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**A year (2014-2015) of plants in Proteomics journal.
Progress in wet and dry methodologies, moving from
protein catalogs, and the view of classic plant
biochemists**

Rosa Sanchez-Lucas, Angela Mehta, Luis Valledor, Francisco Cabello Hurtado, M. Cristina Romero-Rodriguez, Lyudmila Simova Stoilova, Sekvan Demir, Luis E. Rodriguez De Francisco, Ana M. Maldonado Alconada, Ana L. Jorrin-Prieto, et al.

► **To cite this version:**

Rosa Sanchez-Lucas, Angela Mehta, Luis Valledor, Francisco Cabello Hurtado, M. Cristina Romero-Rodriguez, et al.. A year (2014-2015) of plants in Proteomics journal. Progress in wet and dry methodologies, moving from protein catalogs, and the view of classic plant biochemists. *Proteomics*, 2016, 16 (5), pp.866-876. 10.1002/pmic.201500351 . hal-01239816

HAL Id: hal-01239816

<https://univ-rennes.hal.science/hal-01239816>

Submitted on 24 Mar 2016

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1 **A year (2014-2015) of plants in *Proteomics* journal. Progress in wet and dry**
2 **methodologies, moving from protein catalogs, and the view of classic plant**
3 **biochemists**

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27 **Key words:** plant proteomics, proteogenomics, systems biology, signaling, signal
28 transduction

29 Total number of words (including references as well as figure and table legends): 8164

30 **Abstract**

31 The present review is an update of the previous one published in *Proteomics 2015 Reviews* special
32 issue [1] covering the 2014-July 2015 period. It has been written on the bases of the publications that
33 appeared in *Proteomics* journal during that period and the most relevant ones that have been published
34 in other high-impact journals. Methodological advances and the contribution of the field to the
35 knowledge of plant biology processes and its translation to agroforestry and environmental sectors
36 will be discussed. This review has been organized in four blocks, with a starting general introduction
37 (literature survey) followed by sections focusing on the methodology (*in vitro*, *in vivo*, wet and dry),
38 proteomics integration with other approaches (systems biology and proteogenomics), biological
39 information, and knowledge (cell communication, receptors and signaling), ending with a brief
40 mention of some other biological and translational topics to which proteomics has made some
41 contribution.

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57 **INTRODUCTION**

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The topic of plant proteomics was reviewed in 2015 on the basis of the articles published in *Proteomics* journal (from now just *Proteomics*, written in italics) between 2001, when the first issue appeared, and May 2014 [1]. The present review is an update covering the January 2014-July 2015 period in which methodological advances and the contribution of the field to the plant biology knowledge and its translation to agroforestry and environmental sectors will be discussed. It is not intended to be repetitive considering recent reviews but aiming to get to the reader for a deeper discussion of key topics. It was written to be included in the 2016 *Proteomics Review Special Issue*, since it is the last one of the series edited by Prof. Dunn, the editor in chief of *Proteomics* since it appeared and who recently retired. It is a matter of justice to recognize the enormous effort of Prof. Dunn in establishing and consolidating *Proteomics* as a leader journal as well as his scientific contribution to the field. The corresponding author, in particular, and the plant community, in general, should acknowledge the personal consideration and the support he always gave to us reflected, just as an example, in his input to the International Plant Proteomics Organization (INPPO; <http://www.inppo.com/>) society [2], even though plant studies are not within his field of investigation. He, better than nobody else, has been an actor, director, and observer of the evolution of Proteomic Science [3].

According to PubMed, during the period of 2014-July 2015 *Proteomics* published 600 papers distributed in 40 issues, either regular or special including *Reviews 2014 and 2015 Tissue Proteomics and Imaging Mass Spectrometry*, *Top-Down Proteomics*, *Focus on Biomedical Research Trends*, *Proteomics in Chromatin Biology and Epigenetics*, *Proteogenomics*, *Signal Transduction*, *Proteomics Data Visualization*, *Plant-based Foods: Seed, Nutrition and Human Health*, *Virology meets*

79 *Proteomics, Views on Protein N-terminal Biology, and Protein Structure*. These thematic issues
80 perfectly represented the most recent technical advances, the frontier of knowledge in biology, and the
81 interest in translational biomedicine. Of 592 papers published in *Proteomics*, 133 were selected for
82 preparing this review as they dealt with plants (33 original papers, 16 reviews, and 1 meeting report,
83 representing around 10 % of the total papers released by the journal) or presented the state of the art
84 of the field from a biological, methodological or translational point of view.

85 After a PubMed search the terms proteomics or proteome in the title/abstract returned 8553
86 hits. Compared with the other –omics approaches, this figure was slightly or much higher than
87 transcriptomics/transcriptome (7728) or metabolomics/metabolome (3064) searches, respectively.
88 While looking for the combined use of the different omics approaches, PubMed provided 52
89 proteomics, transcriptomics, and metabolomics reports, as well as 113 proteomics and
90 transcriptomics, and 56 proteomics and metabolomics papers. The integration of the different –omics
91 approaches will be discussed in a further section.

92 In the surveyed period, plants continued to be underrepresented in the current proteomics
93 literature, but significantly increasing, with 683 references in PubMed (almost 15% of the total) while
94 for humans, just as a comparative example, the figure was 2795, with quite a few related to the study
95 of cancer, as well as vascular and other diseases [4]. Despite the claimed general worry about food
96 supply, environmental concerns, and climate change, these issues have not been properly reflected in
97 research funding policy in most of the western countries and even less in developing nations. This
98 issue is very well discussed by Donald Kennedy (President Emeritus at Stanford University, and a
99 former editor-in-chief of *Science*) in *Science*'s editorial "Building Agricultural Research" [5]. Human
100 welfare depends on well-connected health and food supply [6-8]. Famine is the major cause of
101 mortality on a worldwide basis, and increasing nutritional value of primary crops would drastically
102 reduce health problems in the less developed countries [9]. The situation seems to be changing, but
103 very slowly; at HUPO (<https://www.hupo.org/>) or general proteomics meetings such as European
104 EuPA (<http://www.eupa.org/>) or Spanish SEProt (<http://www2.cbm.uam.es/~seprot/>), room for non-
105 human proteomics and groups interested in different type of organisms (microorganisms, plant, and

106 animals) has been recently created, or is under discussion. The vision or sensations at the plant
107 specific organization INPPO is that we are surviving within the field as a scientific ghetto. With no
108 doubt, plant and human research can and should benefit from each other. Plants provide an almost
109 unlimited experimental system and despite being far in evolution they share an important number of
110 genes [10], with similar mechanisms of, for example, immunity [11], and host-pathogen interactions
111 [12]. In addition, plants may be sources of drugs for disease treatment [13] and also of allergens [14,
112 15]. Some more examples of common phenomena between plant and animals are totipotency, the
113 property of the cells to be developmentally reprogrammed, the conceptual basis of the stem cell
114 projects [16], and the involvement of telomeres and telomerases in senescence and aging [17].

115 The topic of plant proteomics continues to be extensively reviewed, reflecting its expansion,
116 relevance, and expectations; although we should assume by now that there is a long distance between
117 expectations and realities, and that the full potential of proteomics is far, once again if compared with
118 humans, from being fully exploited in plant biology research and crop science [18-20]. Here, in
119 *Proteomics*, and also in other journals, much attention has been given to proteomics of plant stresses
120 responses, the major topic in plant biology because of its implication in crop protection and
121 biotechnological improvement [21-23]. Up to 15 plant proteomics reviews have been recently
122 published in *Proteomics*, most of them in the Vol. 15, Iss. 5-6 (Reviews 2015) and 10 (Plant-based
123 Foods: Seed, Nutrition and Human Health). Strikingly, the thematic issues covering the most recent
124 technical novelties or the frontier in proteomics and biology knowledge have none or at most two
125 plant papers per issue; it was the case of *Tissue Proteomics and Imaging Mass Spectrometry* (Vol.14,
126 Iss. 7-8, one plant reference by Maciel et al., 2014 [24]), and *OMICS Views on Protein N-terminal*
127 *Biology* (Vol. 15, Iss. 4; two plant original papers by Zhang et al., 2015 [25] and Venne et al., 2015
128 [26]). The review by Walton et al. (2015) [27] on signaling, a topic later discussed, deserves special
129 attention, and is one of the plant reviews we should not miss. Regarding plant reviews in other
130 journals, at least 15 have appeared, with some of them covering topics that will be mentioned later,
131 including software, database and repositories [28] PTMs [29, 30], and signaling [31].

132 Recently, Jorin-Novo (2015) [32] published an opinion article in which scientific standards
133 and MIAPE were discussed in the context of proteomics work and publications. From his experience,
134 the failure to meet proper scientific standards and those requested for a proteomics paper is the most
135 obvious and immediate reason for rejection. As just a simple example, the general terminology
136 employed in the literature included terms such as protein expression, when proteomics approaches
137 actually determine abundance [1, 32]. Abundance is the result not only of gene expression
138 (transcription and translation) but also of protein degradation and PTMs.

139 The list of referenced papers did not pretend to be exhaustive, but limited, according to the
140 author's considerations, to those that presented some methodological, biological or translational
141 novelties. Some of the revised papers fit in the general group of comparative analysis based on 2-DE
142 coupled to MS analysis and they were not included. On the other hand, special attention was given to
143 those using second to fourth generation techniques (labelling, gel-free, SRM/MRM [1]), those
144 integrating classic and -omics approaches, or focused on PTMs and interactomics. Regarding the
145 previous period reviewed, there have not been real novelties in the experimental (plant) system used,
146 the biological process studied or the translational purpose pursued [1]. There is still a general need to
147 go beyond the construction of a protein catalog accompanied by more speculation than real
148 discussion, although better annotations and deeper knowledge is required in some cases.

150 WET AND DRY METHODOLOGIES

151 *In vitro* and *in situ* proteomics analysis

152 Proteomics is a discipline in expansion with continuous novelties and improvements in
153 techniques, protocols, softwares and equipments [33, 34]. This is also true for quite standard and well
154 established protocols in the early proteomics workflow stages prior to MS analysis, as it is important to
155 optimize them to each experimental system (plant, organ, tissue, cell or subcellular fraction). Thus,
156 improved protocols dealing with protein extraction [35], depletion [36, 37], fractionation [38], gel

157 staining [39], multiplexing [40], trypsin protein digestion [41] and sample preparation for shotgun
158 analysis [42, 43] have recently been published.

159 The use of different MS platforms in plant proteomics analysis was previously discussed [1],
160 however it is important to mention that new ion fragmentation procedures as alternative to the current
161 collision induced dissociation, CID, and electron transfer dissociation, ETD, such as
162 photodissociation, have been claimed to produce enhanced sequence coverage and higher confidence
163 in sequence assignment [44, 45].

164 One of the limitations of proteomics, when compared to genomic and transcriptomic analyses,
165 is that proteins cannot be amplified as can DNA and therefore, optimized and efficient extraction
166 methods must be developed. Thus, the very laborious and time consuming used protocols and the
167 number of replicates (the three biological replicates performed as a rule are not enough to perform
168 solid statistical analysis and get confident biological conclusions [46], it is recommended to perform
169 at least 5 biological replicates to allow a reliable estimation of residual variance when more than two
170 treatments are compared) are the main bottleneck that makes proteomics unpopular among plant
171 biologists. The use of an appropriate extraction method is even more relevant for PTMs or
172 interactomics studies that request fractionation steps. These issues have been addressed by Kraemer et
173 al (2015) [47], who compared different alternatives for sample preparation compatible with large
174 numbers of samples in phosphoproteomics analysis.

175 Protein extraction is still challenging and constitutes a big difficulty in the analysis of specific
176 proteins such as integral membrane proteins [48]. Integral membrane proteomics analyses involve
177 complicated protocols that include membrane purification and protein extraction. In order to analyze
178 the symbiosome integral membrane proteins, Clarke et al. (2015) [49] reported a protocol that
179 included isolation of symbiosomes with three-step Percoll gradients and preparation of the membrane
180 system by ultracentrifugation of the broken symbiosomes supernatant, followed by bicarbonate
181 stripping and chloroform-methanol protein extraction prior to MS analysis. Although the excessive
182 manipulation of the sample may interfere in the integral membrane protein analysis it has a great

183 value from a descriptive point of view. Once the protein is identified, the expression of the
184 corresponding genes and the associated phenotype can be assessed by using complementary
185 approaches. As an example, in order to understand intracellular protein trafficking (a hot topic in plant
186 biochemistry), Heard et al. (2014) [50] enriched partially overlapping subsamples of endosomal
187 proteomes associated with endomembrane from *A. thaliana* seedlings and used affinity purification of
188 fluorescent fusion constructs for seven subcellular marker proteins in order to predict its precise or
189 shared location, a strategy that resembles LOPIT [51]

190 From the current literature it is clear that plant proteomics is moving from 2-DE/MS (still the
191 bases of an important number of works published) to shotgun (bottom up gel free label free). Shotgun
192 will be the dominant platform in the near future if accompanied by a powerful and potent MS analysis
193 and equipment. It is simple and does not require much manipulation of the sample. The iTRAQ, quite
194 popular among the Chinese groups, appeared as the third alternative, even though plant proteomicists
195 did not pay much attention in the past to labelling techniques [52-55]. By using this technique, Pan et
196 al. (2015) [56] have reported up to 13,706 and 12,124 proteins identified with a LTQ-Orbitrap hybrid
197 mass spectrometer in *Arabidopsis thaliana* roots and leaves. Changes in abundance between plants
198 supplied with different iron concentrations were observed for 886 proteins with only a low percentage
199 of them showing good correlation with transcript levels.

200 Whatever the employed platform, it is very important that it is validated, as a preliminary step, for
201 the experimental system being analyzed, as for any analytical tool, in order to allow the determination
202 of the number of proteins confidently identified and quantified. Serial dilution of the sample could be
203 enough for validation purposes for those approaches based on label-free [57].
204

205 Fourth generation *in vitro* proteomics, SRM, MRM, or mass-western, sustain hypothesis driven
206 protein analysis. In the surveyed period just one paper, by Rogniaux et al. (2015) [58] was published
207 in *Proteomics* reporting the use of targeted approach for determining the relative abundance of
208 allergens in several wheat varieties, and showing the potential of the technique for translational, and
209 food traceability, purposes. Although the use of these techniques in plants is still limited, targeted

210 proteomics is the way to go for validation of the candidate proteins identified in 2-DE or shotgun
211 analyses. It is certainly a more appropriate follow-up study when compared to qRT-PCR analysis
212 frequently reported in proteomics publications.

213 Imaging has opened new possibilities for *in vivo* or *in situ* proteomics analysis and integration
214 with cellular biology techniques [59], giving, at the same time, the possibility of proteomics analysis
215 at the cellular level [60]. It is a more or less well established technique for metabolomics analysis [61]
216 but under development for proteins [62] and plant tissue samples. The technique has been the focus of
217 the *Proteomics Tissue Proteomics and Imaging Mass Spectrometry* special issue (Vol. 14, Iss. 7-8), in
218 which, two plant studies were reported. Maciel et al. (2014) [24] used the laser ablation imaging
219 technique to evaluate the distribution of selenium and sulfur in leaves of transgenic and non-
220 transgenic *A. thaliana*. One of the limitations of imaging studies is the processing of high amounts of
221 datasets, and therefore attention has been given to data processing methods, as discussed by
222 Wijetunge et al. [63] and Franceschi and Wehrens (2014) [64].

223

224 ***In silico* analysis and data interpretation: software, databases and repositories**

225 Proteomics, as any -omics approach, generates from hundreds to thousands of identified
226 products, being impossible to analyze and interpret all the data in a classic, mind based, manner [65].
227 The high amount of data generated and the costs of the analysis are the main reasons for the low
228 number of samples and biological replicates analyzed. Protein profiling requires software and
229 algorithms for protein identification, quantitation, functional and structural characterization, location,
230 interaction, networks, integration, visualization and deposition in databases and repositories. These
231 softwares are continuously created, improved and updated [66-69]. Recently, Sakata and Komatsu
232 (2014) [28] have surveyed and reviewed plant proteomics databases and websites as well as recent
233 technologies related to data collection and annotation.

234 The use of most algorithms and softwares is far from the current capacity of most plant
235 biochemists working with proteomics. This is the reason why the dry part of the work is in the hands

236 of mass spectrometrists or bioinformaticians usually with no expertise in plant biology. This results
237 in, more often than would be advisable, a blind acceptance of the computer provided data without a
238 minimum rationale and critical evaluation of the data confidence, making it difficult to conclude from
239 a biological point of view. The quite dynamic character of the proteomes and the fixed film provided
240 by our experiments makes most interpretations speculative. The situation for a standard plant
241 biochemist is even worse considering the number of algorithms that is continuously appearing [70].
242 Which one, what for, how to use them and translate to a biological discussion could be hell for most
243 of us [71, 72]. In this regard, *Proteomics* a special issue, *Proteomics Data Visualization* (17, 10). In
244 this and other regular issues a number of reviews discussing these questions have appeared [73-78]

245 The integration of disseminated data through databases is a pursued objective that will benefit
246 the scientific community. In this direction, The BioMart Community Portal (www.biomart.org) is a
247 good example the plant community should follow [79]. It includes over 800 different biological
248 datasets spanning genomics, proteomics, model organisms, cancer data, ontology information and
249 more.

250

251 **PROTEOMICS INTEGRATION WITH OTHER –OMICS**

252 Within the –omics approaches, the advantage, if so, of proteomics, and also metabolomics, is
253 that the information provided is closer to the phenotype [80]. On the other hand, it is much more
254 complicate at the methodological level (i.e. genomics and transcriptomics are favored by PCR and
255 well established microarray techniques). At the molecular level, the phenotype is the result of gene
256 expression through transcriptional and translational events and gene products and metabolites
257 interaction, so proteins are just part of the story. As previously discussed [1], proteomics has
258 limitations, the more relevant being proteome coverage, quantitation, identification in orphan
259 organisms, PTM, and interactomics. Protein identification and quantification cannot always be as
260 confident as we wish because of the organism, experimental design, and the employed techniques and

261 protocols, making our data interpretation conservative or speculative. In comparative proteomics, the
262 high variability in the protein profile among biological samples and the low number of biological
263 replicates employed in most studies hinder the biological interpretation. Thus, it is not easy to establish
264 protein abundance ranges in normal (control) samples, as it has been shown by Higdon et al. (2015)
265 [81]. These authors used data from two human proteome studies featured in Nature, and found that
266 protein abundance among biological replicates varied by ± 4 - to 10-fold for most proteins, with
267 coefficient of variation (CV) in between 62 and 117 %. To control or reduce this CV, it is
268 recommended to compare proteins identified with at least 3 or more unique peptides and normalize
269 abundances against housekeeping proteins with low variability.

270 Just by itself, and more than generating a list or catalog of protein species, proteomics can
271 provide real biological knowledge; however it would require, first, the use of a model systems, in
272 plants mostly *A. thaliana*, with a good collection of mutants for reverse and functional genetics
273 studies and, second, an appropriate experimental system, as for example cell suspensions. A good
274 example of this is the paper published by Smith et al. (2015) [82] in which signal transduction
275 pathways mediating plant programmed cell death was studied. By using isobaric-tagged relative and
276 absolute quantitation of proteins present in the extracellular matrix, 33 proteins were identified as
277 putative cell-death regulatory proteins. One of these proteins, the CYCLASE 1, which had previously
278 no known function, was a negative regulator of cell death in *Arabidopsis*.

279 The integration of the different -omics, in the direction of systems biology, is far from being a
280 reality or was only possible for a reduced number of research groups or through large international
281 consortia [83]. Systems biology, wide genomic approaches, and the recently coined proteogenomics,
282 in combination with image phenotyping, classical molecular, physiological and cell biology
283 approaches [84, 85] will get us to a deeper and better rather than to a partial and biased knowledge of
284 the biological systems. This knowledge is highly relevant for crop improvement aiming at
285 productivity yield increases and survival under extreme environmental conditions through classical,
286 molecular marker assisted, and biotechnological breeding [11, 86-88]

287 Combined proteomics and transcriptomics analysis has been used in the study of the maize
288 response to waterlogging and resulted, in general, in good correlations between both data [54]. But
289 this is not necessarily the rule and it depends on the gene or gene groups, being the consequence of
290 transcriptional, posttranscriptional, translational and posttranslational events. Abundance of both
291 molecules depends not only on their synthesis but also on their stability and degradation. It is also
292 known that different mRNAs possess different translational efficacy [89].

293 At least one paper, by Gupta et al. (2015) [90], has been published in which integrated
294 proteomics and metabolomics have been employed as experimental approaches. They analyzed
295 differences between soybean lines with different color in the seed coat. Differences in flavonoids,
296 which provide the molecular basis of the color, were correlated with levels in pathway-related
297 enzymes. Finally, there are not many papers in which the combined use of the three -omics
298 approaches are reported. In the surveyed period, two papers dealt with the detection of unintended
299 effects in transgenic maize [91, 92].

300 In any case, single and easy multi-omics approach experiments can be always performed and
301 the preliminary steps of the workflow should be evaluated as one of the limitations is just preparative.
302 In order to make confident data comparison or validation, these preparations should come from the
303 same sample. Independent experiments or extractions can originate artefacts or biases, mostly coming
304 from the inherent biological variability. In this direction, recent workflows aimed to extract
305 biomolecules (DNA, RNAs, proteins, and metabolites) have been developed [93]. Despite dedicated
306 workflows showed their efficiency, combined protocols demonstrated their performance in isolating
307 biomolecules suitable to be processed by high-throughput pipelines (gel free label free proteomics,
308 high-throughput metabolomics analyses and next generation sequencing transcriptomic analyses of
309 messenger or regulatory RNAs) while avoiding biases related to the use of not exactly the same
310 sample for the different -omics level analyses. The full overlapping of the datasets makes it easier to
311 interpret and analyze the data when performing a Systems Biology-based integrative approach.

312 Proteogenomics is a relatively new and emerging field at the intersection of genomics and
313 proteomics, being part of the wide genomics approaches. Although it may have multiple derivations
314 (RNA and protein correlation studies, proteomics and data validation, gene sequence analysis, among
315 others), in the simplest way it can be understood as the use of proteomics data and protein MS derived
316 sequences to improve gene sequence annotations [94-96]. From the proteomics perspective it has an
317 important feedback as correct sequence annotation will increase the number and confidence of the
318 protein identification [97], favoring, at the same time the studies of non-model organisms [98]. It will
319 also go deeper into the knowledge of posttranscriptional and translational events giving rise to the
320 different gene products, protein species or proteoforms [99].

321 The topic has been deeply reviewed in *Proteomics* (*Proteogenomics* special issue; Vol. 14,
322 Iss. 23-24). Proteogenomic experiments and prediction tools will differ between genome-sequenced
323 and –unsequenced, as well as eukaryotic or prokaryotic organisms [95, 100].
324

325 FROM PROTEOMICS TO BIOLOGY

326 Cell communication, receptors, and signaling pathways, are good examples of how
327 proteomics can contribute to biological knowledge and be used for translational purposes. This topic
328 has been well presented in a recent, excellent review, by Walton et al. (2015) [27]. Signaling is a hot
329 topic of great relevance in biology mainly approached by using functional and reverse genetics. It is
330 quite challenging as it deals with minor and recalcitrant, membrane bound, proteins, PTMs and
331 interactions, whose investigation requires integration with classic, transcriptomics and metabolomics
332 approaches. In the recent literature surveyed in the reviewed period, this topic is well covered with
333 both review papers and original contributions. Some other topics are also of interest but are somehow
334 related to this or are less represented in the most recent literature. This is the case of plant
335 developmental processes, plant-pathogen interactions and responses to other biotic stresses [101-104].

336 Plants chemically communicate and interact with their biotic and abiotic surroundings,
337 responding and adapting its biology to it. They also complete their developmental program (from seed

338 germination to seed production through vegetative and reproductive phases) in coordination with
339 seasonal changes noticed mainly as variations in light, temperature and water availability. There are
340 also daily changes associated to a circadian clock timed by, among other factors, light regimes. The
341 integration of environmental responses with development is crucial for plant survival, growth, and
342 from an agronomic point of view, productivity. Plants, as sessile organisms, are very versatile, and
343 their genome expression and metabolism is continuously reprogrammed in response to physico-
344 chemical and biological stimuli. Despite the high number of specialized organs, tissues and cell types
345 within a plant, all of them respond coordinately, implicating in good intercellular connexion systems
346 with chemical messages between receptor and effector cells. At the individual cell level, gene
347 expression changes are mediated by receptors, intracellular and interorganule signals, and signal
348 transduction pathways resulting in changes in gene expression through transcriptional, translational
349 and posttranslational events. Quite a number of molecules are known that act as external or internal
350 chemical signals, including phytohormones, and those from microorganisms (symbionts, parasites or
351 pathogens) [101, 103-106]. On the other side, receptors and signal transduction pathways and their
352 components are starting to be characterized [31, 107].

353 Proteomics is contributing to the above processes through descriptive, comparative, PTMs,
354 and interaction studies. Walton et al. (2015) [26] updated the contribution in these areas and the
355 different strategies employed in dissecting plant hormone signaling. Some following papers dealing
356 with PTMs and interactomics related to cell signaling are highlighted. Others, using descriptive or
357 comparative proteomics approaches have been skipped from this review.

358 Protein phosphorylation is the most common mechanism of signal and because of that
359 phosphoproteomics is the immediate strategy to be employed. Silva-Sanchez et al., (2015) [108], and
360 Li et al., (2015) [29] have summarized the current methodologies used in extracting, enriching, and
361 fractionating phosphoproteins, and in their MS analyses for identification, quantitation, and
362 phosphorylation site mapping, conclude on cross-talk between different PTMs (phosphorylation,
363 glycosylation, and redox modifications) and data validation. They provided a list of phosphoproteins,

364 softwares and databases. As an alternative to the current protocols, Han et al. (2015) [109] have
365 proposed a faster workflow analysis in the case of sample amount limitation that increases coverage;
366 it included integrated multistep enzymatic digestion, phosphopeptide enrichment and database search
367 (iMEED). In an attempt to elucidate post-translational processes mediating circadian events,
368 Choudhary et al. (2015) [110] performed a phosphoprotein analysis employing TiO₂ phosphopeptide
369 and MS analysis. They identified 1586 phosphopeptides on 1080 protein groups, out of which 102
370 changed in abundance, including clock proteins (ELF4, PRR3), transcription factors and kinases. By
371 using isotope-assisted quantitative phosphoproteomics, Minkoff et al. (2015) [111] carried out a
372 comparative phosphoproteomics study between wild and mutant ABA-insensitive *A. thaliana* plants.
373 Multiple changes occurred as early as 5 minutes after ABA treatment, with a reduction of the
374 phosphorylation events in the mutant.

375 Reactive oxygen species overproduction is a typical plant response to oxidative stress, these
376 species acting as signals and causing protein redox state changes [30, 112, 113]. The reversible PTM
377 methionine oxidation was studied in *A. thaliana* plants by Jacques et al. (2015) [114] using wild and
378 catalase-2 knock-out mutants. Over 500 sites of oxidation in 400 proteins were reported. They showed
379 that the activity of two specific glutathione-S-transferases was significantly reduced upon oxidation.
380 Konert et al. (2015) [115] have gone deeper into the mechanism of ROS signaling and metabolic
381 adjustment in *A. thaliana*. By using genetic, biochemical, and a combination of MS data-dependent
382 acquisition and SRM, they showed how protein phosphatase 2A, through its regulatory B' γ subunit,
383 interact with a cytosolic aconitase and control mitochondrial alternative oxidases.

384 Interactomics is the most challenging and less developed proteomics approach, but the most
385 concluding one in order to probe *in vitro* and *in situ* better than *in silico* interactions between proteins
386 and proteins and DNA, RNA or metabolites. It will help in developing models from networks
387 mediating receptor capture of signals and gene expression changes. The standard proteomics analysis
388 involves the capture of protein partners (affinity purification or immunoprecipitation) coupled to MS
389 analysis. Other published approaches included *in vivo* cross-linking with para-formaldehyde and

390 immunoaffinity purification of cross-linked protein. It has been used by Obermeyer et al., (2014)
391 [116] in the search for H(+) ATPase complexes in lily pollen. These techniques require high amount
392 of sample and involve excessive manipulation, being subjected to biases or false positives. Microarray
393 technology will be, with no doubt, the future for more feasible experiments in which dynamic
394 interactions could be observed through time course experiments [117]. As an alternative, the parallel
395 analysis of genomic, transcriptomics, and proteomics experiments could be performed [118].

396

397 **Some missed relevant papers**

398 Last but not least in the surveyed period are the publications covering topics that deserve to be
399 mentioned in a general plant proteomics review, either because they report on technical innovations or
400 novel applications to the plant biology research. As we did not intend to be exhaustive, and due to
401 space limitation, we will just mention them.

402 Such is the case of the top-down proteomics [45, 119-121], N-acetylome [122], degradation
403 of proteins with N-termini containing basic amino acids (the Arg/N-end rule) [25], and single cell
404 type proteomics analysis. Plant organs are complex in terms of the different types of tissues and cells
405 that are formed, with each one having its own proteome signature. Thus, for comparative proteomics
406 purposes it is possible that only a discrete number of cells respond to the experimental variable under
407 study and if so, by extracting the whole organ we might be diluting the response. In order to decipher
408 single proteome analysis new methodologies should be developed [86]. Up to now the laser
409 microdissection [123] has been applied, however it has been of very limited use with plants. What is
410 close to be a technical reality for transcriptomics looks impossible for proteomics [124].

411 **Acknowledgements**

412 Thanks to the University of Córdoba (Programas Propio y de Fortalecimiento) and CNPq (AM, and
413 JVJN) for financial support. L.V. was generously funded by the Juan de la Cierva Programme (JCI-
414 2012-12444, Spanish Ministry of Economy and Competitiveness). Rosa Sánchez Lucas is a recipient

415 of a Formación de Profesorado Universitario fellowship (FPU14/00186, Spanish Ministry of
416 Education, Culture and Sport). The funders had no role in the study design, data collection and
417 analysis, decision to publish, or preparation of the manuscript.

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