
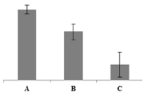
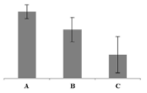
During seed maturation, prior to the desiccation phase, many defense proteins are accumulated and become activated during germination in response to mutated environmental conditions. In this study, various defense proteins were identified as differentially represented among wheat varieties. Among that, some grouped in functional subclasses assisting folding and correct disulfide bridge formation, contributing to face protein misfolding and aggregation during development or environmental stresses, namely protein disulfide isomerase (PDI) (spot 7), peptidyl-prolyl *cis-trans* isomerase (PPI) (spot 35 and 36), 23.2 kDa heat shock protein (spot 26), 18.1 kDa heat shock protein (spot 32), 16.9 kDa heat shock protein (spot 33 and 38), and 17.6 kDa heat shock protein (spot 37). Most of these proteins are believed to assist the massive synthesis of reserve proteins occurring during seed maturation. For example, PDIs catalyze correct formation of intramolecular S-S bonds in proteins in the ER of starchy endosperm, which is essential for their transport to Golgi and final deposition of β-gliadins in protein bodies (Shimoni and Galili, 1996). In cereals, a high expression of PDI members during grain filling has been associated with effective formation of intramolecular disulfide bonds in seed storage proteins, and proper polymerization of gluten (Ewa and Demska, 2016). They are expressed in the aleurone layer of mature grains, where they assist folding of newly synthesized hydrolytic enzymes during germination. They are also strongly induced in wheat grain subjected to abiotic stresses (Mostek et al., 2015). In this study, PDI was strongly over-represented in Saragolla variety as compared to the others, suggesting a strong request of this enzyme during augmented germination of this cultivar. Conversely, PPI was augmented in Svevo cultivar. This protein belongs to the large family of cyclophilins that assist isomerization of proline imidic peptide bond, which is a rate-limiting step in protein folding (Schiene-Fischer and Yu, 2001). They have been reported being induced in response to various abiotic and biotic stresses (Huang et al., 2011; Mostek et al., 2015). Interestingly, it has been demonstrated that the heterologous expression of a wheat cyclophilin TaCypA-1 confers thermotolerance to *Escherichia coli* (Kaur et al., 2017).

Heat shock proteins (HSPs) act as molecular chaperones stabilizing conformation of newly synthesized proteins or assisting recovery of damage proteins after abiotic stresses (Muthusamya et al., 2017). In wheat, 90 kDa HSPs and 70 kDa HSCPs have been reported to increase their concentration during grain development (*T. durum* Desf.) or germination (*T. aestivum* L.) (Dong et al., 2015). Similarly, small heat shock proteins (sHSPs) were observed as over-represented in *T. aestivum* grain in late stages of development (Zhang et al., 2015) or in response to heat stress (Majoul et al., 2004). In this study, all differentially abundant sHSPs detected, namely 23.2 kDa, 18.1 kDa, 17.6 kDa and 16.9 kDa heat shock proteins, were over-represented in the Svevo cultivar, as compared to local varieties. This finding may be associated with the observed extreme sensitivity of the latter ones to environmental abiotic stresses.

A different rationale was associated with variable representation of 1-cys peroxiredoxin (PER1) (spots 27–29) in different wheat cultivars. Peroxiredoxins are ubiquitous antioxidant proteins that regulate cell redox homeostasis by catalyzing the reduction of H_2O_2 and organic hydroperoxides; due to the involvement of H_2O_2 in signal transduction, they also control plant signalling during stress responses. Interestingly, it has been reported that transgenic lines of *Arabidopsis thaliana* overexpressing barley PER1 showed reduced germination when subjected to salt, osmotic or oxidative stress (Haslekas et al., 2003). This finding suggested that PER1 may play a role in the inhibition of germination under non-favourable environmental conditions. This observation is in line with PER1 levels detected in this study, where the representation of PER1 in Saragolla, Senatore Cappelli and Svevo was inversely correlated with the germinating performances of these cultivars, in perfect agreement with the proposed role of H_2O_2 in regulating promotion of germination (Fontaine et al., 1994).

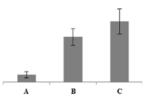
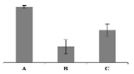
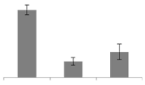
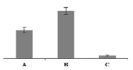
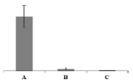
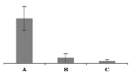
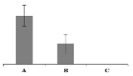
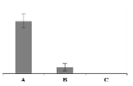
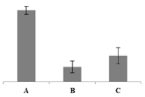
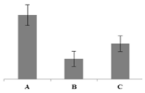
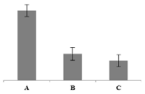
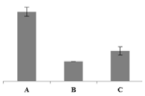
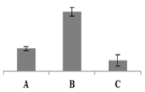
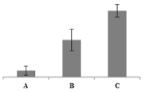
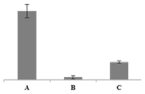
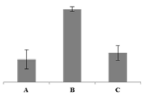
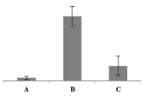
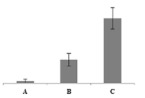
Other differentially represented defense proteins were universal stress protein A (spot 34), late embryogenesis abundant protein (LEAP) 31 (spot 21 and 23) and group 3 (spot 22), basic endochitinase C or A (spot 20), and α-amylase inhibitor 0.19 (spot 41 and 43), subtilisin (spot 31) and trypsin (spot 40 and 42) inhibitors. While universal stress protein A and LEAPs showed a variable quantitative representation between the wheat cultivars, the other proteins involved in biotic stress response were all over-represented in the Svevo cultivar. This finding may be associated with the observed high sensitivity of Saragolla and Senatore Cappelli varieties to biotic stresses (Buerstmayr et al., 2009). LEAPs are highly hydrophilic proteins playing key roles in the response to environmental challenges. Representation analysis of LEAPs in seedling leaves from wheat genotypes subjected to drought stress demonstrated that quantitative levels of these proteins contribute to drought tolerance (Lia et al., 2018). On the other hand, endochitinases are pathogenesis-related proteins that catalyze degradation of the cell wall of fungal pathogens. Endochitinase A and C levels have been shown to be affected by environmental stresses during grain filling in *T.*

Table 1
Differentially represented proteins in durum wheat grains collected three days after germination from Svevo (A), Senatore Cappelli (B) and Saragolla (C) cultivars, as deduced by 2-DE analysis of the corresponding extracts and nanoLC-ESI-LIT-MS/MS. See the experimental section for grain handling, protein extraction and proteomic analysis. Spot number, UniProtKB code, protein name, Mascot score, theoretical Mr and pI values, number of peptides identified, number of unique peptides identified and sequence coverage (%), together with the corresponding representation values in the different cultivars are reported.

Spot	UniProtKB code	Protein name	Mascot score	Theor. Mass	Theor. pI	Peptides	Unique peptides	Sequence coverage (%)	Relative representation
Metabolism and energy production									
5	A0A1D5ZG58	Enolase 2	632	46717	5.7	11	10	34.9	
9	A0A1D5VE13	Fructose-bisphosphate aldolase	341	39185	6.85	7	6	22.9	
10	A3KLL4	Malate dehydrogenase, cytoplasmic	743	35805	5.75	19	13	46.8	
12	A0A096UTL2	Glyceraldehyde-3-phosphate dehydrogenase	463	36840	6.4	10	7	28.5	
24	A0A1D5VIX2	Triosephosphate isomerase, cytosolic	557	32672	8.58	25	9	34.4	
25	A0A1D5VIX2	Triosephosphate isomerase, cytosolic	1107	32672	8.58	32	15	63.6	
16	W5A6D0	Aldose reductase	569	36206	6.65	13	10	33.8	
Stress and defense									
7	A0A024FR39	Protein disulfide-isomerase	1264	53988	5.1	47	24	60.6	
20	Q4Z8L8	Basic endochitinase C	198	28599	8.66	6	3	23.3	
	A0A1D6BR50	Basic endochitinase A	174	34383	8.28	5	3	17	
21	A0A1D5YGW1	Late embryogenesis abundant protein 31	1011	27014	5.06	38	13	76.3	
22	W4ZN68	Late embryogenesis abundant protein, group 3	462	23182	8.81	18	8	39.7	
23	A0A1D5YGW1	Late embryogenesis abundant protein 31	968	27014	5.06	28	14	77.4	
26	A0A1D5T6F6	23.2 kDa heat shock protein	696	21818	5.44	26	13	61.7	
27	Q6W8Q2	1-Cys peroxiredoxin PER1	628	24178	6.08	23	12	56.9	
28	Q6W8Q2	1-Cys peroxiredoxin PER1	335	24178	6.08	9	6	43.5	
29	Q6W8Q2	1-Cys peroxiredoxin PER1	305	24178	6.08	12	6	43.1	

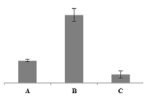
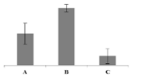
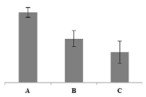
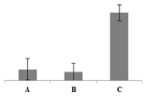
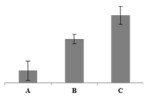
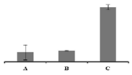
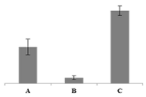
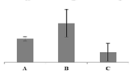
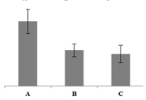
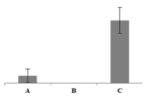
(*continued on next page*)

Table 1 (*continued*)

Spot	UniProtKB code	Protein name	Mascot score	Theor. Mass	Theor. pI	Peptides	Unique peptides	Sequence coverage (%)	Relative representation
31	P16347	Alpha-amylase/subtilisin inhibitor	523	19849	6.77	22	9	57.2	
32	W5E0N3	18.1 kDa class I heat shock protein	91	17497	5.52	2	2	16.5	
33	A0A077RPI9	16.9 kDa class I heat shock protein 1	303	16885	5.83	7	5	41.7	
	A0A077RX64	17.6 kDa heat-shock protein	230	17596	5.95	6	5	31,3	
34	A0A1D5TFS6	Predicted protein – similar to Universal stress protein A	175	17891	6.3	6	3	17.9	
35	A0A1D5T4V4	Peptidyl-prolyl cis-trans isomerase	185	18692	9.68	3	3	24	
36	A7LM55	Peptidyl-prolyl cis-trans isomerase	326	18618	8.59	12	6	42.7	
37	A0A077RX64	17.6 kDa heat-shock protein	212	17596	5.95	5	4	30	
38	A0A077RPI9	16.9 kDa class I heat shock protein 1	179	16885	5.83	2	2	23.2	
40	A0A1S6KXP9	Alpha amylase/trypsin inhibitor	188	15430	7.67	3	3	36.1	
41	A0A1D5WWL8	Alpha-amylase inhibitor 0.19	340	17240	6.06	10	5	45.5	
42	A0A1S6KXP9	Alpha amylase/trypsin inhibitor	200	15430	7.67	3	3	36.8	
43	A0A077RSX3	Alpha-amylase inhibitor 0.19	442	16910	5.28	16	6	52.6	
Storage									
1	V9TQ99	HMW glutenin x-type subunit Bx7	209	86229	9.26	5	4	9.2	
2	V9TQ99	HMW glutenin x-type subunit Bx7	554	86229	9.26	29	9	17	
3	A0A1D5XS09	63 kDa globulin-like protein	581	69208	7.17	17	12	24.4	
4	A0A1D5XS09	63 kDa globulin-like protein	1321	69208	7.17	60	22	44.7	
6	A0A1D5YEH1	Globulin-1 S allele	881	55488	6.63	20	15	33.3	
11	A0A1D5S0Z9	12 S seed storage globulin 1	809	64227	6.64	47	14	30.9	

(*continued on next page*)

Table 1 (*continued*)

Spot	UniProtKB code	Protein name	Mascot score	Theor. Mass	Theor. pI	Peptides	Unique peptides	Sequence coverage (%)	Relative representation
13	A0A173DQZ1	Avenin-like b1	256	33788	8.08	14	5	34	
14	A0A173DQZ1	Avenin-like b1	337	33788	8.08	14	6	37.5	
15	A0A1D6CWE1	Avenin-like a1	155	22790	8.5	5	2	13.9	
18	Q7XYC3	Globulin 1	340	26699	7.66	7	6	37.8	
30	A7XDG3	Gamma gliadin	181	16537	8.88	8	2	21.4	
Miscellaneous									
44	A0A1D5RUP2	Nucleoside diphosphate kinase	480	16607	6.3	17	8	48.7	
8	A0A1D5XF01	Uncharacterized protein	324	37603	6.5	6	6	19.7	
17	A0A1D6SDR0	Uncharacterized protein	597	28044	4.19	20	8	49.5	
19	A0A1D6RWC3	Uncharacterized protein	643	27090	4.72	18	9	51.8	
45	A0A1D5XMK2	Uncharacterized protein	213	15295	8.33	11	5	40.5	

aestivum (Sancho et al., 2008). Finally, protease inhibitors act as a part of the plant defense machinery by inhibiting the action of proteases from insects fungi and nematodes (Ruan et al., 2017). It has been proposed that these inhibitors also function in the modulation of the activity of seed endogenous proteases to prevent premature degradation of storage proteins during grain development, or even as storage proteins themselves (Smid et al., 2015). Many of them have allergenic properties in humans, such as the α-amylase inhibitors 0.19, which is one of the most active allergens associated with wheat flour allergies by inhalation (Sander et al., 2011). It is worth noting that ancient varieties exhibited a reduced amount of this allergen, as compared to Svevo, a trait which could represent a favourable quality factor for wheat derivatives production.

3.6. Metabolism and energy production

In plants, carbon metabolism plays a central role in energy supply during seed germination and early stages of seedling growth. Various enzymes related to glycolysis and tricarboxylic acid cycle (TCA) were here identified as differentially represented, namely fructose-bisphosphate aldolase (FBA) (spot 9), enolase 2 (ENO2) (spot 5), cytosolic triosephosphate isomerase (TPIS) (spot 24 and 25), cytoplasmic malate dehydrogenase (MDH) (spot 10) and NADP-dependent non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN) (spot 12). FBA, TPIS and ENO2 are key enzymes of the initial phase of glycolysis in which carbohydrates, mostly sucrose, are converted into hexose phosphates and then split in triose phosphates, to be oxidized in the energy-conserving phase of glycolysis. In our conditions, FBA was over-represented both in Saragolla and Senatore Cappelli varieties, as compared to Svevo one, whereas TPIS and ENO2 were particularly abundant in Senatore Cappelli. On the overall, these data may suggest that local landraces should be particularly efficient in the transfer of carbohydrates from various substrate pools to triose phosphates, thus triggering energy production through glycolysis during germination and early phases of growth. Moreover, Senatore Cappelli showed an increased expression also of cytosolic MDH, a key enzyme for energy production in plants, since it connects glycolysis to TCA cycle. In fact, this protein functions to convert the cytoplasmic oxaloacetate produced by glycolytic phosphoenolpyruvate into malate, to be oxidized in the TCA cycle. GAPDN was over-represented in the Saragolla cultivar. In addition to chloroplast-located, NADP-dependent photosynthetic GAPDH and cytoplasm-located NAD-dependent glycolytic GAPDH, both phosphorylating enzymes, plant cells contain a NADP-dependent, non-phosphorylating GAPDH (GAPDN) that generates NAPDH in the cytosol as result of the oxidation of photosynthetic Ga3P exported from chloroplasts (Habenicht et al., 1994). Results obtained in *Arabidopsis* GAPDN null mutants, which showed inhibition of glycolysis, reduced CO_2 fixation and increased oxidative stress (Rius et al., 2006), strongly suggest that this enzyme is fundamental to the efficiency of both glycolysis and Calvin cycle, thus playing a pivotal role in plant growth and development.

On the overall, analysis of differentially represented proteins involved in carbon metabolism pointed out that local cultivars presented a higher representation of enzymes essential to provide a proper

energetic supply to assist the observed augmented germinating performances of these cultivars.

3.7. Miscellaneous

Some other proteins that do not classify in the above categories were also identified in this study, namely nucleoside diphosphate kinase (NDK) (spot 44) and four uncharacterized proteins (spot 8, 17, 19 and 45). NDK was over-represented in the Saragolla cultivar, as compared to the other two varieties. NDKs are cytosolic, mitochondrial and plastidic enzymes regulating the homeostasis of the cellular nucleoside triphosphates pool, but also involved in heat stress, UV-B, reactive oxygen species and phytochrome signalling (Johansson et al., 2008). Among uncharacterized proteins, two (spot 8 and spot 45) were over-represented in the Saragolla cultivar. As far as their molecular function, spot 8 referred to a polypeptide with high sequence similarity to serine peptidase inhibitors, whereas spot 45 was homologous to a member of the 70 kDa HSP family.

4. Conclusions

The present study was aimed at describing major agronomic performances and differences in the protein repertoire of three-days germinated Saragolla and Senatore Cappelli grains, as compared to the elite cultivar Svevo. Analysis highlighed interesting differences in the protein pattern between local and elite cultivars, which can be potentially related to corresponding differences in growth habit or nutritional and food quality traits. In particular, Senatore Cappelli and Saragolla showed an increased amount of storage proteins as compared to Svevo, a fact that is in accordance with data attributing to the total protein content of grains a major role in determining pasta-cooking quality (D'Egidio et al., 1990). In Saragolla and Senatore Cappelli cultivars, a higher content of HMW glutenin and gamma-gliadin forms than Svevo occurred, a fact that fairly correlates with the reported very good rheological properties of doughs prepared from grains of these two cultivars (Cornish et al., 2006). Moreover, the consistent over-representation of PDI in the Saragolla cultivar, an enzyme that is essential to proper polymerization of gluten (Ewa and Demska, 2016), seems as well in accordance with the high-quality semolina obtainable from this variety. Different enzymes of glycolysis and TCA cycle resulted on the overall over-represented in Senatore Cappelli and Saragolla cultivars as compared to Svevo, suggesting that the observed better germination performances of these local varieties may be due to a corresponding higher efficiency in the energy production from carbohydrate metabolism. On the other hand, the general down-representation of proteins involved in the response to environmental and biotic stress observed in the old cultivars may be tentatively correlated to the reduction of the fitness cost of expression of resistance (Herms and Mattson, 1992) in the absence of stress, thus potentially contributing to increase corresponding germination rate and speed up early growth.

In conclusion, data reported here underline how, in a next future, the integration of high throughput phenotyping methodologies with high throughput platforms for molecular characterization can greatly impact punctual description of local and ancient cultivars, highly contributing to address corresponding major agronomic problems of cultivation and to identify molecular markers for amelioration of nutritional and sensory quality in breeding programs.

CRediT authorship contribution statement

Mariapina Rocco: Formal analysis. **Maria Tartaglia:** Investigation, Formal analysis. **Francesco Paolo Izzo:** Data curation, Validation. **Ettore Varricchio:** Project administration. **Simona Arena:** Formal analysis, Validation. **Andrea Scaloni:** Resources, Funding acquisition, Supervision. **Mauro Marra:** Conceptualization, Writing – review & editing.

Acknowledgments

This work was supported by funds from the Italian Ministry of University and Research to the National Research Council for the project "InterOmics – Tecnologie *omiche* per la valorizzazione del germoplasma di frumento duro".

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2018.12.010.

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