Journal of Proteomics 143 (2016) 390–400

Contents lists available at ScienceDirect

Journal of Proteomics

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

The variations in the nuclear proteome reveal new transcription factors and mechanisms involved in UV stress response in *Pinus radiata*

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ARTICLE INFO

Article history:
Received 15 January 2016
Received in revised form 25 February 2016
Accepted 1 March 2016
Available online 4 March 2016

Keywords:
Pinus
UV stress
Nuclei
SEQUEST
MAPA
Transcription factor

ABSTRACT

The importance of UV stress and its side-effects over the loss of plant productivity in forest species demands a deeper understanding of how pine trees respond to UV irradiation. Although the response to UV stress has been characterized at system and cellular levels, the dynamics within the nuclear proteome triggered by UV is still unknown despite that they are essential for gene expression and regulation of plant physiology. To fill this gap this work aims to characterize the variations in the nuclear proteome as a response to UV irradiation by using state-of-the-art mass spectrometry-based methods combined with novel bioinformatics workflows. The combination of SEQUEST, *de novo* sequencing, and novel annotation pipelines allowed cover sensing and transduction pathways, endoplasmic reticulum-related mechanisms and the regulation of chromatin dynamism and gene expression by histones, histone-like NF-Ys, and other transcription factors previously unrelated to this stress source, as well as the role of alternative splicing and other mechanisms involved in RNA translation and protein synthesis. The determination of 33 transcription factors, including NF-YB13, Pp005698_3 (NF-YB) and Pr009668_2 (WD-40), which are correlated to stress responsive mechanisms like an increased accumulation of photoprotective pigments and reduced photosynthesis, pointing them as strong candidate biomarkers for breeding programs aimed to improve UV resistance of pine trees.

Significance: The description of the nuclear proteome of *Pinus radiata* combining a classic approach based on the use of SEQUEST and the use of a mass accuracy precursor alignment (MAPA) allowed an unprecedented protein coverage. This workflow provided the methodological basis for characterizing the changes in the nuclear proteome triggered by UV irradiation, allowing the depiction of the nuclear events involved in stress response and adaption. The relevance of some of the discovered proteins will suppose a major advance in stress biology field, also providing a set of transcription factors that can be considered as strong biomarker candidates to select trees more tolerant to UV radiation in forest upgrade programs.

1. Introduction

The environmental stresses are the main factors affecting tree productivity. The importance of drought and temperature tolerance has been studied for years [1–4]. However, in the actual climate change scenario, UV radiation claims for attention. UV radiation has significantly increased in the last decades [5] and is expected to continue to raise in the near future, according to the current UV irradiance prediction models [6]. Since this stress affects growth and development of plants [7], as well as reproduction and survival [8], which in the end determines plant productivity, the increase of the levels of UV reaching Earth's surface will have an important economic impact in the forestry sector.

Previous studies have demonstrated the effects of UV stress over tree physiology in Poplar [9] and Laurus [10] including well known photomorphogenic alterations, physiological effects on photosynthesis performance and water and pigment contents, as well as metabolomics profiling describing metabolism rearrangement under UV to promote phenolics and isoprenoid accumulation. In addition, UV causes damage to macromolecules and the generation of reactive oxygen species [11–13]. Nonetheless, most of this responses and adaptation mediating mechanisms have been reported in the model species *Arabidopsis thaliana* [9,14,15] or in crop species, like grapevine [16], rice [17–19] and maize [20,21]. These studies were based on the application of high UV dosages to obtain clear responses, but the magnitudes of these dosages which are unlikely to be reached in the field, make

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http://dx.doi.org/10.1016/j.jprot.2016.03.003
1874-3919/

difficult to conclude the actual impact of the radiation over plant productivity, as well as its scope, thus reducing the translational application of these knowledge to the field [22].

To overcome these limitations, in our group we studied the systemic response to UV stress in *Pinus radiata* applying a realistic UV dosage based on existing climate models. We combined metabolomic and proteomic analyses, finding that adaptation to UV stress courses through a rearrangement of central metabolism to prevent ROS production and support biosynthetic processes leading to the accumulation of photoprotective pigments mediated by a coordinated integration of UV-, ROS- and light-dependent signals and a deep proteome remodelling and having a payback in productivity as a consequence [23].

In the current context, in which environment is changing and new tools need to be developed in order to assist breeding programs, the knowledge of the nuclear regulation of stress adaptive responses is of special interest since long term adaptive processes including proteome and metabolome remodelling reflect, in last term, very specific changes in gene regulation modulated by precise signalling pathways.

Nuclear proteins play a role in many crucial cellular functions, such as signalling, gene regulation and protein degradation. Moreover, there are increasing numbers of functions related to regulation of translation and RNA-dependent mechanisms. In fact, last estimations say that around one-fourth of the total proteins are localized in the nucleus [24]. However, quantitatively speaking they are not abundant compared to the total protein amount and their study faces some difficulties related to their structural redundancy and biological significance. Thus, there are many families and individual nuclear proteins that have been described, but the functions of most of them remain unknown in model species [25] and are almost undescribed in orphan species. This fact, together with the opposite biological effects have been described for very similar nuclear factors [26], hinders the interpretation of plant nuclear proteomes.

Despite the paramount role of the nucleus the number of studies focusing on nuclear protein dynamics under UV or other environmental stress is scarce. To fill this gap, and considering that previous studies focused on total proteome (Pascual et al., unpublished data) were not sensitive enough to decipher nuclear protein dynamics, we performed a time course experiment employing realistic UV dosages aimed to characterize the quick variations in the nuclear proteome triggered by short exposition to UV (2 h) and those changes observed after a two days UV exposure (16 h).

The variations in the nuclear proteome were defined by using organelle purification and a gel- and label-free approach. Protein identification and quantification followed a combined strategy, including in-house databases, MapMan ontology and specific algorithms for the prediction of nuclear protein functions and the analysis of the data in a comprehensive manner following targeted and untargeted approaches, emphasizing the discovery of involved transcription factors. The integration of these results with physiological measurements and previously generated UV metabolomics profile enhanced the biological meaning of this work and its usefulness for providing a novel set of markers for tree selection and breeding programs.

2. Material and methods

2.1. Plant material and experimental design

The assay was conducted on one-year-old seedlings. After cold stratification, the seeds were germinated and grown in peat:perlite:vermiculite (3:1:1) in 1.5 L pots under a greenhouse-controlled environment and long photoperiod (16 h light/8 h darkness). Plants were irradiated with 0.33 W m^{-2} UV light (250–350 nm lamp peaking at 300 nm) in independent blocks of 3 individuals each. Samples were taken at different intervals: after 2 h of irradiation (2 h samples) and after 16 h of irradiation during two consecutive days (8 h each day, 16 h samples). Control plants were maintained under the same greenhouse conditions without UV radiation (Control). To avoid biases related to circadian clock, all the plants were sampled at the same time (Fig. 1). Plant material was sampled, frozen in liquid nitrogen immediately and stored at −80 °C until analysis.

2.2. Photosynthetic performance measurements

Photosynthetic performance was measured immediately after the end of UV treatment. Chlorophyll fluorescence-based measurements were employed to monitor induced damage with an imaging/pulse-amplitude modulation fluorimeter (OS1-FL, Opti-Sciences, Hudson, USA) in the three replicates per experimental situation. Maximal efficiency of the PSII (Fv/Fm) in needles pre-adapted to darkness for 30 min and the PSII quantum yield (QY) in light-adapted needles were calculated according to Maxwell et al. [27].

2.3. Needle pigment contents

Pigment contents were quantified in three replicates of each treatment following the protocol by Escandón et al. [28]. In brief, the needles were ground (100 mg) to a fine powder with liquid nitrogen before adding extraction solutions. Chlorophylls *a* (Chl *a*) and *b* (Chl *b*), and carotenoids were extracted with 2 mL of cold acetone:Tris-HCl (80:20, v/v, pH 7.8) and anthocyanins with methanol:HCl:water (90:1:1, v/v/v). The concentration of Chl *a*, Chl *b*, carotenoids and anthocyanins was determined spectrophotometrically according to the equations provided by Sims et al. [29].

2.4. Nuclei isolation and protein extraction

Nuclei were isolated following the protocol described by Valledor et al. [30] with minor modifications (described in the companion article [31]). Once isolated, proteins in nuclei samples were extracted in 300 μL of 1% SDS with the help of a sonication period of 15 s. Proteins were dissolved in 1 v of 1.5 M sucrose, 10 mM DTT and 1% protease inhibitor cocktail (Sigma, P9599) and purified with phenol. Dry protein pellets were dissolved in 1.5% SDS, 8 M Urea and quantified by BCA assay [32]. The detailed protein extraction procedure is described in Ref. [31]. The enrichment in nuclear proteins was assessed by 1-DE SDS-PAGE (see Fig. 1 in Ref. [31]).

2.5. Protein digestion and mass spectrometry

Proteins were processed and analysed as described by Valledor et al. [33] with minor modifications. In brief protein samples (60 μg) were run 0.5 cm in 12% SDS-PAGE gels, and then protein bands were cleaned, trypsin digested and desalted [31].

The nLC-Orbitrap (Thermo) system was maintained as previously specified, with only a slight modification in the effective gradient, which was set to 90 min from 5 to 45% acetonitrile/0.1% formic acid (v/v) with a later column regeneration step of 27 min.

We followed database dependent and independent approaches for quantifying protein abundance. The abundance of the proteins obtained after applying SEQUEST algorithm was estimated from the peak areas of the three most abundant peptides assigned to each protein employing Proteome Discoverer 1.4 software (Thermo). A database independent strategy based on ProtMax software [34] was also used to reduce biases related to the poor presence of Pine nuclear proteins in databases. The individual *m/z* and abundances were determined and quantified using the ion count method following the recommendations previously given by Egelhofer et al. [34].

2.6. Protein identification

The identification of proteins in our database dependent approach followed a sequential strategy. Firstly, three in-house protein databases

Fig. 1. Scheme of the experimental system employed. The photoperiods, as well as the UV radiation times are indicated in the two days the experiment lasted.

were constructed from *Pinus pinaster* transcriptome v. 3.0 [35], *Pinus taeda* genome v. 1.01 [36], and *P. radiata* sequences available in public databases and in house libraries [37] following the procedure described by Romero-Rodríguez et al. [38]. Databases were annotated against UniProtKB/Swiss-Prot and UniProtKB/TrEMBL Viridiplantae using sma3s script [39] and functionally classified through Mercator pipeline annotation [40] according to MapMan ontology [41] (Databases and MapMan maps are available at www.valledor.info; detailed information is provided in [31]) SEQUEST algorithm included in Proteome Discoverer 1.4 was used for the identification of proteins employing a 5% false discovery rate (FDR), two peptides per protein, medium confidence, and an XCorr score above 1.8 as identification threshold. Unknown and unannotated proteins were analysed with PlantTFcat tool [42] and blasted against PlnTFDB [43] transcription factors database in order to identify transcription factors and nuclear regulators. Contaminant proteins not belonging to the nuclei or endoplasmic reticulum were dropped from analysis based on their annotation.

Database independent approach relied on the previous identification since those *m/z* showing statistical significance were, in first place, assigned to peptides in proteins groups previously identified by SEQUEST searching algorithm. For those *m/z* not present in previously described dataset, Novor *de novo* algorithm [44] included in DeNovoGUI [45] was applied and confidence thresholds were established according to Ma [44]. In a second step, the identified sequences were annotated by homology using BLAST against UniProtKB/Swiss-Prot, UniProtKB/TrEMBL Viridiplantae, and our custom databases using the settings recommended for "blastp-short" program in BLAST+ guide [46].

2.7. Analysis of proteome dataset

Data preprocessing of both targeted and untargeted strategies was performed following the recommendations given by Valledor and Jorrín [47]. Data were normalized following a sample-centric approach and log transformed. To avoid biases caused by estimation of non-nuclear proteins abundance values were corrected by using the average value of the two most stable nuclear proteins present in all samples (Pp003257_2-Pp003276_2 and PITA_000008893-RA, annotated as histones). The stability of these proteins during experiment was determined by a pairwise comparison approach considering all detected protein species following the method described by Vandesompele et al. [48].

Centered and scaled values (*z*-scores) were subjected to multivariate (Principal Component Analysis (PCA) and Heatmap clustering) and univariate (one-way ANOVA; 5% FDR with an alpha = 0.05) analyses. The abundance data of the proteins identified with SEQUEST were integrated with photosynthetic performance and pigment content data (Supplemental Table 1) and metabolomics results from a previous study (Supplemental Table 2) by Sparse Partial Least Squares (sPLS) regression and the construction of sPLS-based interaction networks.

All the preprocessing and analysis described were performed in R environment v.3.1.1 [49] using mixOmics v.4.0.2 [50], SLqPCR v.1.30.0 [51] and specific scripts developed in our lab (available upon request).

2.8. RNA extraction and quantitative real time PCR

RNA was extracted from three replicates of each situation according to Valledor et al. [52] and then quantified in a spectrophotometer. RNA integrity was checked by agarose gel electrophoresis, and potential DNA contaminations by PCR employing the *UBIQUITINE* (*UBI*) primer pair. Later, 500 ng of RNA was reversed transcribed using the RevertAid kit (Thermo Scientific) and random hexamers as primers following the manufacturer's instructions.

qPCR reactions were performed in a CFX Connect Real Time PCR machine (Bio-Rad) with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad); three biological and two analytical replicates were made. Normalized Relative Quantities (NRQ) and Standard Errors of RQ were determined according to Hellemans et al. [53]. Expression levels of *ACTIN* (*ACT*) and *UBIQUITINE* (*UBI*) were used as endogenous controls. Detailed information about the primers used for qPCR experiments is available in Supplemental Table 3.

3. Results

3.1. Applied UV dosages induced changes in photosynthetic performance and pigment contents

The applied UV dosage, 0.33 W m^{-2} led to a significant reduction of both fluorescence-based photosynthetic performance parameters measured: maximal efficiency of photosystem II (Fv/Fm) and quantum yield (QY) (Supplemental Table 1). Specifically, we observed a significant reduction in both parameters after 16 h of radiation (Fig. 2a). In addition, in the measured pigment contents we observed transient changes in Chl *a* and carotenoids in 2 h plants (Fig. 2b), being in both cases recovered contents similar to those observed in the control after 16 h of radiation. Surprisingly, no significant changes were observed in the cases of Chl *b* and anthocyanins after 2 h of UV (Fig. 2b).

3.2. Needle nuclear proteome dynamically responded to UV radiation

GeLC-Orbitrap/MS analysis of the nuclei-enriched fractions from the needles sampled after the UV assay allowed the identification of 388 proteins and the quantification of 374. After the removal of the non-nuclear proteins based on their *in silico* annotation, 267 proteins were identified, of which 145 were considered as differentially accumulated (see Supplemental Table 1 in Ref. [31]; ANOVA, 5% FDR).

MapMan categorization of the identified proteins showed longer-term increases in protein, RNA and amino acid metabolism proteins in average, consistent with active protein remodelling under UV, while DNA metabolism was reduced with the UV, likely as a consequence of the damage caused by the UV. Surprisingly, stress and redox bins showed an average decrease (Fig. 3).

Quantified proteins showed different accumulation patterns during experiment course (Fig. 4a; Supplemental Fig. 1). Heatmap revealed the existence of 6 protein clusters regarding their accumulation profile during the experiment: proteins overaccumulated with the UV,

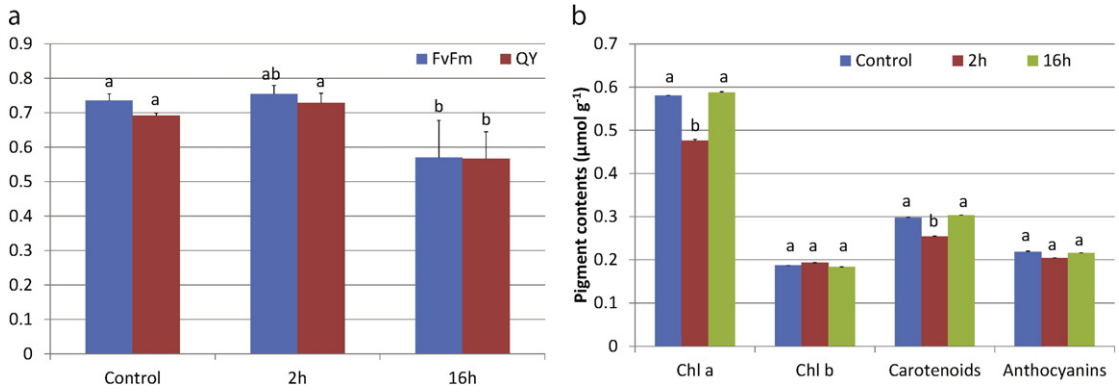

Fig. 2. (a) Photosynthetic performance measured as maximal efficiency of PSII (FvFm) and Quantum Yield (QY) in the control and the treated plants. The error bars show SD, and the letters indicate significant differences (Kruskal-Wallis, $p < 0.05$). (b) Needle contents of chlorophylls *a* and *b*, carotenoids and anthocyanins. In all cases, the error bars show SE, and the letters indicate significant differences (Kruskal-Wallis, $p < 0.05$).

depleted, not changing, depleted in 2 h plants, accumulated in 2 h plants and depleted after 16 h of UV.

Multivariate analysis based on PCA also revealed specific dynamics of the responses to UV at the nuclear proteome (Fig. 4b, Supplemental Table 4). Considering the distribution of the samples along PC1 and the proteins showing higher loadings, like redox-related proteins (negatively correlated) and amino acid metabolism-related proteins such as aspartate aminotransferases (positively correlated), it could be hypothesized that this component gathers the variance of the samples related to the UV irradiation or dosage. On the other hand, PC2 showed high loadings (positive and negative) to RNA binding proteins (PITA_000008378-RA, PITA_000008612-RA) and ribosomal proteins (RPL11, RPL26, RPS12, RPS16, RPS17), and may be representing the changes in the machinery required for the stress survival and active proteome remodelling triggered after first irradiation period.

3.3. Untargeted approach revealed a new set of proteins involved in UV stress response and sensing

A database independent approach, based on the untargeted analysis of all parent ions present in our samples, was the solution we chose to reduce the biases and limitations consequence of the poor coverage of Pine nuclear proteins in the available databases.

Untargeted analysis of the MS proteomics results using ProtMax accounted 16,638 ions, of which 9094 was above the established background signal. Out of these, 6098 ions were differentially accumulated (see supplemental Table 2 in [31]). PCA of the quantified ions (Fig. 5b, Supplemental Table 5) showed a good separation between treatments and the expected grouping between replicates following a similar pattern to protein dataset (Fig 4b). Heatmap clustering analysis of the quantified ions showed the existence of 6 clusters (Fig. 5a): proteins depleted with the UV, not changing, overaccumulated in 16 h plants, depleted after 2 h of UV, accumulated mainly in 16 h plants and accumulated with the UV. After statistical analyses 300 *m/z* corresponding to those ions showing lower *p*-values in ANOVA and/or showing the highest loadings considering its absolute value to the first two principal components allowed the confident determination of 57 sequences, some of them revealed as relevant also in the targeted approach (Supplemental Table 6).

Interestingly 17 of the 300 *m/z* were annotated as transcription factors or as other proteins involved in transcription regulation, like Zinc finger and SNF2 domain containing proteins or RNA-binding (Supplemental Table 6). In addition, a set of Ser/Thr protein kinases and phosphatases of the 2C family of unknown function in both cases, although these families are related to stress [54–56], were identified, as well as several unknown proteins categorized in the light signalling MapMan category (Supplemental Table 6).

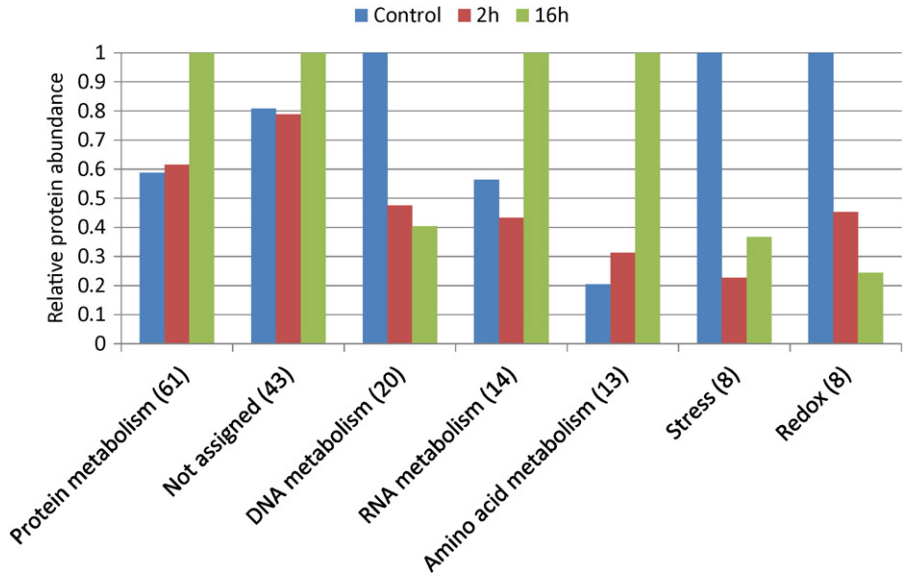

Fig. 3. Overview of protein abundance of protein metabolism, not assigned, DNA, RNA and amino acid metabolism, stress and redox MapMan functional bins in each experimental situation. Average protein abundances are expressed over a maximum value of 1. The number of proteins in each bin is indicated in brackets.

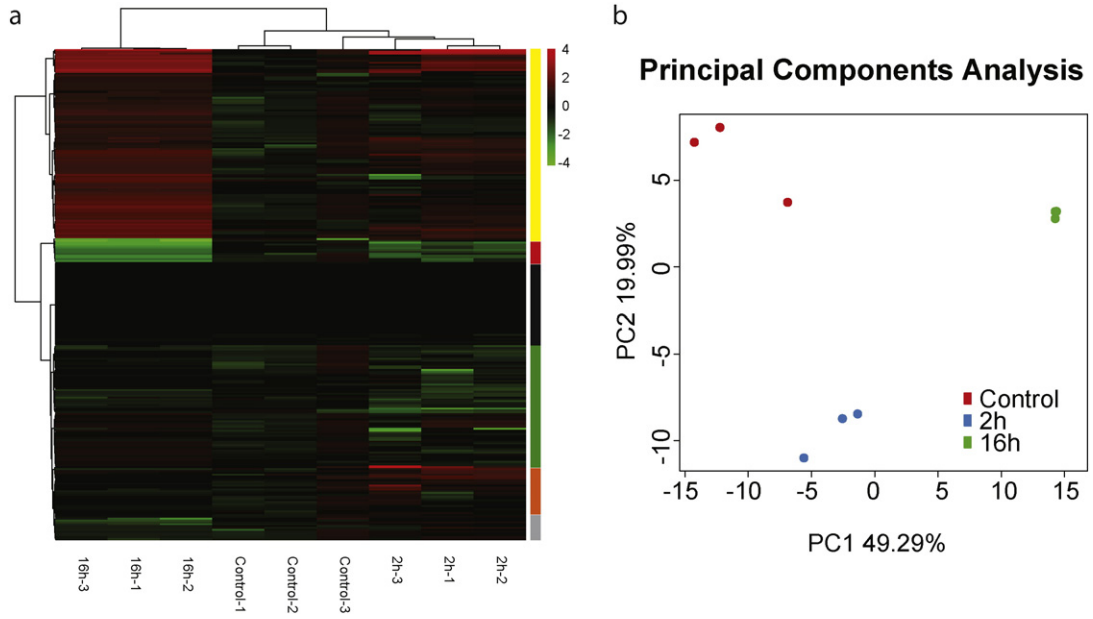

Fig. 4. Targeted approach. (a) Hierarchical clustering analysis and heatmap of the quantified proteins. Data were log2-transformed. Coloured bars represent protein clusters: yellow, proteins overaccumulated with the UV; red, depleted; black, not changing; green, depleted in 2 h plants; orange, accumulated in 2 h plants; and grey, depleted after 16 h of UV. (b) Principal Component Analysis (PCA) plot showing the first two principal components (PCs), explaining more than 50% of the observed variance.

3.4. Combined targeted and untargeted approaches revealed new transcription factors involved in UV stress in pine

One of the aims of this work was deepening into the mechanisms regulating UV stress adaption in Pinus genre, being targeting DNA and RNA interacting proteins one of our main objectives. The use of in-house constructed databases coupled to DNA-interacting proteins searching algorithms combined with a database independent strategy and *de novo* sequencing allowed the identification of a set of transcription factors and other transcription regulators of different families, which are reported here to be involved in UV stress response in *P. radiata* for the first time. Table 1 showed the summary of the 34 different transcription regulators found by SEQUEST search and in the *de novo* sequenced most relevant *m/z* from the untargeted approach. Specifically, transcription factors and regulators from the APFI, ARR, bHLH, bZIP, C2C2-CO-like, HB, HSF, MYB, NF-YB, SNF2, TCP, Trihelix and WRKY families and a WD40-repeat domain containing protein were found, showing different accumulation patterns after UV irradiation (Fig. 6).

The biological relevance of the transcriptional regulators with a significant differential accumulation was explored by integrating their abundances, physiological parameters measured in the experiment (Supplemental Table 1), and metabolomics results from a previous study (Supplemental Table 2). After testing that sPLS approach was capable to distinguish the different treatments (Supplemental Fig. 2) interaction networks were plotted considering edges with correlations

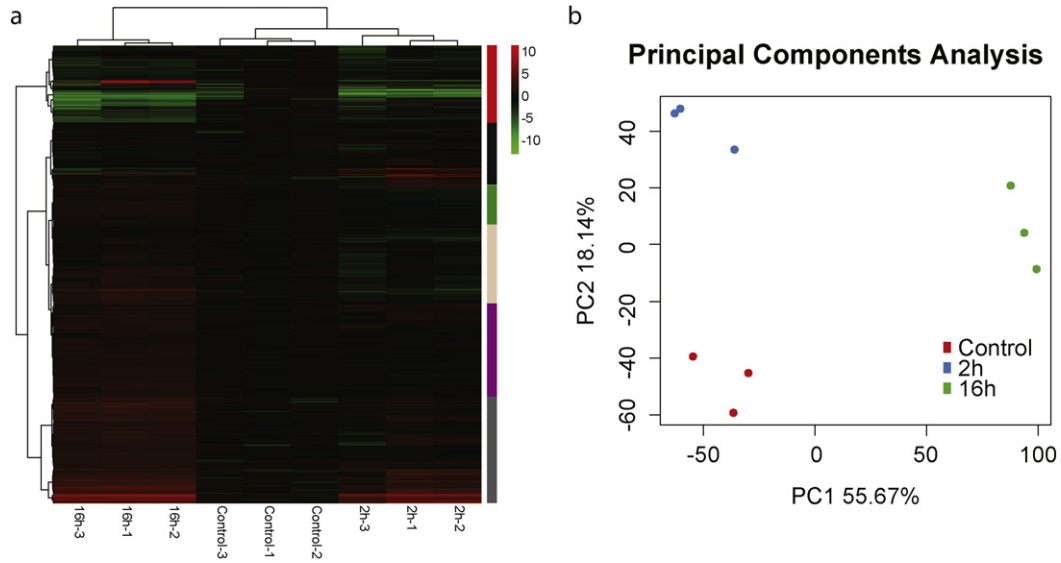

Fig. 5. Untargeted approach. (a) Hierarchical clustering analysis and heatmap of the quantified proteins. Data were log2-transformed. Coloured bars represent protein clusters: red, proteins depleted with the UV; black, proteins not changing; green, overaccumulated in 16 h plants; pink, depleted after 2 h of UV and accumulated after 16 h; violet, accumulated mainly in 16 h plants; and grey, accumulated with the UV. (b) Principal Component Analysis (PCA) plot showing the first two principal components (PCs), explaining more than 50% of the observed variance.

Table 1
Summary of transcription factors and other transcription regulators found in the combined targeted and untargeted approaches. The accessions (UniProt in italics), *m/z*, when corresponding, mean abundances ± SD, algorithm used for the identification, score, ANOVA significance (5% FDR), transcription factor family following PlnTFDB classification and BLAST annotations are indicated in each case.

Accession	*m/z*	Control		2 h		16 h		Algorithm	Score	ANOVA	Transcription factor family	BLAST annotation	
		Mean	SD	Mean	SD	Mean	SD			5% FDR		Protein symbol	Protein name
PITA_000042515-RA		4.01E-03	5.25E-04	6.47E-03	7.89E-04	9.62E-03	2.44E-12	SEQUEST	6.19	*	APFI	CAG3	Transcription factor APFI
Q75HW2, PITA_000011845-RA	1090.51	8.93E-04	1.61E-04	7.82E-04	1.24E-04	6.01E-03	2.04E-04	NOVOR	24.7	*	ARR	RR27	Two-component response regulator ORR27 (OsRRA16)
Q9CAA9–2, Pr009222_1	773.72	8.29E-03	1.33E-03	2.21E-03	5.15E-04	4.49E-03	7.69E-04	NOVOR	10.3	*	bHLH	BHLH49	Transcription factor bHLH49 (basic helix-loop-helix protein 49) (AtbHLH49) (bHLH 49) (Protein ACTIVATOR FOR CELL ELONGATION 1) (Transcription factor EN 82) (bHLH transcription factor bHLH049)
Q99142, Pr009259_2	452.23	2.97E-03	2.79E-04	2.68E-03	2.22E-04	9.83E-03	1.53E-03	NOVOR	30.2	*	bZIP	GBF3	bZIP G-box
Q9SID1, Pr000415_1	579.62	1.37E-01	3.02E-02	1.24E-01	2.92E-02	2.76E-02	2.03E-03	NOVOR	32.6	*	C2C2-CO-like	BBX25	B-box zinc finger protein 25 (protein SALT TOLERANCE HOMOLOG 1) (Salt tolerance-like protein)
Q9M9P4, PITA_000025316-RA	361.91	2.10E-04	1.13E-04	1.24E-02	7.43E-03	6.30E-03	3.34E-03	NOVOR	24.5	*	HB	HDG8	Homeobox-leucine zipper protein HDG8 (HD-ZIP protein HDG8) (Homeodomain GLABRA 2-like protein 8) (Homeodomain transcription factor HDG8) (Protein HOMEODOMAIN GLABROUS 8)
Q9FFK0, PITA_000041531-RA	516.77	0.00E + 00	0.00E + 00	2.26E-03	7.20E-04	5.57E-03	6.30E-04	NOVOR	23.1	*	HB	WOX7	WUSCHEL-related homeobox 7
Q6H6Q7, PITA_000031067-RA	711.88	9.17E-03	2.01E-04	7.93E-03	2.41E-04	1.91E-02	3.37E-04	NOVOR	53.6	*	HSF	HSFA3	Heat stress transcription factor A-3 (Heat stress transcription factor 7) (OsHsf-07)
Q6A333–3, PITA_000004378-RA	626.3	1.58E-02	1.03E-03	9.41E-03	3.57E-04	1.17E-02	1.01E-03	NOVOR	39.2	*	MYB		Unknown protein
Q0JIC2–2, PITA_000011057-RA	827.08	1.88E-03	4.82E-04	2.53E-03	4.74E-04	7.29E-05	6.31E-06	NOVOR	15.7	*	MYB		Unknown protein
Q9LDE1, PITA_000030235-RA	759.01	8.75E-03	1.00E-03	8.40E-03	1.19E-03	1.95E-02	1.80E-03	NOVOR	29.8	*	MYB	MYB108	Transcription factor MYB108 (Myb-related protein 108) (AtMYB108) (Protein BOTRYTIS-SUSCEPTIBLE 1)
P27898, PITA_000016280-RA	526.88	7.69E-05	2.63E-05	0.00E + 00	0.00E + 00	8.17E-03	6.84E-03	NOVOR	19.3	*	MYB	P	Myb-related protein P
P49592, PITA_000067106-RA	494.26	5.32E-03	1.03E-03	3.78E-03	9.41E-04	1.90E-02	1.59E-03	NOVOR	52	*	NF-YB	NF-YB13	Nuclear factor Y, subunit 13
Pp005698_3		2.94E-01	7.56E-02	1.21E-01	3.99E-03	5.75E-02	8.96E-12	SEQUEST	65.47	*	NF-YB		
Pp004797_2		1.03E + 00	7.00E-03	1.02E + 00	2.68E-03	1.02E + 00	2.11E-10	SEQUEST	69.69		NF-YB		
Pr002465_1–Pp016823_1		3.16E-01	1.73E-01	6.64E-01	4.59E-02	5.64E-01	1.07E-02	SEQUEST	19.33		NF-YB		
Pp003764_3		1.27E + 00	4.04E-01	1.77E + 00	5.95E-01	9.51E-01	5.85E-11	SEQUEST	35.77		NF-YB		
Pp016390_2		1.32E + 00	6.68E-02	1.34E + 00	2.75E-02	1.29E + 00	3.66E-10	SEQUEST	99.96		NF-YB		
PITA_000013079-RA		3.13E-01	7.77E-02	1.24E-01	1.15E-02	4.05E-02	6.57E-12	SEQUEST	60.72	*	NF-YB		
PITA_000046208-RA		9.93E-02	7.77E-03	1.15E-01	6.92E-03	1.31E-01	3.75E-11	SEQUEST	16.91	*	NF-YB		
PITA_000059035-RA		4.33E-01	1.54E-01	8.57E-01	3.68E-02	7.99E-01	3.10E-02	SEQUEST	31.88		NF-YB		
PITA_000040049-RA		1.15E + 00	3.19E-01	1.49E + 00	4.41E-02	1.81E + 00	4.10E-01	SEQUEST	46.84		NF-YB		
PITA_000067325-RA		1.43E + 00	1.81E-01	1.47E + 00	5.19E-02	1.36E + 00	2.60E-10	SEQUEST	204.52		NF-YB		
F4I8S3, PITA_000079040-RA	492.25	4.84E-03	1.09E-03	5.19E-03	1.75E-03	1.47E-04	7.31E-05	NOVOR	33.5	*	SNF2	CLSY3	SNF2 domain-containing protein CLASSY 3 (EC 3.6.4.-) (Protein CHROMATIN REMODELLING 31) (AtCHR31)
Q7X9V2, PITA_000037058-RA	812.93	4.57E-03	4.17E-04	4.15E-03	3.77E-04	1.31E-02	4.17E-04	NOVOR	23.4	*	SNF2	PIE1	Protein PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1 (EC 3.6.4.12) (Independent early flowering 1 protein) (Protein CHROMATIN REMODELLING 13) (AtCHR13)
O82277, PITA_000037058-RA	732.32	4.05E-03	3.41E-04	3.67E-03	1.73E-04	8.53E-03	1.81E-04	NOVOR	30.7	*	TCP	TCP10	Transcription factor TCP10
Q9SDW0, PITA_000047138-RA	506.29	2.58E-04	1.24E-04	4.22E-02	1.94E-02	6.03E-02	3.62E-03	NOVOR	52.3	*	Trihelix	GT-3 A	Trihelix transcription factor GT-3a (Trihelix DNA-binding protein GT-3a)
Pr009668_2		2.40E-03	5.78E-04	6.09E-03	4.31E-03	1.41E-02	1.72E-12	SEQUEST	3.7		WD40		
Q9LY00, PITA_000055692-RA	626.84	4.67E-02	9.67E-03	4.48E-02	6.44E-03	1.22E-02	1.64E-04	NOVOR	33.9	*	WRKY	WRKY70	Probable WRKY transcription factor 70 (WRKY DNA-binding protein 70)

higher than |0.8| (Fig. 7). These networks suggested a pivotal role of these proteins in the response to the UV stress. NF-YBs showed high correlations with polyamine metabolism (specifically putrescine) and several sugars, while the identified APFI and WD40-repeat transcription factors were highly correlated to Fv/Fm and QY, as well as, intermediaries of flavonoid pathway, like epicatechin and epigallocatechin. In all the cases a strong link with C supply/demand balance was found, as intermediaries of the myo-inositol pathway, like inosose or myo-inositol, suggested. Moreover, RT-qPCR analysis showed an upregulation concomitantly with the radiation in the cases of PITA_000042515-RA (APFI), Pp005698_3 (NF-YB) and Pr009668_2 (WD40-repeat) (Fig. 8), supporting also their importance for UV response.

Additional transcriptional regulators were discovered after *de novo* sequencing of those precursor ions quantified using the MAPA approach that showed a significant differential accumulation. Furthermore, the qPCR analysis of these transcripts (Fig. 8) showed that most of them were overexpressed after UV irradiation. Importantly, the results from proteomic and qPCR analysis demonstrated that some correlation trend exists between the differential expression profiles of the gene and the accumulation of the transcription factors.

These results, although do not necessarily imply a straight functional connection between transcript-protein levels and specific biological processes, represent a promising set of targets to be further studied in new experiments aimed to unveil specific transcriptional regulatory mechanisms.

4. Discussion

The present study focuses on nuclei proteomics under UV stress, this organelle being not included in stress studies very often, despite its central role in the regulation of gene expression. Nuclear proteins are, in general, underrepresented in databases, even in model species, so this is an important fact when studying orphan species. Therefore, an untargeted approach, independent of any bias and limitation as consequence of the database/s used for protein identification, shows itself useful in order to extract as much information as possible from a nuclear proteomics study.

4.1. Combined targeted and untargeted approach enhanced the unravelling of key proteins in UV stress nuclear responses

In the present study, focusing on nuclear proteome profiling by MS, we identified 388 proteins, improving previous result in the model species rice [57] or Arabidopsis [58] and a recent work in wheat [59]. Interestingly, these three contributions employed a 2-DE based approach, describing up to 700 spots and identifying <200. This means that despite our efforts for gathering all available genomic information and the use of *de novo* sequencing algorithms both of them must be improve in order to gain a complete coverage of the nuclear proteome in plant species. Protein identification from tandem MS data classically depends on the availability of the sequenced genome of the studied species. In this aspect the use of a gel free approach together with our custom databases [38] increased the number of identified proteins in an orphan species such as *Pinus radiata*. We were able to identify a higher number of proteins than previously described in this species in works dealing with complete needle proteome and following a 2-DE approach [37,60]. However, these numbers are still far from the predicted number of nuclear proteins or the number of proteins that can be seen in 2-DE gels. To overcome database-derived limitations, the use of an untargeted approach using ProtMax showed itself as convenient, since 57 new proteins, otherwise would had not been unravelled, were assessed by *de novo* sequencing and identified from the 300 most significant *m/z*. Out of these, 18 *m/z* were identified as transcription factors, which accumulation changed during the experiment. Identified peptides were initially blasted against UniProt and Pine custom databases, showing perfect matches between database and experiment sequences. qPCR analyses also proved that most of their corresponding gene products also followed a similar trend. These results supported the suitability of the employed workflow in scenarios of low protein database coverage.

4.2. Nuclear regulation of UV-induced proteome remodelling

An active remodelling of the proteome is crucial for stress response and adaptation [61]. Regarding the nucleus, gene transcription and protein translation are key processes driving to an eventual and complete remodelling of the whole proteome, including the nuclear proteome itself.

In this sense, several RNA-binding proteins (RBPs), known to govern many aspects of RNA metabolism, including pre-mRNA processing, splicing, and RNA stability [62], showed differential accumulation under UV stress. However, their specific roles are not completely understood, although they are emerging as important proteins for rapid response to environmental cues [63]. Between all of them, HEN4 (Pp009885_1), known to be involved in methylation-mediated silencing of sRNAs [64] and found in nuclear speckles, enriched in splicing factors [65], was specifically accumulated in UV-treated plants and relevant according to PCA, suggesting an important role of splicing in UV stress response.

Regarding translation, the high number of ribosomal proteins identified, as well as the number of them showing high loadings in PC2, hypothesized to represent the changes in the machinery required for the stress survival and active proteome remodelling triggered after first irradiation period, supports the ribosomal rearranging as an important mechanism for stress response through protein translation regulation [66,67]. We found some, like RPS10 and RPL28, which were depleted with the stress, while others followed an opposite trend and were accumulated either short- (RPS17) or longer-term (RPS8). There were also ribosomal proteins specific of the UV-treated plants (RPS24, RPL35, RPL21 and RPS5). Considering that more experiments are required to define their specific molecular function, still unknown, their high importance in ANOVA and PCA models supports their crucial implication in the regulation and control of the proteome remodelling.

4.3. Increased protein folding machinery and Endoplasmatic Reticulum regulation is required for stress adaption

Under stressful conditions, protein folding machinery in endoplasmic reticulum is overwhelmed as the demand for protein folding reaches a maximum [68], particularly in the case of UV due to its photodamaging effect over macromolecules, including proteins [13]. The need of an increased rate of protein production was supported by the accumulation of HSP70 (only found in stressed plants), HSP90 and a J family protein (+2.6-fold and +2.38-fold after 16 h of UV, respectively). These proteins are known to be translocated to the nucleus under abiotic stress conditions [69] and to be part of a multichaperone involved in the stability of transcriptional complexes [70,71]. Furthermore, HSFA3 (Q6H6Q7; PITA_000031067-RA), a heat shock transcription factor, significantly increased after 16 h of UV at protein (+2.08-fold) and transcription (+2.26-fold) level being a main regulator of HSPs expression, including some HSP70 family proteins [72,73]. Interestingly BIP3, a chaperone which is also a master regulator of specific bZIP transcription factors (*i.e.* bZIP28) in Arabidopsis by retaining them in the endoplasmic reticulum [74], was also overaccumulated (+5.88-fold in 16 h plants). It is proposed that BIP3 is competed away from bZIP28 by the high abundance of misfolded proteins [74] that are accumulated in the reticulum. This is in concordance with the overaccumulation of a peptide corresponding to one bZIP transcription factor and GBF3 (Q99142; Pr009259_2; +3.3-fold and +2.53-fold in 16 h plants at protein and transcription level, respectively), also a bZIP, reported to have a COP1- and HY5-dependant expression [75]

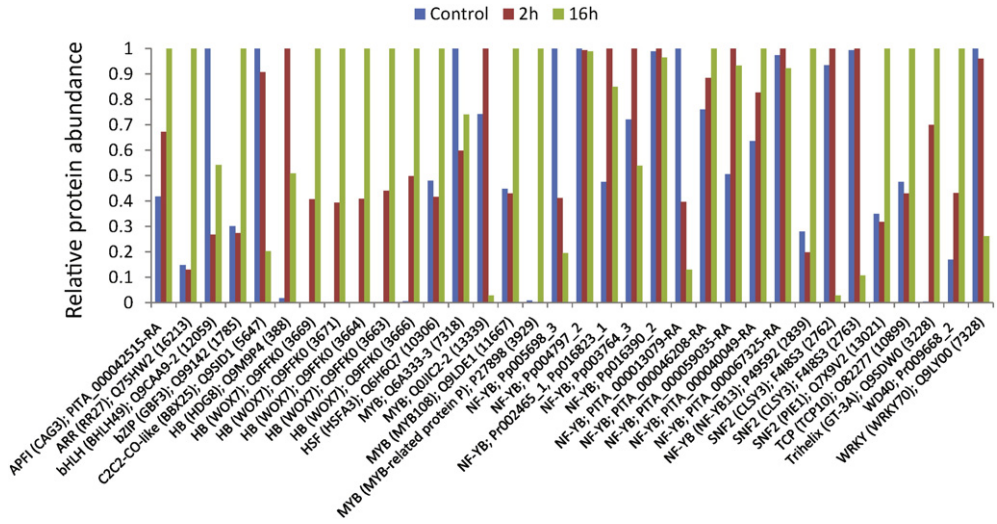

Fig. 6. Relative protein abundance of the transcription factors and regulators identified in the study in the different treatments. Protein abundances are expressed over a maximum value of 1. The accession and, when corresponding, the protein name and ID are indicated.

and required in ABA mediated stress responses [76], both elements of the UVR8 signalling pathway [15,77].

4.4. Nucleosome remodelling through the differential accumulation of histone isoforms and histone-like transcription factors is triggered after UV irradiation

Chromatin regulation is essential to regulate gene expression and relies on the interaction between many epigenetic factors, including histone modifications, DNA methylation and the incorporation of specific histone variants into the nucleosomes [78]. We found that some histone variants (H1, H4, H2B, H2B.2, HTB11, and HTA5) were reduced during the experiment, while others were either longer-term, like H2A.1 and a histone superfamily protein (PITA_000040049-RA), or short-term accumulated, like HTA6, HTA3, HTA9, and other histone superfamily proteins (Pr002465_1–Pp016823_1, PITA_000059035-RA, Pr007105_2, PITA_000067325-RA). Interestingly, some of these histones were identified as members of the heterotrimeric NF-Y family of

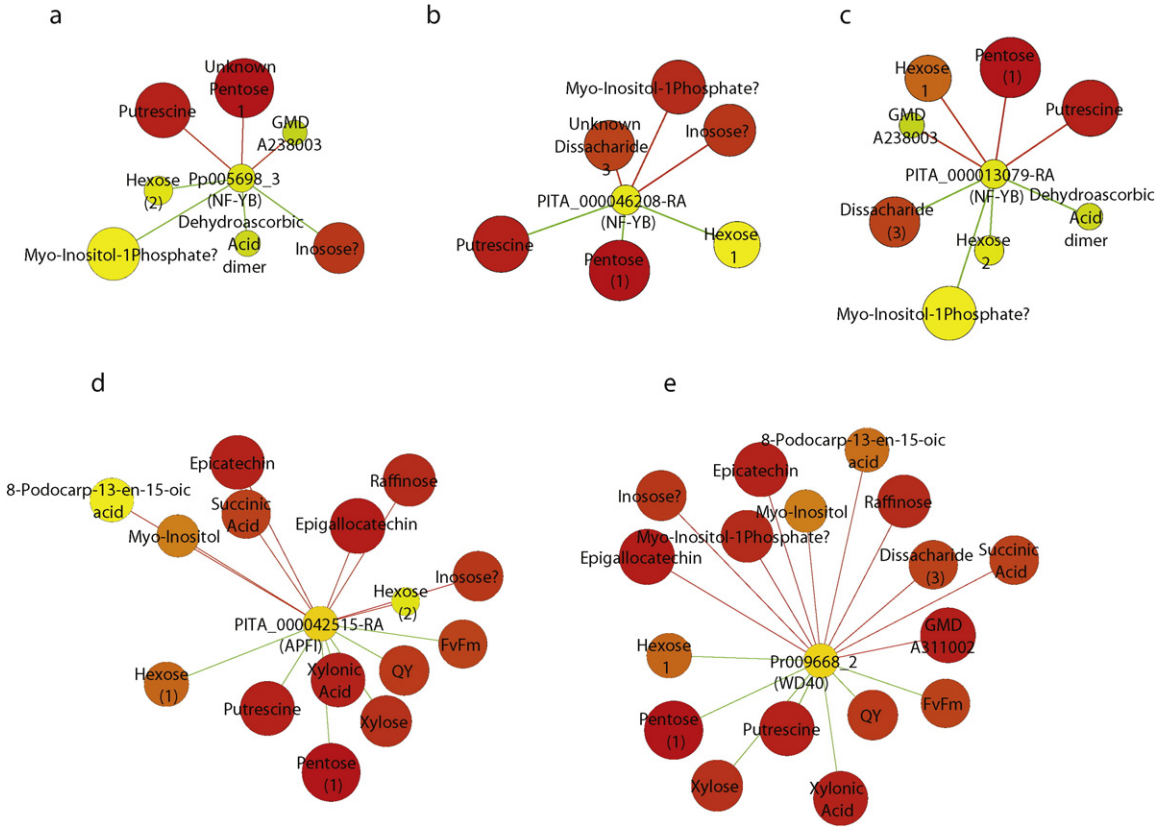

Fig. 7. sPLS-based networks of the transcription factors found to differentially accumulate in the experiment. The first neighbours are shown only in all the cases. (a) Pp005698_3 (NF-YB). (b) PITA_000046208-RA (NF-YB). (c) PITA_000013079-RA (NF-YB). (d) PITA_000042515-RA (APFI transcription factor family protein). (e) Pr009668_2 (WD40-repeat transcription factor). Red edges represent positive correlations, while the green ones represent negative correlation values. Only those correlations equal or higher, in absolute value, than 0.8 are shown. Node size and colour represent Radiality, corresponding low values to smaller sizes and brighter colours and high values to larger sizes and darker colours.

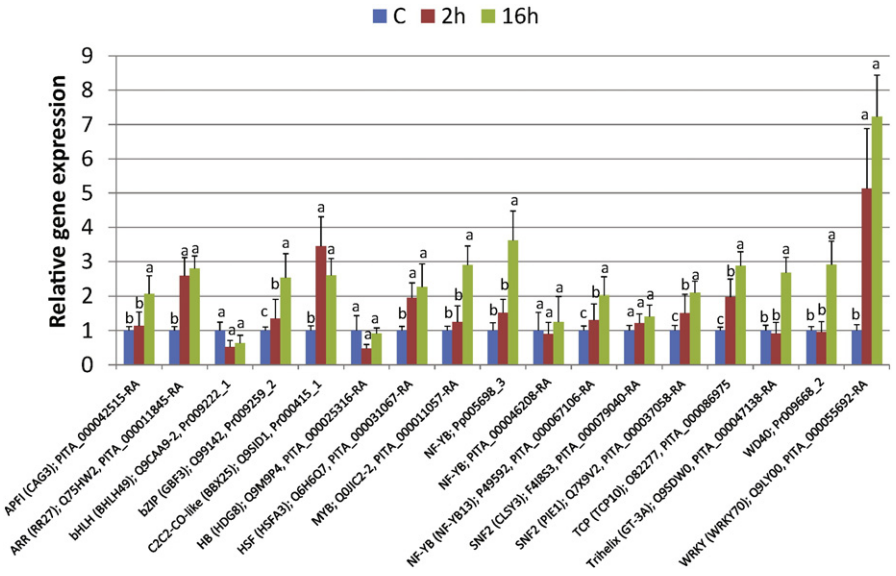

Fig. 8. Analysis of the relative quantity (RQ) in the different experimental situations of the transcripts of some transcription factors found to differentially accumulate under UV and revealed as relevant by PCA and/or ANOVA. Expression levels are shown regarding the Control and were normalized using *ACT* and *UBI* as housekeeping genes. Error bars show the SE of normalized RQ for each gene and each sample scaled to the control. Different letters indicate statistically significant differences (*t*-test, $p < 0.05$). The accessions corresponding to UniProt and/or our custom Pine database, as well as the family and, in some cases, the name of the transcription factor in brackets are indicated.

transcription factors (Pp005698_3, Pp004797_2, Pp016823_1, Pp003764_3, Pp016390_2, PITA_000013079-RA, PITA_000046208-RA, PITA_000059035-RA, PITA_000040049-RA, PITA_000067325 and P49592) that upregulate stress responsive genes [68,79,80], including NF-YB subunits, regulated their expression by BIP3-bZIP. Pp005698_3, PITA_000013079-RA, PITA_000046208-RA and P49592 were considered as relevant players of the UV adaptive mechanisms as it was revealed by ANOVA and multivariate analyses.

Interestingly, HTA9 (Pp003764_3; +1.38-fold in 2 h plants), an H2A.Z histone that we annotated as NF-YB subunit, showed a high positive loading in PC2, proposed to represent the mechanisms involved in the coordinated remodelling of the proteome so as to survive to the stress, showing its importance in UV stress responses. This was also the case of Pp005698_3, found to be upregulated at transcriptional (+3.6-fold) level after 16 h of UV and being relevant in PCA and sPLS models, although it protein abundance decreseased (−5.26-fold) pointing to a possible post transcriptional regulation that reduced the abundance of this protein. HTA9 levels have been correlated to stress response genes and their responsiveness and regulation under stressful conditions [81]. H2A.Z are known to recruit RNA Pol II and enhance its access to chromatin [82]. NF-YBs do in a similar way in transcription complexes with different bZIPs [83].

H2AX isoforms (HTA6 and HTA3, short-term accumulated, +1.23-fold and +1.38-fold respectively) have different roles in gene regulation. HTA6 and HTA3 are related to promote chromatin condensation [84] and DNA break repair [85] respectively. The initial accumulation may be necessary for reducing the initial damage that UV is causing to the DNA until polyphenolic pigments and ELIP proteins [23] constitute an effective sunscreen.

NF-YB13 expression is upregulated in *Brassica* [86] and *Populus* [87] under salt stress and ABA-treatment, and drought stress, respectively. In the same way, under UV we observed a longer-term (+3.7-fold in 16 h plants) accumulation of NF-YB13 (P49592; PITA_000067106-RA) at protein level, supported by its increased accumulation observed at transcription level. Its biological function remains undetermined, although everything points its involvement in stress responses, as the other members of NF-YB family.

These results reinforced our hypothesis about the crucial role of this family in UV resistance in *Pinus radiata*, also supported by the correlations that were found between some NF-YBs and myo-inositol and polyamines pathways, known to be related to stress response [88,89]; the relevance of this transcription factors for UV stress is strongly suggested and determine their specific biological function in the regulation of chromatin remodelling would be of special interest.

Interestingly APFI and WD40-repeat transcription factors seemed to link two of the most known phenomena triggered by UV irradiation [13], the reduction in photosynthesis (showing a negative correlation to Fv/Fm and QY) and the increased accumulation of photoprotective pigments, most of them phenolic compounds (positive correlation to epicatechin and epigallocatechin). These results, considering only three families of transcription factors, showed the complexity behind a systemic stress response in a plant system [13], and provide new candidates for establishing new regulatory pathways responsive to this stress.

5. Conclusions

This work showed for first time the dynamics of the nuclear proteome responding to UV stress. The depth of this study, considering the number of identified and annotated proteins, allowed the depict of this process, covering sensing and transduction pathways, endoplasmic reticulum mechanisms, and the regulation of chromatin dynamism and gene expression by histones, histone-like NF-Y, and other transcription factors previously unrelated to this stress source, as well as the role of alternative splicing and other mechanism involved in RNA translation and protein synthesis. Altogether these mechanisms drove, in last term, to an active remodelling of the proteome, a crucial process for response and adaptation to UV stress in Pine that was previously uncharacterized.

Considering that the exploit of natural variation in Pine species [90] as one of the most promising tools for tree upgrading programs, the determination of NF-YB13, Pp005698_3 (NF-YB) and Pr009668_2 (WD-40) as specific gene products involved in stress responsive mechanisms strongly correlated to the increased accumulation of photoprotective pigments and to the reduced photosynthesis will introduce a new set of novel biomarkers for selecting an industry-demanded trait. Moreover, the importance of a whole set of transcription factors that can be subject of further research were unravelled.

In conclusion, this work showed an improved analytical workflow for analysing proteome datasets in conditions of low database representation

that allowed the first profile of the UV nuclear proteome of a tree species, contributing to gain a deeper and wider view of nuclei proteome dynamics and its biological relevance as well as providing a novel set of clear biomarkers aimed to help breeding programs focused to increase pine tolerance to UV stress.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2016.03.003.

Transparency Document

The Transparency document associated with this article can be found, in the online version.

Acknowledgements

The authors wish to thank Dr. Mónica Meijón for critically reading the manuscript. This publication is an output of the Projects financed by the Spanish Ministry of Economy and Competitiveness (AGL2014-54995-P) and by the Government of the Principality of Asturias (FC-15-GRUPIN14-055). J.P., M.E., and L.V. were respectively supported by FPU (AP2010-5857, Ministry of Education, Spain), Severo Ochoa (BP11117, Government of the Principality of Asturias), and Juan de la Cierva (JCI-2012-12444; Spanish Ministry of Economy and Competitiveness) fellows.

References

[1] J. Krasensky, C. Jonak, Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks, J. Exp. Bot. (2012).
[2] J. Zhu, Salt and drought stress signal transduction in plants, Annu. Rev. Plant Biol. 53 (2002) 247–273.
[3] T. Zhu, N.J. Provart, Transcriptional responses to low temperature and their regulation in Arabidopsis, Can. J. Bot. 81 (2003) 1168–1174.
[4] C. Guy, F. Kaplan, J. Kopka, J. Selbig, D.K. Hincha, Metabolomics of temperature stress, Physiol. Plant. 132 (2008) 220–235.
[5] R.L. McKenzie, P.J. Aucamp, A.F. Bais, L.O. Bjorn, M. Ilyas, S. Madronich, Ozone depletion and climate change: impacts on UV radiation, Photochem. Photobiol. Sci. 10 (2011) 182–198.
[6] S. Montzka, S. Reimann, A. Engel, K. Kruger, W. Sturges, D. Blake, et al., Scientific assessment of ozone depletion: 2010, Global Ozone Research and Monitoring Project-Report No 51, 2011.
[7] D.T. Krizek, Influence of ultraviolet radiation on germination and early seedling growth, Physiol. Plant. 34 (1975) 182–186.
[8] T. Day, C. Ruhland, C. Grobe, F. Xiong, Growth and reproduction of Antarctic vascular plants in response to warming and UV radiation reductions in the field, Oecologia 119 (1999) 24–35.
[9] M. Kaling, B. Kanawati, A. Ghirardo, A. Albert, J.B. Winkler, W. Heller, et al., UV-B mediated metabolic rearrangements in poplar revealed by non-targeted metabolomics, Plant Cell Environ. (2014) n/a-n/a.
[10] M. Bernal, D. Verdaguer, J. Badosa, A. Abadía, J. Llusià, J. Peñuelas, et al., Effects of enhanced UV radiation and water availability on performance, biomass production and photoprotective mechanisms of *Laurus nobilis* seedlings, Environ. Exp. Bot. 109 (2015) 264–275.
[11] M. Brosche, A. Strid, Molecular events following perception of ultraviolet-B radiation by plants, Physiol. Plant. 117 (2003) 1–10.
[12] B.A. Brown, C. Cloix, G.H. Jiang, E. Kaiserli, P. Herzyk, D.J. Kliebenstein, et al., A UV-B-specific signaling component orchestrates plant UV protection, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 18225–18230.
[13] M.A.K. Jansen, V. Gaba, B.M. Greenberg, Higher plants and UV-B radiation: balancing damage, repair and acclimation, Trends Plant Sci. 3 (1998) 131–135.
[14] W.-X. Li, Y. Oono, J. Zhu, X.-J. He, J.-M. Wu, K. Iida, et al., The Arabidopsis NFYA5 transcription factor is regulated transcriptionally and posttranscriptionally to promote drought resistance, Plant Cell 20 (2008) 2238–2251.
[15] K. Tilbrook, A.B. Arongaus, M. Binkert, M. Heijde, R. Yin, R. Ulm, The UVR8 UV-B photoreceptor: perception, signaling and response, The Arabidopsis Book 2013, p. e0164.
[16] J. Martínez-Lüscher, M. Sánchez-Díaz, S. Delrot, J. Aguirreolea, I. Pascual, E. Gomès, Ultraviolet-B radiation and water deficit interact to alter flavonol and anthocyanin profiles in grapevine berries through transcriptomic regulation, Plant Cell Physiol. (2014).
[17] H. Du, Y. Liang, K. Pei, K. Ma, UV radiation-responsive proteins in rice leaves: a proteomic analysis, Plant Cell Physiol. 52 (2011) 306–316.
[18] I. Fedina, J. Hidema, M. Velitchkova, K. Georgieva, D. Nedeva, UV-B induced stress responses in three rice cultivars, Biol. Plant. 54 (2010) 571–574.
[19] X.C. Wu, C.X. Fang, J.Y. Chen, Q.S. Wang, T. Chen, W.X. Lin, et al., A proteomic analysis of leaf responses to enhanced ultraviolet-B radiation in two rice (*Oryza sativa* L.) cultivars differing in UV sensitivity, J. Plant Biol. (54) (2011) 251–261.
[20] P. Casati, M. Campi, D. Morrow, J. Fernandes, V. Walbot, Transcriptomic, proteomic and metabolomic analysis of UV-B signaling in maize, BMC Genomics 12 (2011) 321.
[21] P. Casati, A. Stapleton, J. Blum, V. Walbot, Genome-wide analysis of high-altitude maize and gene knockdown stocks implicates chromatin remodeling proteins in response to UV-B, Plant J. 46 (2006) 613–627.
[22] J.J. Wargent, B.R. Jordan, From ozone depletion to agriculture: understanding the role of UV radiation in sustainable crop production, New Phytol. 197 (2013) 1058–1076.
[23] L. Valledor, M.J. Canal, J. Pascual, R. Rodriguez, M. Meijon, Early induced protein 1 (PrELIP1) and other photosynthetic, stress and epigenetic regulation genes are involved in *Pinus radiata* D. don UV-B radiation response, Physiol. Plant. 146 (2012) 308–320.
[24] N. Pandey, S. Pandey-Rai, Short term UV-B radiation-mediated transcriptional responses and altered secondary metabolism of in vitro propagated plantlets of *Artemisia annua* L, Plant Cell Tissue Org. Cult. 1-15 (2013).
[25] L. Pruneda-Paz Jose, G. Breton, H. Nagel Dawn, S.E. Kang, K. Bonaldi, J. Doherty Colleen, et al., A genome-scale resource for the functional characterization of Arabidopsis transcription factors, Cell Rep. 8 (2014) 622–632.
[26] S. Danisman, F. van der Wal, S. Dhondt, R. Waites, S. de Folter, A. Bimbo, et al., Arabidopsis Class I and Class II TCP transcription factors regulate jasmonic acid metabolism and leaf development antagonistically, Plant Physiol. 159 (2012) 1511–1523.
[27] K. Maxwell, G.N. Johnson, Chlorophyll fluorescence—a practical guide, J. Exp. Bot. 51 (2000) 659–668.
[28] M. Escandón, M. Meijón, J. Pascual, L. Valledor, B. Correia, M. Dias, et al., Response to heat stress in plants and embryogenic callus of *Pinus radiata* D. Don, XX Reunión de la Sociedad Española de Fisiología Vegetal (SEFV) and XIII Congreso Hispano-Luso de Fisiología Vegetal 2013, p. 371 Abstract book.
[29] D.A. Sims, J.A. Gamon, Relationships between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages, Remote Sens. Environ. 81 (2002) 337–354.
[30] L. Valledor, J. Pascual, M. Meijón, M. Escandón, M.J. Cañal, Conserved epigenetic mechanisms could play a key role in regulation of photosynthesis and development-related genes during needle development of *Pinus radiata*, PLoS One 10 (2015), e0126405.
[31] Alegre S, Pascual J, Naggler M, Escandón M, Annacondia ML, Weckwerth W, et al. Analysis of the UV-stress nuclear proteome of *Pinus radiata* by mass spectrometry (GeLC-Orbitrap/MS). Data in Brief. submitted for publication.
[32] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, et al., Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.
[33] L. Valledor, W. Weckwerth, in: J.V. Jorrin-Novo, S. Komatsu, W. Weckwerth, S. Wienkoop (Eds.), An Improved Detergent-Compatible Gel-Fractionation LC-LTQ-Orbitrap-MS Workflow for Plant and Microbial Proteomics, Plant ProteomicsHumana Press 2014, pp. 347–358.
[34] V. Egelhofer, W. Hoehenwarter, D. Lyon, W. Weckwerth, S. Wienkoop, Using ProtMAX to create high-mass-accuracy precursor alignments from label-free quantitative mass spectrometry data generated in shotgun proteomics experiments, Nat. Protoc. 8 (2013) 595–601.
[35] J. Canales, R. Bautista, P. Label, J. Gomez-Maldonado, I. Lesur, N. Fernandez-Pozo, et al., De novo assembly of maritime pine transcriptome: implications for forest breeding and biotechnology, Plant Biotechnol. J. 12 (2014) 286–299.
[36] D. Neale, J. Wegrzyn, K. Stevens, A. Zimin, D. Puiu, M. Crepeau, et al., Decoding the massive genome of loblolly pine using haploid DNA and novel assembly strategies, Genome Biol. 15 (2014) R59.
[37] L. Valledor, Jorrín JV, Rodríguez JL, C. Lenz, Meijón Mn, R. Rodríguez, et al., Combined proteomic and transcriptomic analysis identifies differentially expressed pathways associated to *Pinus radiata* needle maturation, J. Proteome Res. 9 (2010) 3954–3979.
[38] M.C. Romero-Rodríguez, J. Pascual, L. Valledor, J. Jorrín-Novo, Improving the quality of protein identification in non-model species: characterization of *Quercus ilex* seed and *Pinus radiata* needle proteomes by using SEQUEST and custom databases, J. Proteome (2014).
[39] A. Muñoz-Mérida, E. Viguera, M.G. Claros, O. Trelles, A.J. Pérez-Pulido, Sma3s: a three-step modular annotator for large sequence datasets, DNA Res. 21 (2014) 341–353.
[40] M. Lohse, A. Nagel, T. Herter, P. May, M. Schroda, R. Zrenner, et al., Mercator: a fast and simple web server for genome scale functional annotation of plant sequence data, Plant Cell Environ. (2013) n/a-n/a.
[41] O. Thimm, O. Bläsing, Y. Gibon, A. Nagel, S. Meyer, P. Krüger, et al., mapman: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes, Plant J. 37 (2004) 914–939.
[42] X. Dai, S. Sinharoy, M. Udvardi, P. Zhao, PlantTFcat: an online plant transcription factor and transcriptional regulator categorization and analysis tool, BMC Bioinform. 14 (2013) 321.
[43] D. Riano-Pachon, S. Ruzicic, I. Dreyer, B. Mueller-Roeber, PlnTFDB: an integrative plant transcription factor database, BMC Bioinform. 8 (2007) 42.
[44] B. Ma, Novor: real-time peptide de novo sequencing software, J. Am. Soc. Mass Spectrom. 26 (2015) 1885–1894.
[45] T. Muth, L. Weilnböck, E. Rapp, C.G. Huber, L. Martens, M. Vaudel, et al., DeNovoGUI: an open source graphical user interface for de novo sequencing of tandem mass spectra, J. Proteome Res. 13 (2014) 1143–1146.
[46] C. Camacho, G. Coulouris, V. Avagyan, N. Ma, J. Papadopoulos, K. Bealer, et al., BLAST+: architecture and applications, BMC Bioinforma. 10 (2009) 421.
[47] L. Valledor, J. Jorrín, Back to the basics: maximizing the information obtained by quantitative two dimensional gel electrophoresis analyses by an appropriate experimental design and statistical analyses, J. Proteome 74 (2011) 1–18.

[48] J. Vandesompele, K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, et al., Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, Genome Biol. 3 (2002) research0034.1 - research.11.
[49] R Development Core Team, R: a language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria, 2012.
[50] I. González, Lé Cao K-A, S. Déjean, mixOmics: Omics Data Integration Project, 2011.
[51] M. Kohl, SLqPCR: functions for analysis of real-time quantitative PCR data at SIRS-Lab GmbH, R Package, 2007 Jena, Germany.
[52] L. Valledor, M. Escandón, M. Meijón, E. Nukarinen, M.J. Cañal, W. Weckwerth, A universal protocol for the combined isolation of metabolites, DNA, long RNAs, small RNAs, and proteins from plants and microorganisms, Plant J. (2014) n/a-n/a.
[53] J. Hellemans, G. Mortier, A. De Paepe, F. Speleman, J. Vandesompele, qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data, Genome Biol. 8 (2007) R19.
[54] A. Schweighofer, H. Hirt, I. Meskiene, Plant PP2C phosphatases: emerging functions in stress signaling, Trends Plant Sci. 9 (2004) 236–243.
[55] S. Fuchs, E. Grill, I. Meskiene, A. Schweighofer, Type 2C protein phosphatases in plants, FEBS J. 280 (2013) 681–693.
[56] A. Kulik, I. Wawer, E. Krzywińska, M. Bucholc, G. Dobrowolska, SnRK2 protein kinases—key regulators of plant response to abiotic stresses, OMICS 15 (2011) 859–872.
[57] M.K. Choudhary, D. Basu, A. Datta, N. Chakraborty, S. Chakraborty, Dehydration-responsive nuclear proteome of rice (*Oryza sativa* L.) illustrates protein network, novel regulators of cellular adaptation, and evolutionary perspective, Mol. Cell. Proteomics 8 (2009) 1579–1598.
[58] M.S. Bae, E.J. Cho, E.-Y. Choi, O.K. Park, Analysis of the Arabidopsis nuclear proteome and its response to cold stress, Plant J. 36 (2003) 652–663.
[59] T. Bonnot, E. Bancel, C. Chambon, J. Boudet, G. Branlard, Martre P., Changes in the nuclear proteome of developing wheat (*Triticum aestivum* sL.) grain, Front. Plant Sci. (2015) 6.
[60] L. Valledor, M.A. Castillejo, C. Lenz, R. Rodríguez, M.J. Cañal, Jorrín JS, Proteomic analysis of *Pinus radiata* needles: 2-DE map and protein identification by LC/MS/MS and substitution-tolerant database searching, J. Proteome Res. 7 (2008) 2616–2631.
[61] C.J. Nelson, A.H. Millar, Protein turnover in plant biology, Nat. Plants 1 (2015).
[62] H. Kang, S.J. Park, K.J. Kwak, Plant RNA chaperones in stress response, Trends Plant Sci. 18 (2013) 100–106.
[63] A. Ambrosone, A. Costa, A. Leone, S. Grillo, Beyond transcription: RNA-binding proteins as emerging regulators of plant response to environmental constraints, Plant Sci. 182 (2012) 12–18.
[64] N.G. Bologna, O. Voinnet, The diversity, biogenesis, and activities of endogenous silencing small RNAs in Arabidopsis, Annu. Rev. Plant Biol. 65 (2014) 473–503.
[65] A.S.N. Reddy, I.S. Day, J. Göhring, A. Barta, Localization and dynamics of nuclear speckles in plants, Plant Physiol. 158 (2012) 67–77.
[66] R. Sormani, C. Masclaux-Daubresse, F. Daniele-Vedele, F. Chardon, Transcriptional regulation of ribosome components are determined by stress according to cellular compartments in *Arabidopsis thaliana*, PLoS One 6 (2011) e28070.
[67] S. Xue, M. Barna, Specialized ribosomes: a new frontier in gene regulation and organismal biology, Nat. Rev. Mol. Cell Biol. 13 (2012) 355–369.
[68] J.-X. Liu, S.H. Howell, Endoplasmic reticulum protein quality control and its relationship to environmental stress responses in plants, Plant Cell 22 (2010) 2930–2942.
[69] T. Langer, S. Rosmus, H. Fasold, Intracellular localization of the 90 kDA heat shock protein (HSP90alpha) determined by expression of a EGFP-HSP90alpha-fusion protein in unstressed and heat stressed 3T3 cells, Cell Biol. Int. 27 (2003) 47–52.
[70] Y. Guo, T. Guettouche, M. Fenna, F. Boellmann, W.B. Pratt, D.O. Toft, et al., Evidence for a mechanism of repression of heat shock factor 1 transcriptional activity by a multichaperone complex, J. Biol. Chem. 276 (2001) 45791–45799.
[71] J. Li, J. Soroka, J. Buchner, The Hsp90 chaperone machinery: conformational dynamics and regulation by co-chaperones, Biochim. Biophys. Acta Mol. Cell Res. 1823 (2012) 624–635.
[72] F. Schramm, J. Larkindale, E. Kiehlmann, A. Ganguli, G. Englich, E. Vierling, et al., A cascade of transcription factor DREB2A and heat stress transcription factor HsfA3 regulates the heat stress response of Arabidopsis, Plant J. 53 (2008) 264–274.
[73] T. Yoshida, Y. Sakuma, D. Todaka, K. Maruyama, F. Qin, J. Mizoi, et al., Functional analysis of an Arabidopsis heat-shock transcription factor HsfA3 in the transcriptional cascade downstream of the DREB2A stress-regulatory system, Biochem. Biophys. Res. Commun. 368 (2008) 515–521.
[74] R. Srivastava, Y. Deng, S. Shah, A.G. Rao, S.H. Howell, Binding protein is a master regulator of the endoplasmic reticulum stress sensor/transducer bZIP28 in Arabidopsis, Plant Cell 25 (2013) 1416–1429.
[75] A. Oravecz, A. Baumann, Z. Máté, A. Brzezinska, J. Molinier, E.J. Oakeley, et al., Constitutively photomorphogenic1 is required for the UV-B response in Arabidopsis, Plant Cell Online 18 (2006) 1975–1990.
[76] G. Lu, A.L. Paul, D.R. McCarty, R.J. Ferl, Transcription factor veracity: is GBF3 responsible for ABA-regulated expression of Arabidopsis Adh? Plant Cell 8 (1996) 847–857.
[77] J.-J. Favory, A. Stec, H. Gruber, L. Rizzini, A. Oravecz, M. Funk, et al., Interaction of COP1 and UVR8 regulates UV-B-induced photomorphogenesis and stress acclimation in Arabidopsis, EMBO J. 28 (2009) 591–601.
[78] Y. Zhu, A. Dong, W.-H. Shen, Histone variants and chromatin assembly in plant abiotic stress responses, Biochim. Biophys. Acta Gene Regul. Mech. 1819 (2012) 343–348.
[79] J.-X. Liu, S.H. Howell, bZIP28 and NF-Y transcription factors are activated by ER stress and assemble into a transcriptional complex to regulate stress response genes in Arabidopsis, Plant Cell 22 (2010) 782–796.
[80] J.-X. Liu, R. Srivastava, P. Che, S.H. Howell, An endoplasmic reticulum stress response in Arabidopsis is mediated by proteolytic processing and nuclear relocation of a membrane-associated transcription factor, bZIP28, Plant Cell. 19 (2007) 4111–4119.
[81] D. Coleman-Derr, D. Zilberman, Deposition of histone variant H2A.Z within gene bodies regulates responsive genes, PLoS Genet. 8 (2012), e1002988.
[82] M. Adam, F. Robert, M. Larochelle, L. Gaudreau, H2A.Z is required for global chromatin integrity and for recruitment of RNA polymerase II under specific conditions, Mol. Cell. Biol. 21 (2001) 6270–6279.
[83] T. Laloum, S. De Mita, P. Gamas, M. Baudin, A. Niebel, CCAAT-box binding transcription factors in plants: Y so many? Trends Plant Sci. 18 (2013) 157–166.
[84] R. Yelagandula, H. Stroud, S. Holec, K. Zhou, S. Feng, X. Zhong, et al., The histone variant H2A.W defines heterochromatin and promotes chromatin condensation in Arabidopsis, Cell 158 (2014) 98–109.
[85] C. Charbonnel, M.E. Gallego, C.I. White, Xrcc1-dependent and Ku-dependent DNA double-strand break repair kinetics in Arabidopsis plants, Plant J. 64 (2010) 280–290.
[86] L. Xu, Z. Lin, Q. Tao, M. Liang, G. Zhao, X. Yin, et al., Multiple nuclear factor Y transcription factors respond to abiotic stress in *Brassica napus* L. PLoS One 9 (2014), e111354.
[87] D.-H. Yan, X. Xia, W. Yin, NF-YB family genes identified in a poplar genome-wide analysis and expressed in *Populus euphratica* are responsive to drought stress, Plant Mol. Biol. Report. 31 (2013) 363–370.
[88] R. Valluru, W. Van den Ende, Myo-inositol and beyond — emerging networks under stress, Plant Sci. 181 (2011) 387–400.
[89] R. Alcázar, F. Marco, J.C. Cuevas, M. Patron, A. Ferrando, P. Carrasco, et al., Involvement of polyamines in plant response to abiotic stress, Biotechnol. Lett. 28 (2006) 1867–1876.
[90] M. Meijón, I. Feito, M. Oravec, C. Delatorre, W. Weckwerth, J. Majada, et al., Exploring natural variation of *Pinus pinaster* Aiton using metabolomics: is it possible to identify the region of origin of a pine from its metabolites? Mol. Ecol. (2016).