Analysis of durum wheat proteome changes under marine and fungal biostimulant treatments using large-scale quantitative proteomics: A useful dataset of durum wheat proteins

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ABSTRACT

Durum wheat requires high nitrogen inputs to obtain the high protein concentration necessary to satisfy pasta and semolina quality criteria. Optimizing plant nitrogen use efficiency is therefore of major importance for wheat grain quality. Here, we studied the impact on grain yield, protein concentration, and for the first time on protein composition of a marine (DPI4913) and a fungal (AF086) biostimulants applied to plant leaves. A large-scale quantitative proteomics analysis of wheat flour samples led to a dataset of 1471 identified proteins. Quantitative analysis of 1391 proteins revealed 26 and 38 proteins with a significantly varying abundance after DPI4913 and AF086 treatment, respectively, with 14 proteins in common. Major effects affected proteins involved in grain technological properties like grain hardness, in storage functions with the gluten protein gamma-gliadin, in regulation processes with transcription regulator proteins, and in stress responses with biotic and abiotic stress defense proteins. The involvement of biostimulants in the abiotic stress response was further suggested by an increase in water-use efficiency for both DPI4913 (15.4%) and AF086 (9.9%) treatments. Overall, our work performed in controlled conditions showed that DPI4913 and AF086 treatments promoted grain yield while maintaining protein concentration, and positively affected protein composition for grain quality.

Data are available via ProteomeXchange with identifier PXD012469.

Significance

We performed a large-scale quantitative proteomics study of the total protein extracts from flour samples to determine the effect of biostimulant treatment on the protein composition of durum wheat grain. To our knowledge, only a few studies in the literature have applied proteomic approaches to study durum wheat grains and even less to the effect of biostimulants on the protein composition of durum wheat grain. Moreover, most approaches used fractional extraction of proteins to target reserve proteins followed by two-dimensional electrophoresis (2-DE) or two-dimensional differential gel electrophoresis (2D-DIGE) that suffers from a low identification rate of proteins. We identified and quantified a large protein dataset of about 1400 proteins and determined molecular functions of proteins affected by biostimulants treatments.

1. Introduction

Durum wheat (*Triticum durum*) is a major agricultural crop that is primarily grown to produce mostly pasta and semolina. A total of 39.9 million tons' are produced per year and it is mainly cultivated in the Mediterranean basin and in North America [1]. The main crop characteristics sought by producers are yield and protein concentration, requiring high nitrogen inputs [2,3]. However, with increasing fertilizer

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prices and the risk of nitrogen leaching, farmers have to modify their fertilizing practices. Thus, to meet both industrial and environmental requirements, complementary processes have to be developed to optimize plant growth efficiency and environmental adaptability.

New strategies to enhance nitrogen uptake are frequently based on the use of biological molecules that act as biostimulants. According to the European Biostimulants Industry Council (EBIC), biostimulants are defined as follows: "Plant biostimulants contain substance(s) and/or micro-organisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality. Biostimulants have no direct action against pests, and therefore do not fall within the regulatory framework of pesticides" [4]. Biostimulants were considered by Zhang and Schmidt [5] as "materials, other than fertilizers, that promote plant growth when they are applied in small quantities." In addition, they can be used to enhance the effectiveness of conventional mineral fertilizers [6]. Among the different categories of biostimulants, substances extracted from seaweeds are the most frequently studied, and substances extracted from fungi are receiving increasing attention [7]. Marine and fungal bioactive substances are widely studied for their effect on yield, growth and nutrient absorption. This effect depends on many factors such as species or experimental conditions [8]. Only a few studies in the literature refer to durum wheat crops [9–11] and even less to the effect of biostimulants on the protein composition of wheat grain [12]. Among them, experiments showed that wheat grain protein concentration was increased by the application of biostimulants based on amino acids [13] or fungal biostimulants [14]. It has also been shown that nitrogen accumulation in wheat grains was increased by seaweed extracts [15]. Moreover, biostimulants are reported to improve fruit quality for various crops such as grapevine with seaweed extract application or vine-shoot extract [16,17], tomato with protein hydrolysate, plant and seaweed extracts [18,19], orange with plant extract and yeast [20], and cherry with seaweed extract [21].

The increase in nitrogen inputs has been shown to lead to changes in the protein composition of the grain, which depends mainly on the final quantity of nitrogen accumulated during grain filling [4,5]. Mature wheat grains contain 60 to 75% starch, 6 to 20% proteins, about 10% moisture, and 1.5 to 2% lipids [22]. According to the Osborne classification [23], proteins are divided into four main groups: albumins (soluble in water), globulins (soluble in a dilute salt solution), gliadins (soluble in 70% ethyl alcohol), and glutenins (soluble in a dilute acidic or basic solution). When the total amount of N in grain increases, the glutenin fraction remains stable while the gliadin fraction increases and the albumin-globulin fraction decreases [24,25]. Moreover, nitrogen concentration is linked to protein concentration through a nitrogen-to-protein conversion factor specific to each cereal product [26]. Gliadins and glutenins are referred to as prolamins. They represent major storage proteins [8,11,27], accounting for 70–80% of the total proteins, and are responsible for the technological quality of flour [28,29]. More precisely, the rheological properties of durum wheat depend on the balance between gliadins and glutenins. Gliadins are known to confer extensibility to the dough [30]. Glutenins are divided into low-molecular weight glutenin subunits (LMW-GS) and high-molecular weight glutenin subunits (HMW-GS). HMW-GS is considered to have an impact on bread-making quality [31], whereas LMW-GS is considered to contribute to pasta-making quality [32]. Albumins and globulins account for 15–20% of the total proteins and are involved in important functions in grain metabolism and regulation processes [33]. They include various enzymes and polypeptide inhibitors [9] such as alpha and beta-amylase, protease, oxidoreductase, and enzyme inhibitor [33] that affect many metabolism pathways and cellular processes such as redox homeostasis, cell cycle, cell defense and photosynthesis.

Mass spectrometry-based proteomics is a very efficient strategy to identify proteins in various matrices. In addition, a quantitative differential analysis allows the determination of protein variations under several conditions. In the literature, research has been conducted to investigate protein synthesis and accumulation in developing wheat grains using proteomics [34]. However, few studies have applied proteomic approaches to study durum wheat grains [9,32,35]. Most approaches used fractional extraction of proteins to target reserve proteins [10,36,37] followed by two-dimensional electrophoresis (2-DE) [10,11] or two-dimensional differential gel electrophoresis (2D-DIGE). However, these approaches suffer from several limitations such as a low identification rate of proteins [38] and a lack of accurate protein quantification.

The objective of the present study was to determine the effects of two foliar biostimulants, the seaweed extract DPI4913 and the fungal extract AF086, on a durum wheat crop, focusing on the plant nitrogen uptake and the variations in the protein composition in the grain on biostimulant treatments.

We used a large-scale label-free quantitative proteomics approach to analyze the total protein extract from flour. This unbiased method allowed the identification and quantification of about 1400 proteins in mature durum wheat grain. The analysis of protein variations showed several important biological pathways affected by both biostimulant treatments, including technological grain properties, storage functions, regulation processes and stress responses.

2. Materials and methods

2.1. Experimental design and plant material

A greenhouse experiment was carried out from January to June 2016 in Toulouse, France (43°52′72" N, 1°50′14″ E). The experiment consisted of durum wheat var. Anvergur is a variety with good gluten quality and good protein concentration. One factor was studied: the foliar application of biostimulants. Treatments were applied as foliar sprays at the second node stage (stage 32 of the Zadoks scale [39]). The following products were tested: DPI4913 containing *Ascophyllum nodosum* extract and a mix of amino acids; AF086 containing enzymes extracted from *Trichoderma*, manganese sulfate and sulfur. Three treatments were compared: a Control, with no foliar treatment; DPI4913, with a foliar application at a rate of 1 l/ha; and AF086, with a foliar application at a rate of 5 l/ha. Both DPI4913 and AF086 were provided by Agronutrition (a nutritional supplement company, a subsidiary of the De Sangosse Group, Carbonne, France).

Seeds of durum wheat var. Anvergur were germinated in plastic goblets filled with sand for one week in a growth chamber (25 °C/20 °C day/night; light intensity: 200 µmol $m^{-2} s^{-1}$ PAR; photoperiod: 12 h) and then for two weeks in the greenhouse (temperature > 10 °C, ambient light, fertigated with a modified Coïc-Lesaint solution). The nutrient solution composition was the following: NO_3^- 9.03 mM, NH_4^+ 1.25 mM, PO_4^{3-} 0.88 mM, K+ 3.49 mM, Ca^{2+} 2.70 mM, Mg^{2+} 0.96 mM, SO_4^{2-} 0.96 mM. Seedlings were transferred to 2 l plastic pots containing 2.2 kg of sandy soil (pH 5.0, 86.4% sand, 10.6% silt, and 3.0% clay). Each pot contained four one-tiller plants and received 50 ml of nutrient solution two to three times a week depending on plant needs.

The experiment used a randomized complete block design in order to minimize the variability due to external factors such as illumination, temperature or humidity. The experiment was divided into nine blocks containing two replications of each treatment, leading to 18 biological replicates per condition. One pot containing four plants was a replication.

The soil water retention capacity (SWC) was determined as follows: five 2 l pots were saturated with water. After the complete percolation of free water, the soil water content reached field capacity. The soil samples were then weighed, placed in an oven at 105 °C for 48 h and then weighed again. The soil water content at field capacity was calculated as the difference between the two weights: 20.6%. From the second node stage until harvest, pots were weighed three times a week

and soil water content was adjusted to 75% of field capacity. However, before watering, the moisture content in the soil was sometimes lower than 60%, indicating a slight water stress.

Weight loss between two weighing operations was considered to be due to transpiration. Water-use efficiency (WUE) was determined as follows:

$$WUE = \frac{total\ biomass\ per\ plant\ at\ harvest\ (g)}{water\ transpired\ from\ second\ node\ stage\ until\ harvest\ (l)}$$

Grains from four plants contained in one pot (ie from one replication) were harvested at maturity, mixed, and dried (60 °C for 48 h) in a drying oven for DW determination. Then, they were ground into flour for total N determination and for proteomics analysis. Flour samples from each replication was prepared independently.

2.2. Nitrogen-to-protein quantification

Total N content was determined by Continuous-Flow Mass Spectrometry using a Euro-EA Eurovector elemental analyzer coupled with an IsoPrime mass spectrometer (GV Instruments, Crewe, UK), at the Biochemistry and Plant Molecular Physiology Laboratory (BPMP) in Montpellier, France.

Protein concentration was calculated using the nitrogen-to-protein conversion factor for hard durum wheat flour: 5.81 [26].

2.3. Sample preparation for proteomics analysis

Wheat flour samples were obtained by finely grinding wheat grains from each plant replication. 1 g of flour from each of these 18 biological replicates corresponding to one condition (either control, DPI4913-treated, or AF086-treated) were then pooled to average biological variability. For each condition, the biochemical sample preparation was performed three times independently. A quantity of 50 mg flour was mixed with 1.5 ml lysis buffer (SDS 2%, 80 mM TrisHCL pH 8.5) and vortexed for 2 h at room temperature. After centrifugation (8000 *g*, 4 °C, 15 min) the supernatant was collected. Protein concentration was determined by DC protein assay (Biorad). A total of 100 µg of proteins was reduced in Laemmli buffer containing 25 mM dithiothreitol (DTT) at 95 °C for 5 min, and cysteines were alkylated by the addition of 90 mM iodoacetamide for 30 min at room temperature. Protein samples were loaded onto a 12% SDS-polyacrylamide gel and subjected to short electrophoresis. After Instant Blue (Invitrogen) staining of the gel, one gel band was excised, washed twice with 50 mM ammonium bicarbonate-acetonitrile (1:1, v:v) and then washed once with acetonitrile. Proteins were in-gel digested by the addition of 50 µl of a solution of modified sequencing grade trypsin in 25 mM ammonium bicarbonate (30 ng/µl, sequence grade, Promega, Charbonnières, France). The mixture was incubated at 37 °C overnight. The resulting peptides were extracted from the gel by one round of incubation (15 min, 37 °C) in 1% formic acid–acetonitrile (40%) and two rounds of incubation (15 min each, 37 °C) in 1% formic acid–acetonitrile (1:1). The three extracted fractions were pooled and air-dried. Tryptic peptides were resuspended in 100 µl of 2% acetonitrile and 0.05% trifluoroacetic acid for further MS analysis. A mix of standard synthetic peptides (iRT Kit; Biognosys, 0.2×) was spiked in all of the samples to monitor the stability of the nanoLC-MS/MS system during the analytical sequence.

2.4. NanoLC-MS/MS analysis

Peptide mixtures at 1 µg/µl were analyzed by nanoLC-MS/MS using a nanoRS UHPLC system (Dionex, Amsterdam, The Netherlands) coupled to an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Each biochemical replicate of flour samples was analyzed twice. Two microliters of each sample (2 µg) were loaded on a C18 pre-column (5 mm × 300 µm; Dionex) at 20 µl/min in 2% acetonitrile, 0.05% trifluoroacetic acid. After 5 min of desalting, the pre-column was switched online with the analytical C18 column (50 cm × 75 µm inner diameter; in-house packed with Reprosil C18) equilibrated in 95% of solvent A (5% acetonitrile + 0.2% formic acid in water) and 5% of solvent B (80% acetonitrile + 0.2% formic acid in water). Peptides were eluted using a 5–50% gradient of B for 105 min at a 300 nL/min flow rate. The LTQ-Orbitrap was operated in data-dependent acquisition mode with Xcalibur software. Survey scan MS spectra were acquired in the Orbitrap on the 300–2000 *m/z* range with the resolution set to a value of 60,000. The 20 most intense ion survey scans were selected for CID (collision-induced dissociation) fragmentation, and the resulting fragments were analyzed in the linear trap (LTQ). Dynamic exclusion was used within 60 s to prevent repetitive selection of the same peptide.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [102] partner repository with the dataset identifier PXD012469.

2.5. Bioinformatics analysis of nanoLC-MS/MS data

Acquired MS and MS/MS data were processed using MaxQuant software (version 1.5.3). Derived peak lists were submitted to the Andromeda search engine and data were searched against the UNIPROT protein database release march 2017 (http://uniprot.org) with a mixed database composed of annotations for *T. aestivum* and *T. durum* (146, 782 sequences; 65, 059, 445 residues) and a list of potential contaminant sequences. The precursor mass tolerance was set to 20 ppm for initial searches and 6 ppm for main Andromeda database searches. The fragment ion mass tolerance was set to 0.8 Da. Trypsin/P was chosen as the enzyme and two missed cleavages were allowed. Oxidation of methionine and protein N-terminal acetylation were defined as variable modifications, and carbamidomethylation of cysteine was defined as a fixed modification. The minimum peptide length was set to seven amino acids. The minimum number of unique peptides was set to one. The maximum false discovery rate (FDR), calculated by employing a reverse database strategy, was set to 1% for peptides and proteins.

For label-free relative quantification of the samples, the "match between runs" option of MaxQuant was enabled to allow cross-assignment of MS features detected in the different runs. The minimal ratio count was set to 1 for calculation of LFQ intensities. The LFQ metric was used to perform relative quantification between proteins identified in different biological conditions (reflecting a normalized protein quantity deduced from all peptide intensity values). Protein entries identified as potential contaminants by MaxQuant were eliminated from the analysis. Missing values were replaced by a constant noise value determined independently for each analytical run as the 1% percentile of the total protein population.

2.6. Statistical and functional data analysis

For each comparison of quantitative proteomics data, only proteins quantified in a minimum of three nanoLC-MS/MS runs (out of six per flour sample) for at least one of the flour sample used for comparison were subjected to statistical analysis. A *p*-value and a ratio of the average normalized area were calculated. Proteins are considered variants when the p-value < 0.05 and the ratio < 0.5 or > 2. Volcano plots were drawn to visualize significant protein abundance variations between two conditions. They represent log10 (p-value) according to the log2 ratio.

Functional data analysis was performed using Gene Ontology (GO) annotations referring to biological processes and cellular components in Uniprot (https://www.uniprot.org/uploadlists/).

Table 1
Grain dry biomass per plant, protein concentration and amount of protein in grains per plant with different foliar treatments in a greenhouse experiment; southwest France.

	Grain dry biomass per plant (g)				Protein concentration in grains per plant (%)				Amount of protein in grains per plant (mg)			
Average ± SD												
CONTROL	1.13	±	0.33	a	19.51	±	2.25	a	214.9	±	50.2	a
DPI 4913	1.40	±	0.35	b	19.95	±	1.58	a	268.2	±	64.9	b
AF086	1.44	±	0.36	b	19.30	±	1.59	a	270.6	±	72.7	b
ANOVA P-value												
Treatment	0.007				NS				0.001			
Block	0.05				0.01				NS			

Note: The letters (a and b) indicate a statistical difference ($P < 0.05$) between treatments.

3. Results and discussion

3.1. Grain dry biomass and protein accumulation

For both biostimulant treatments, the grain biomass and the amount of protein accumulated in grains per plant (one-tiller plants) was statistically higher for treated plants (Table 1). It was increased by 24.8% with DPI4913 and by 25.9% with AF086. Grain dry biomass was also significantly higher for treated plants (23.9% for DPI4913 and 27.4% for AF086, respectively). The grain dry biomass and the amount of protein in grains per plant were positively affected by biostimulant application, but the protein concentration in grains per plant was not affected. These results contrast with the inverse correlation between grain protein concentration and yield, as commonly shown by Baker et al. and Simmonds [40,41]. This deviation from the protein dilution curve (grain protein concentration as a function of yield) is a required criterion for wheat varietal selection [42]. In our results, this positive deviation can be obtained by the use of biostimulants, leading to an increase in protein yield.

The increase in the amount of protein in grains per plant, and thus of the amount of nitrogen, has been shown to be correlated with a variation of protein composition [43]. To study the impact of biostimulants on this variation, proteomic analyses were performed to identify the differentially-represented proteins under biostimulant treatment.

3.2. Protein composition of durum wheat flours

In order to identify the largest number of durum wheat proteins and to determine the variations in the protein composition in the grain using biostimulants treatment, we used a large-scale label-free quantitative proteomics approach based on the analysis of the total protein extract from flour samples obtained from milled grains. The workflow is presented in Fig. 1. Proteins were extracted from flour samples using an optimized protocol. Protein separation using one-dimensional SDS PAGE was used to verify that the complexity of the protein mixtures and the distribution of proteins along the gel between 20 kDa and 100 kDa were similar for each sample (data not shown). For further proteomics analyses, only a short electrophoretic migration was performed and one gel band was excised. Proteins were in-gel digested with trypsin and subjected to nanoLC-MS/MS analysis for protein identification, validation and quantification.

In our study, three conditions were considered: a Control sample, without biostimulant treatment of the plant, and two flour samples from plants treated, by either DPI4913 or AF086 as described (see material and methods section). For each condition, 24 plant culture replicates have been obtained leading to 72 samples in total. The corresponding flour samples were then pooled for each condition to average culture variations in samples. Three flour replicates per conditions and three technical replicates per flour sample were further analyzed to allow statistical analysis.

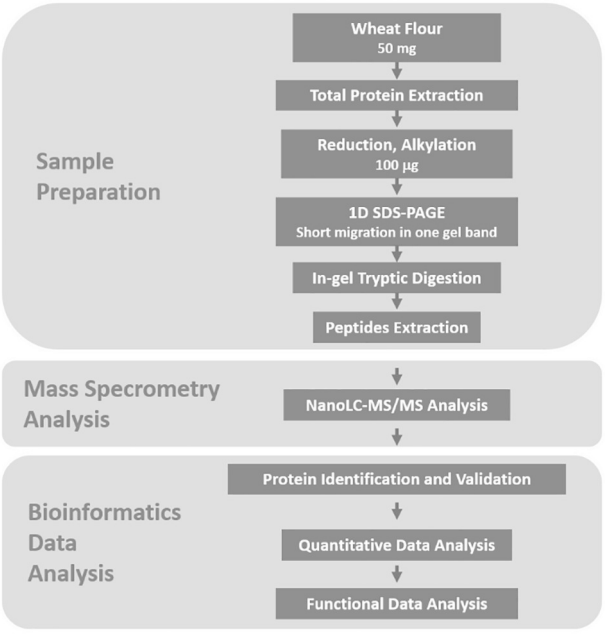


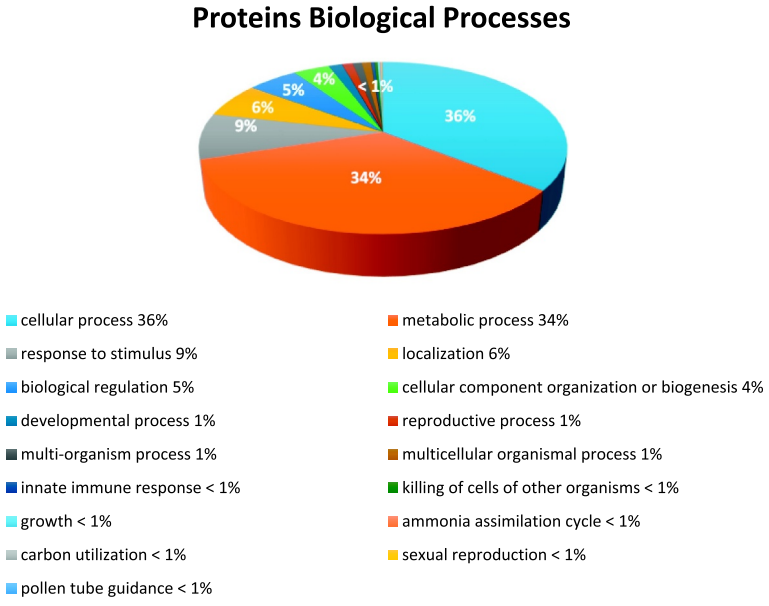
Fig. 1. Proteomics analysis workflow of durum wheat flour samples.

For each condition, almost 1500 proteins were identified (1459 for the Control sample, 1449 for the AF086-treated sample, and 1456 for the DPI4913-treated sample) leading to 1471 proteins identified in total. We used a mixed *Triticum* database composed of *T. aestivum* and *T. turgidum* ssp. *durum* (146,782 sequences; 65,059,445 residues) since there were only two proteins in the SwissProt database for durum wheat and only 715 proteins were translated from the genomic EMBL database. However, only 40% of the identified proteins were characterized. To our knowledge, this represents the largest dataset of proteins identified for durum wheat.

In order to characterize better this dataset, a functional analysis based on Gene Ontology (GO) terms for molecular functions and biological processes was conducted. The resulting 1471 identified proteins (for all conditions) were used, after removing redundant protein codes, and only 50–60% of them were associated with a GO category (Fig. 2). This analysis showed that the main molecular functions associated to durum wheat flour were catalytic activity (41%) and binding (37%), followed by structural molecule activity (7%), nutrient reservoir activity (4%), and transporter activity (3%). When considering the role of proteins in biological processes, the main categories were cellular (36%) and metabolic processes (34%), followed by response to stimulus (9%), localization (6%), biological regulation (5%), and cellular components organization or biogenesis (4%). These categories have already been described in previous proteomics studies of wheat grains [44,45] and reflect the protein composition of the mature grains.

The same functional analysis based on GO terms was also performed

A



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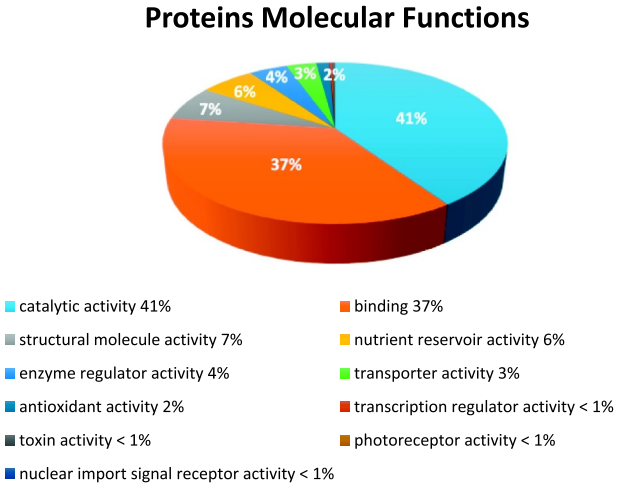


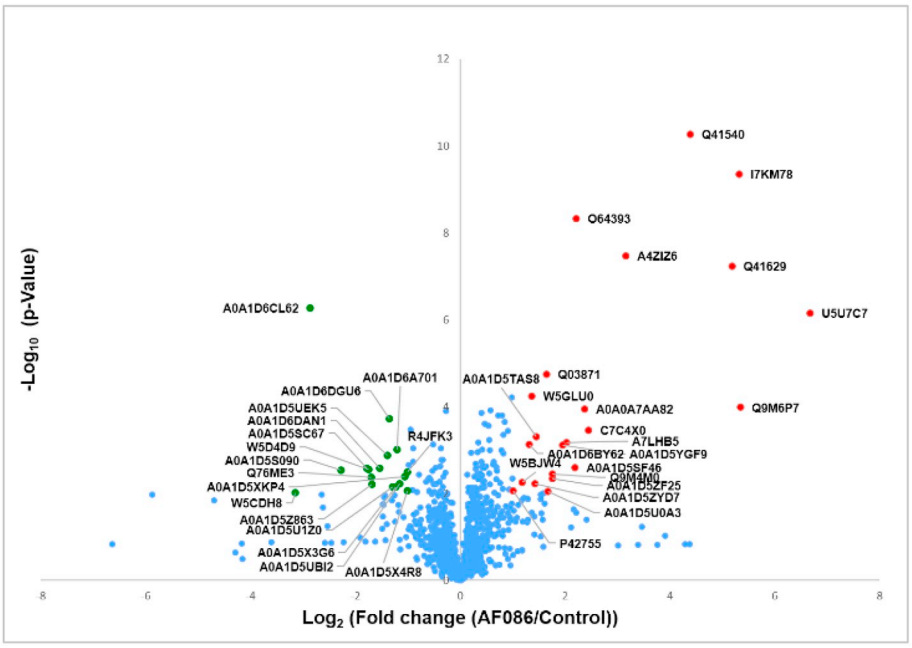
Fig. 2. Pie charts of the distribution of proteins from durum wheat grains based on their predicted: (A) biological processes; (B) molecular functions. This involves all the proteins identified in flour samples in the three conditions having a GO term associated, i.e. a total of 962 proteins for Molecular Functions and 655 proteins for Biological Processes.

using the proteins identified in each condition independently (the Control samples, the AF086 and the DPI4913-treated samples). It showed a similar protein distribution for all conditions (data not shown). It can thus be concluded that there is no effect of the biostimulants used on the overall protein categories distribution in the grain.

Quantitative analyses were then performed to determine the effect of biostimulants on the abundance of each protein in wheat grain. Comparison of DPI4913-treated and AF086-treated samples with Control samples allowed the quantification of 1391 proteins. Among these, 20 and 22 were over-represented after DPI4913 and AF086 treatments of the plant, respectively; and 6 and 16 were under-represented after DPI4913 and AF086 treatments of the plant respectively (Fig. 3). Thus, only a small set of proteins seems to be affected by biostimulants treatments. However, abundance ratios were rather high for some proteins, like the gamma-gliadin increased 100 times.

Grain proteins with varying abundances after biostimulants treatment were classified in four functional categories: grain technological properties, storage function, regulation processes and stress/defense responses. A total of 14 proteins, including nine defense proteins, four storage proteins and one protein involved in grain technological properties, were found to be differentially represented for both DPI4913 and AF086-treated samples as compared to Control (Table 2). 12 proteins were found to be differentially represented specifically in DPI4913-treated samples, of which two defense proteins, two storage proteins, six regulation proteins and two unclassified proteins (Table 3). We found 24 proteins differentially represented specifically in AF086-treated samples, of which 12 defense proteins, three regulation proteins, three storage proteins, one protein involved in technological properties and five unclassified proteins (Table 4).

A



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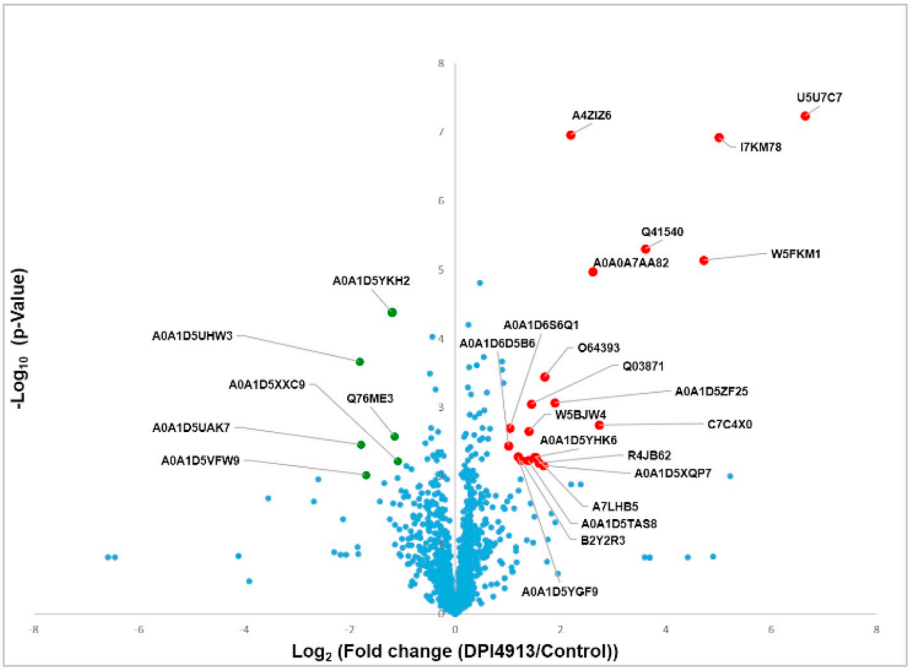

Fig. 3. Quantitative proteomics analysis of flour samples from durum wheat grains after (A) AF086 and (B) DPI4919 biostimulant treatments of the plant. Volcano plot [−log10 (p-value) vs. log2 (fold change)] highlighting proteins with significantly varying abundance in the samples. Statistical analysis was performed from three biological replicates with a two-sided Student *t*-test, variance correction and permutation-based false discovery rate (FDR). Proteins with an adjusted *p*-value ≤0.05 were considered to be significantly regulated if they presented a log2-fold change ≥1 (in red) or ≤ −1 (in green) (corresponding to a 2-fold change in protein abundance). Those proteins considered to be significant are shown with their corresponding accession names.

3.3. Proteins involved in grain technological properties

Among the technological properties of durum wheat described by Troccoli et al. [46], grain hardness and protein concentration are the main traits taken into account.

In our study, two proteins differentially represented by biostimulants have these traits. A grain softness protein from the puroindoline-like family, Gsp1D, was found to be five times more abundant after both DPI4913 and AF086 treatments, compared to the Control treatment. Its role is uncertain but puroindolines may enhance grain hardness, having an effect on the adhesion between the constituent polymers (starch granules and protein network) of endosperm cells, impacting milling behavior and flour yield. In the same way, Heinze et al. have shown that increased grain hardness may occur due to the failure of PUIA-encoding gene expression or mutations in the PUIB-encoding gene [47]. This result suggests that DPI4913 and AF086 would increase grain hardness.

A protein ubiquitin-protein transferase (A0A1D5S090, uncharacterized protein) was specifically under-represented by AF086 (five time less abundant than for the Control treatment). It has been shown that ubiquitin-related genes are differentially represented in isogenic lines contrasting for pericarp cell size and grain weight in

Table 2
List of significantly over- or under-represented proteins identified in mature grains of durum wheat both after DPI4913 and AF086 treatments.

Gene name	UniProt Accession number	Protein name	Molecular function	Fold change Biostimulant vs Control	
				AF086	DPI4913
Defense					
	Q41540	CM 17 protein	Alpha-amylase inhibitor activity	20.9	12.2
	A4ZIZ6	Monomeric alpha-amylase inhibitor (Fragment)	Alpha-amylase inhibitor activity	8.9	4.6
wtai-CM1	C7C4X0	Alpha amylase inhibitor CM1 (Fragment)	Alpha-amylase inhibitor activity	5.5	6.7
PR4B	O64393	Wheatwin-2	Antimicrobial. Fungicide. Pathogenesis-related protein	4.6	3.2
	A0A1D5YGF9	Peptidylprolyl isomerase	Interconversion of cis-trans isomers	3.9	2.3
	A0A1D5ZF25	Calcium-transporting ATPase	Roles in transport systems and stress signaling in cellular homeostasis	3.4	3.7
	A0A1D5TAS8	Uncharacterized protein	ATP-binding	2.7	2.6
	W5BJW4	Uncharacterized protein	GTP-binding	2.3	2.6
arf	Q76ME3	ADP-ribosylation factor	GTP-binding	0.3	0.4
Storage					
	U5U7C7	Gamma-gliadin	Nutrient reservoir activity	102.0	99.3
GID-HE1	I7KM78	Gamma-gliadin	Nutrient reservoir activity	39.9	32.2
gli-wE12	A7LHB5	Alpha gliadin	Nutrient reservoir activity	4.1	2.9
Glu-1By9	Q03871	HMW glutenin subunit 1By9	Nutrient reservoir activity	3.1	2.7
Technological properties					
Gsp-1D	A0A0A7AA82	Grain softness protein (Fragment)	Puroindoline like	5.2	6.1

hexaploid wheat [48]. Thus, AF086 would increase cell size and grain weight.

3.4. Proteins involved in storage functions

Storage proteins accumulation depends on regulatory proteins. Indeed, in the developing endosperm cells, prolamins are folded and assembled in the endoplasmic reticulum, where the enzyme protein disulfide isomerase may catalyze disulfide bond formation and exchange, affecting the functional properties of gluten [49,50].

The ratios between the different components of storage proteins has been associated with technological performances [51]. In our study, gluten components proteins were differentially represented by both the DPI4913 and AF086 treatments.

Gliadin family proteins differentially represented by DPI4913 and AF086 were substantially over-represented (maximum fold change: 102.0). Glutenin fractions differentially represented by DPI4913 and AF086 were less over-represented (maximum fold-change: 3.1) or under-represented. It appears that the gliadin-to-glutenin ratio was increased by biostimulants. Similarly, nitrogen supply has been shown to be accompanied by an increase in gliadin-to-glutenin ratio [52]. In our experiment, the effect of DPI4913 and AF086 on the increase of the amount of proteins in grains per plant (see Section 3.1) led to an increase in the gliadin-to-glutenin ratio.

An increase in the expression of gamma-gliadin and alpha-gliadin was observed in grains of plants treated with DPI4913 and AF086. Previous studies reported alpha-gliadin to be over-represented under water or heat stress [53,54] and under water stress for gamma-gliadin [10]. HMW-GS expression was also increased by DPI4913 and AF086. Like gamma-gliadin and alpha-gliadin, HMW-GS has been reported by

Table 3
List of significantly over- or under-represented proteins identified in mature grains of durum wheat specifically for the DPI4913 treatment.

Gene name	UniProt Accession number	Protein name	Molecular function	Fold change DPI4913 vs Control
Defense				
	A0A1D5UAK7	Cysteine proteinase inhibitor	Cysteine-type endopeptidase inhibitor activity	0.3
	A0A1D5UHW3	Uncharacterized protein	GTP binding (small GTPase mediated signal transduction)	0.3
Regulation				
	W5FKM1	Uncharacterized protein	Zinc ion binding	26.3
	A0A1D5XQP7	Protein disulfide-isomerase	Cell redox homeostasis	3.2
	A0A1D6S6Q1	Uncharacterized protein	DNA binding/ transcription factor activity. Sequence-specific DNA binding	2.1
	A0A1D5XXC9	Beta-amylase / Amylopectin maltohydrolase	Amylopectin maltohydrolase activity/ beta-amylase activity	0.5
	A0A1D5YKH2	Histone H2A	DNA binding/ protein heterodimerization activity	0.4
	A0A1D5VFW9	Uncharacterized protein	Hydrolase activity; hydrolyzing O-glycosyl compounds (carbohydrate metabolic process)	0.3
Storage				
LMW-GS	R4JB62	Low-molecular-weight glutenin subunit	Nutrient reservoir activity	3.0
GluB3–6	B2Y2R3	Low molecular weight glutenin subunit	Nutrient reservoir activity	2.4
Unclassified				
	A0A1D5YHK6	Uncharacterized protein		2.8
	A0A1D6D5B6	Uncharacterized protein		2.0

Table 4
List of significantly over- or under-represented proteins identified in mature grains of durum wheat specifically for the AF086 treatment.

Gene name	UniProt Accession number	Protein name	Molecular function	Fold change AF086 vs Control
Defense				
ANT-G1	Q41629	ADP.ATP carrier protein 1; mitochondrial	Catalyzes the exchange of ADP and ATP across the mitochondrial inner membrane	36.3
	A0A1D5SF46	Uncharacterized protein	Unfolded protein binding	4.6
	A0A1D5U0A3	Uncharacterized protein	Unfolded protein binding	3.2
	A0A1D5ZYD7	Uncharacterized protein	Oxidoreductase activity/ zinc ion binding	2.7
	W5GLU0	Uncharacterized protein	Co and Zn ion binding / proton-transporting ATP synthase activity. Rotational mechanism	2.6
	A0A1D6BY62	Peroxidase	Hydrogen peroxide catabolic process/ response to oxidative stress	2.5
EMH5	P42755	Em protein H5	Stress response	2.0
	A0A1D5XKP4	Uncharacterized protein	Response to freezing	0.5
	A0A1D5UBI2	Uncharacterized protein	Response to cold/oxidative stress	0.4
	A0A1D6DGU6	Uncharacterized protein	ATP binding	0.4
	A0A1D5SC67	Uncharacterized protein	Unknown (belongs to LEA protein family)	0.3
TRAES_3BF063600070CFD_c1	W5D4D9	Uncharacterized protein	Glutathion S-transferase activity	0.3
Regulation				
	A0A1D5X4R8	Histone H2B	Regulation of transcription. Replication and repair of DNA	0.5
	A0A1D6CL62	Uncharacterized protein	Phosphoenolpyruvate carboxylase activity	0.1
	A0A1D5X3G6	Uncharacterized protein	Aspartic-type endopeptidase activity	0.4
Storage				
	Q9M6P7	Gamma-gliadin (fragment)	Nutrient reservoir activity	40.5
	Q9M4M0	Alpha-gliadin	Nutrient reservoir activity	3.4
LMW-GS	R4JFK3	Low-molecular-weight glutenin subunit (fragment)	Nutrient reservoir activity	0.5
Technological properties				
	A0A1D5S090	Uncharacterized protein	Ubiquitin-protein transferase activity	0.2
Unclassified				
	A0A1D5U1Z0	Malate synthase	Malate synthase activity	0.4
	A0A1D6A701	Uncharacterized protein		0.4
	A0A1D5UEK5	Uncharacterized protein		0.4
	A0A1D6DAN1	Uncharacterized protein		0.3
	A0A1D5Z863	Uncharacterized protein		0.3

Pompa et al. [54] to be over-represented under heat stress. It appears that mechanisms similar to those implied in response to water stress or heat stress are activated by biostimulant application.

Furthermore, HMW-GS and gamma-gliadin are interesting proteins able to store nitrogen when the supply of nitrogen increases [38,55]. Thus, the over-representation of HMW-GS and gamma-gliadin by biostimulants can be linked with a need to store nitrogen, and thus to an increase in the amount of nitrogen in grains.

LMW-GS expression was affected by both biostimulants but not in the same way. It was over-represented by DPI4913 and under-represented by AF086. As mentioned above, HMW-GS is considered to have an impact on bread-making quality [31], whereas LMW-GS is considered to contribute to pasta-making quality [32]. DPI4913 would then have a greater influence on pasta-making quality than AF086.

However, since HMW-GS and LMW-GS are respectively over-represented and under-represented by AF086, it increases the HMW-GS-to-LMW-GS ratio, which has been associated with a better technological performance [51]. Moreover, the observed under-representation of LMW-GS with AF086 is similar to that observed in response to water stress [10] or heat stress [54]. Then, AF086 – as DPI4913 – impacts metabolic pathways involved in response to water stress or heat stress and contributes to improve wheat technological quality.

3.5. Proteins involved in regulation processes

Among the proteins involved in regulation processes, transcription factors are DNA binding proteins that control the rate of transcription of genetic information from DNA to messenger RNA by binding to a specific DNA sequence [56]. Histones are also involved in the regulation of transcription since they are responsible for DNA condensation, organization and regulation in the nucleus, impacting accessibility and effectiveness of the transcriptional machinery [57,58].

Regulation pathways concerning information within the individual cell and throughout the plant are activated at the cellular level when subjected to biotic and abiotic stresses. In this study, grain proteins involved in regulation processes were differentially represented by DPI4913 and AF086, but none was differentially represented by both biostimulants.

DNA-binding protein expression was influenced by DPI4913 application. Indeed, proteins belonging to the DNA-binding protein family were differentially represented by DPI4913: a histone H2A was under-represented, and an uncharacterized DNA-binding protein was over-represented. One DNA-binding protein, a histone H2B protein, was down-regulated by AF086. Zhou et al. [59] discussed the role of two H2A-H2B dimers, constituting the entry/exit point of nucleosomal DNA access. It thus appears that DPI4913 and AF086 have an impact on the transcriptional machinery regulation.

Moreover, an uncharacterized protein belonging to the zinc-binding protein was over-represented by DPI4913. Forty percent of the Zn-binding proteins are transcription factors and it has been shown that the number of zinc-binding proteins is linearly correlated with the total number of proteins encoded by the genome of vegetal or animal organisms [60,61]. The over-representation of a zinc-binding protein is correlated with the observed increase of the total amount of proteins in grains for plants treated with DPI4913.

A protein-disulfide isomerase was over-represented by DPI4913. This enzyme is necessary for synthesis, polymerization and the accumulation of storage proteins in many tissues [62,63], this result can be

linked to the previously described modification of storage proteins expression by DPI4913.

A beta-amylase was under-represented by DPI4913. During grain development, beta-amylase is a starch-degrading enzyme that cleaves a 1,4-D-glucosidic bond from polyglucans [64]. It is considered to be one of the major proteins in the starchy endosperm. The under-representation of beta-amylase by DPI4913 might lead to starch accumulation in grains.

Similarly, an uncharacterized protein with hydrolase activity, hydrolyzing O-Glycosyl compounds, was down-regulated by DPI4913. O-Glycosyl hydrolases hydrolyze the glycosidic bond between two or more carbohydrates. Inactivating the sucrose degradation pathways is linked to an increased accumulation of soluble carbohydrates [65].

An uncharacterized protein described as having a phosphoenolpyruvate carboxylase activity was down-regulated by AF086. Phosphoenolpyruvate carboxylase was identified by González et al. in the protein bodies of immature durum wheat grains [66] and reported to contribute to amino acid and protein biosynthesis during grain development [67]. Our results suggest that AF086 influences amino acid and protein biosynthesis during grain development.

Aspartic-type endopeptidase was under-represented by AF086. It was suggested by Darabi and Seddigh that it participates in storage protein degradation during the mobilization of reserve proteins in seed germination of wheat [68]. Aspartic-type endopeptidase also regulates other plant mechanisms such as senescence, stress responses, programmed cell death, reproduction, and antimicrobial defenses [69–74]. The level of expression of this enzyme is of great importance since it may be potentially damaging when over-represented or present in high concentrations [75]. Thus, by down-expressing aspartic-type endopeptidase activity, AF086 may reduce protein degradation and have an impact on senescence, stress response, programmed cell death, reproduction, and antimicrobial defenses.

Variations of regulatory proteins after treatments may impact important cellular pathways and functions like protein synthesis and protein degradation that may have direct effect on protein accumulation in the grain or that may be related to responses to various abiotic and biotic stresses.

3.6. Proteins involved in stress and defense response

Some proteins involved in stress and defense responses are differentially represented by both DPI4913 and AF086, while others are differentially represented specifically either by DPI4913 or by AF086.

Three proteins belonging to the alpha-amylase inhibitor family were over-represented by both DPI4913 and AF086: CM 17 protein, monomeric alpha-amylase inhibitor and alpha-amylase inhibitor CM1. Alpha-amylase inhibitor proteins are key natural players in plant defense against insect and microorganisms [76]. Among defense proteins, alpha-amylase inhibitor proteins were identified a s defense-related enzymes. Alpha-amylase inhibitors are also considered to be storage proteins that compensate for amino acid insufficiency during seedling growth [77]. The multiple forms of proteins in this family, and their abundance during grain development is linked to a need to protect starch, deposited in the endosperm from degradative enzymes [78]. Therefore, DPI4913 and AF086 may have an impact on grain filling by protecting starch deposition or by storing amino acids.

A cysteine-type endopeptidase inhibitor was specifically under-represented by DPI4913. Cysteine-type endopeptidases are enzymes involved in storage protein maturation [79,80] and can be induced in seeds for stress tolerance such as drought [81,82] or damage by pathogens [83,84]. Even if DPI4913 has a positive effect on grain filling and stress defense through the over-representation of certain types of proteins, it appears that it has a more debatable effect on protein maturation and stress defense through the under-representation of a cysteine-type endopeptidase.

Two uncharacterized proteins belonging to the unfolded protein binding family were specifically over-represented by AF086. Unfolded protein-binding families are molecular chaperones, binding proteins that are in unstable, non-native structural states [85]. AF086 would help to prevent protein denaturation in the case of a stressful environment.

One ATP-binding protein belonging to the Heat Shock Protein 70 family (HSP 70) was over-represented by both DPI4913 and AF086, whereas another one was specifically under-represented by AF086. HSP70 protein expression increases in response to high temperature during grain filling [86]. The mechanisms of sHSP action are not fully understood, even if they are highly inducible by stress. They have an ATP-independent chaperone activity and prevent thermal aggregation by binding to denatured proteins. They help in the proper folding of proteins in association with other chaperones [87,88]. Two other heat-stress-induced proteins, peptidylprolyl isomerase [89] and wheatwin-2 protein [90] were over-represented by both DPI4913 and AF086. Thus, our results show that DPI4913 and AF086 differentially express some heat-stress-responsive proteins.

A GTP-binding protein, which is an ADP-ribosylation factor affected by environmental stresses [91], was under-represented by both DPI4913 and AF086. In addition, an uncharacterized protein belonging to the GTP-binding protein family was specifically under-represented by DPI4913. Grover et al. [92] observed that GTP-binding proteins led to the regulation of a cascade of kinases that play a substantial role in environmental stress signal transduction. High temperatures would also increase the production of GTP-binding proteins, leading to the enhancement of kinase activities. Contrary to what has been reported above, DPI4913 and AF086 under-represented some heat-responsive proteins.

The expression of a calcium-transporting ATPase that plays a role in transport systems and stress signaling in cellular homeostasis [93] was increased by both DPI4913 and AF086. Therefore, DPI4913 and AF086 also had a role on stress signaling pathways.

In addition, calcium-transporting ATPases are defense-related enzymes that play a role in transport systems and stress signaling in cellular homeostasis [93]. Calcium is known as a secondary messenger in many signaling pathways. It mediates responses to environmental stresses such as heat and cold stresses, salinity, osmotic and oxidative stress, drought, plant hormones and pathogens [94].

An ATP carrier protein 1 was specifically over-represented by AF086. This protein catalyzes the exchange of ADP and ATP across the mitochondrial inner membrane and may control reactive oxygen species generation by means of energy-dissipating systems [95]. A peroxidase, also reported to respond to oxidative stress [96], was also specifically over-represented by AF086. Moreover, the protein peroxidase is described as having an antifungal function [37,97]. Thus, AF086 had a positive effect on plant response to oxidative stress and enhanced plant response to biotic stress.

A protein from the Glutathione S-transferase family was under-represented by AF086. Glutathione S-transferase proteins were reported to have different enzymatic properties and to be differentially regulated by xenobiotics and by pathogen attacks [98]. This implies that AF086 was involved in biotic stress response.

An uncharacterized protein belonging to the late embryo abundant (LEA) protein family was over-represented by AF086. Two uncharacterized proteins involved in response to freezing and to cold were also under-represented by AF086. An Em protein, H5, was over-represented by AF086. The Em gene of wheat encodes the first LEA protein group [99], playing a key role in environmental stress response [100]. They help other proteins to fold after denaturation during water stress [101]. Thus, AF086 induced the differential expression of proteins involved in abiotic stress responses.

In our results, the determination of water-use efficiency, which refers to the ratio of biomass produced to the cumulative transpiration, was improved by 15.4% by DPI4913 and by 9.9% by AF086 application (Table 5). A high water-use efficiency is usually considered as a trait of

Table 5
Water-use efficiency with different foliar treatments in a greenhouse experiment; southwest France.

		Water-use efficiency ($g.L^{-1}$)			
Average ± SD					
	CONTROL	0.71	±	0.11	a
	DPI 4913	0.82	±	0.14	b
	AF086	0.78	±	0.12	b
ANOVA P-value					
Treatment		< 0.001			
Block		0.06			

Note: The letters (a and b) indicate a statistical difference.
($P < 0.05$) between treatments.
(WUE = total dry biomass per plant at harvest/water transpired from second node stage until harvest)

water deficit tolerance. The total dry biomass produced per unit of water transpired was significantly higher for plants treated with both DPI4913 and AF086. This result is in agreement with the observed effect of biostimulants on grain protein expression. DPI4913 and AF086 appeared to increase the expression of some proteins involved in abiotic stress defense.

4. Conclusions

This study provides clues about the effects of DPI4913 and AF086 on wheat grain production and quality. Biostimulants induce an increase in grain yield and protein quantity. The increase in the protein quantity is associated with a modification of the protein composition in the grains. To investigate the changes in protein composition of wheat grain after DPI4913 and AF086 biostimulants treatments of the plant, we performed a comparative total proteome analysis of the flour from milled grains of durum wheat. Fifty proteins were found to be differentially represented after DPI4913 and AF086 treatments, including 14 in common to both treatments.

These identified proteins were involved in metabolic pathways and processes, including storage, regulation processes, and defense response against abiotic and biotic stresses. Among them, four proteins were particularly differentially represented: i) gamma-gliadin, which is a storage protein that is substantially over-represented by both DPI4913 and AF086, implied in technological properties and water stress response; ii) ADP/ATP carrier protein-1, which is over-represented by AF086, implied in plant defense mechanisms against biotic stress; iii) the Zn-ion binding protein, which is over-represented by DPI4913 implied in transcription regulation processes; and iv) CM 17, which is over-represented by both DPI4913 and AF086, implied in starch degradation inhibition, amino acid storage and plant defense mechanisms against biotic stress.

As for CM 17 and ADP/ATP carrier protein-1, their implication in biotic stress has provided a foundation for further studies. Moreover, characterizing the transcription regulation processes affected by the Zn-ion binding protein would also allow us to further understand and describe biostimulant action mechanisms.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

EAL and SV oversaw the greenhouse experiment. CP and AG oversaw proteomic analysis. AG, CP and EAL collected and analyzed the data. CP and EAL wrote the first draft of the manuscript. CD provided expertise on biostimulants and helped design the experiment. OBS, PG and TL supervized the research work, helped design the experiment, analyzed data and improved the manuscript content.

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