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Role of the proteome in phytohormonal signaling☆

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ABSTRACT

Phytohormones are orchestrators of plant growth and development. A lot of time and effort has been invested in attempting to comprehend their complex signaling pathways but despite success in elucidating some key components, molecular mechanisms in the transduction pathways are far from being resolved. The last decade has seen a boom in the analysis of phytohormone-responsive proteins. Abscisic acid, auxin, brassinosteroids, cytokinin, ethylene, gibberellins, nitric oxide, oxylipins, strigolactones, salicylic acid — all have been analyzed to various degrees. For this review, we collected data from proteome-wide analyses resulting in a list of over 2000 annotated proteins from *Arabidopsis* proteomics and nearly 500 manually filtered protein families merged from all the data available from different species. We present the currently accepted model of phytohormone signaling, highlight the contributions made by proteomic-based research and describe the key nodes in phytohormone signaling networks, as revealed by proteome analysis. These include ubiquitination and proteasome mediated degradation, calcium ion signaling, redox homeostasis, and phosphoproteome dynamics. Finally, we discuss potential pitfalls and future perspectives in the field. This article is part of a Special Issue entitled: Plant Proteomics — a bridge between fundamental processes and crop production.

1. Introduction

The phytohormones are small-molecule regulators that collectively regulate all aspects of the life of a plant cell. Growth and development, integration of environmental cues including biotic and abiotic stressors, regulation of circadian clock output, seed formation, and senescence — these are just few examples from a long list of important functions. Phytohormones have been intensively studied, but the signaling mechanisms underlying their complex modes of action are a long way from being resolved. In this review we summarize recent advances in the characterization of phytohormone signaling pathways, with a focus on phytohormone-responsive proteome analyses.

1.1. Signaling mechanisms

In general, any signaling cascade comprises the perception, transduction and response processes. The fastest responses, which are mediated via allosteric control, occur within milliseconds (Fig. 1). This reversible binding of small signal molecules is an important means by which the activity of many proteins is controlled. For example, a metabolic pathway product can bind to a key enzyme and inhibit its activity by inducing conformational changes that are transmitted to the enzyme's active site. A calcium ion flux can act as a regulator in this way, as can a number of other small molecules. In fact, over fifty ions and over 70,000 organic compounds have been annotated as being allosteric modulators (AlloSteric Database, [1]). The second level of regulation is governed by post-translational modifications (PTMs). The time required for PTM regulation to take place is typically minutes, and these forms of regulations are highly complex and far from being fully understood. We have previously reviewed in detail the more important PTMs and the methods of choice for isolation of modified proteins [2]; the most common PTMs in plant hormonal signaling are phosphorylation, ubiquitination and redox modifications and these will be addressed in the relevant parts of this review. The final level of regulation is that mediated by gene expression and the transcription-translation machinery. Though it is the slowest process, with an effective time span of hours, it is the best understood component of plant hormone signaling, and most of the current signaling pathway models described below are based predominantly on transcriptional analyses and studies of candidate gene mutants.

2. Phytohormone-responsive proteome

For this review, we have collected data from over one hundred proteome analyses conducted in the last fifteen years (i.e. since the year 2000), and nearly 6000 protein entries from diverse plant species are

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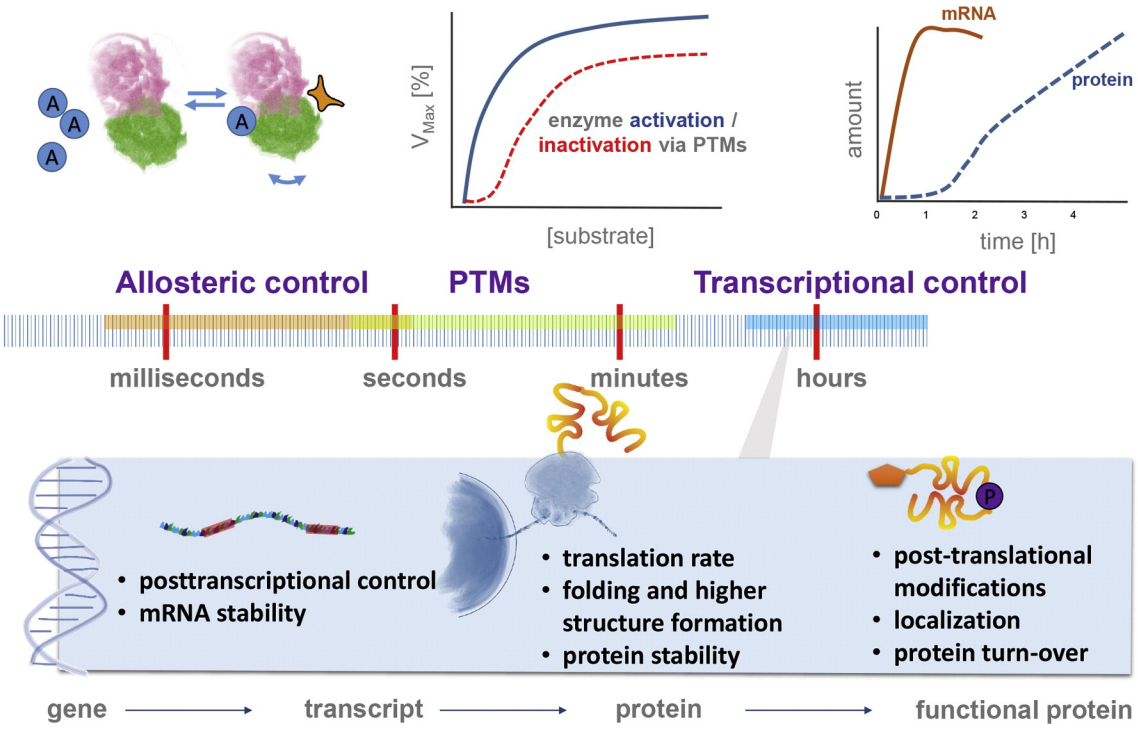

Fig. 1. Signaling mechanisms. Based on [3,4].

listed in the supplementary material. Where available, the *Arabidopsis* gene identifier was used. In total, there are 2157 reported *Arabidopsis* phytohormone-responsive proteins which represent a wide range of functions and cover all major metabolic pathways, with nearly four hundred unique enzymes (on the basis of EC numbers) (Fig. S1). The stress-related hormones abscisic acid, jasmonic acid and salicylic acid have been the most intensively studied substances, together representing more than 50% of all experiments (Table 1), and this is reflected in the number of *Arabidopsis* proteins annotated as being responsive to specific phytohormones: abscisic acid (955), cytokinin (690), brassinosteroids (375), jasmonic acid/oxylipins (336), auxin (137), salicylic acid (100), strigolactone (38), ethylene (13). The average overlap between the different phytohormone treatments among the identified *Arabidopsis* proteins is relatively high (13%), ranging up to 29% (jasmonates/abscisic acid). The shared biological processes include carbohydrate metabolism and photosynthesis, energy metabolism, response to stress and redox processes, and amino acid and protein metabolism (Fig. 2A). To provide an interspecies overview and utilize the whole dataset, we manually filtered the data and combined individual protein entries into protein families. The resulting list contains 70 protein families, each of which was found at least once in response to more than four different phytohormones (Fig. 2B, Supplementary tables). As well as the similarities identified from *Arabidopsis* annotations, this list highlights the common roles of cytoskeletal components (tubulin, actin), protein phosphorylation (kinases, phosphatases), membrane transport (ABC transporters), and the proteasome (26S regulatory subunit). The whole set is available from the Supplementary material.

Although the majority of the analyses employed a gel-based proteomics approach (Table 1), nearly half of the reported phytohormone-responsive entries were identified *via* a gel-free shotgun LC–MS protocol. 2-DE is still the method of choice for the analysis of PTMs, especially in redox proteomics, but the increased availability of state-of-the-art mass spectrometry is shifting the field towards peptide-based analyses. The efficiency of the LC–MS platform seemingly outperforms that of 2-DE separation, but its sensitivity is still compromised by loss

Table 1
Phytohormone proteomics overview. Numbers indicate the number of proteome-wide analyses that have been published since the year 2000; two-dimensional gel electrophoresis, 2-DE; liquid chromatography–mass spectrometry, LC–MS; two-dimensional liquid chromatography, 2D-LC.

Stimulus	Response			Method			
	Early 0–30 min	Delayed 1–8 h	Long-term >12 h	2-DE	LC–MS	2D-LC	PTM analysis
Abscisic acid (ABA)	4	12	18	20	13	1	8
Auxin (AUX)	0	4	5	6	3	0	2
Brassinosteroids (BR)	0	3	6	6	1	1	1
Cytokinin (CK)	3	2	9	11	3	0	3
Ethylene	0	1	3	3	1	0	1
Gibberellins (GA)	0	1	11	10	1	1	1
Nitric oxide, protein nitration (NO)	0	2	3	4	1	0	2
Oxylipins (jasmonates) (OL, JA)	0	5	14	15	2	0	3
Polyamines (PA)	0	0	1	1	0	0	1
Salicylic acid (SA)	0	2	14	14	1	0	0
Strigolactone (SL)	0	0	2	1	1	0	1

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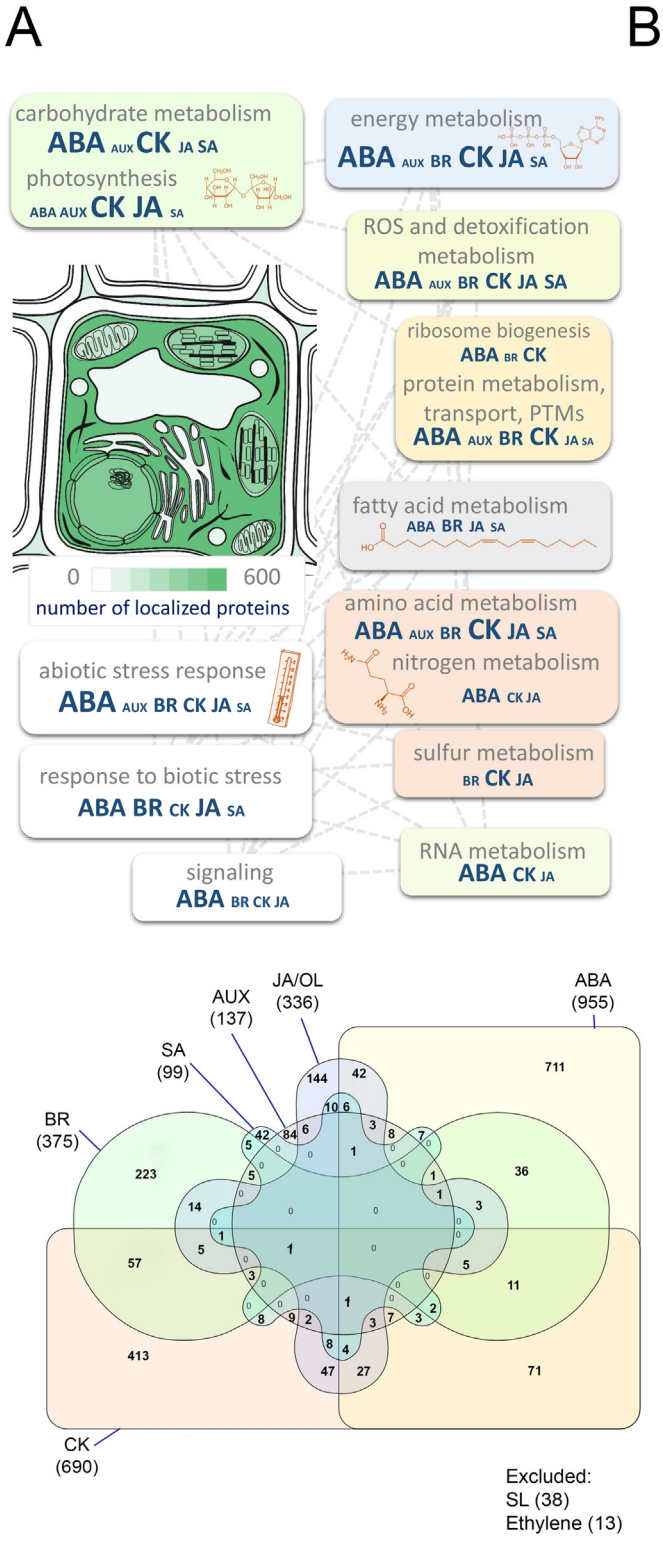

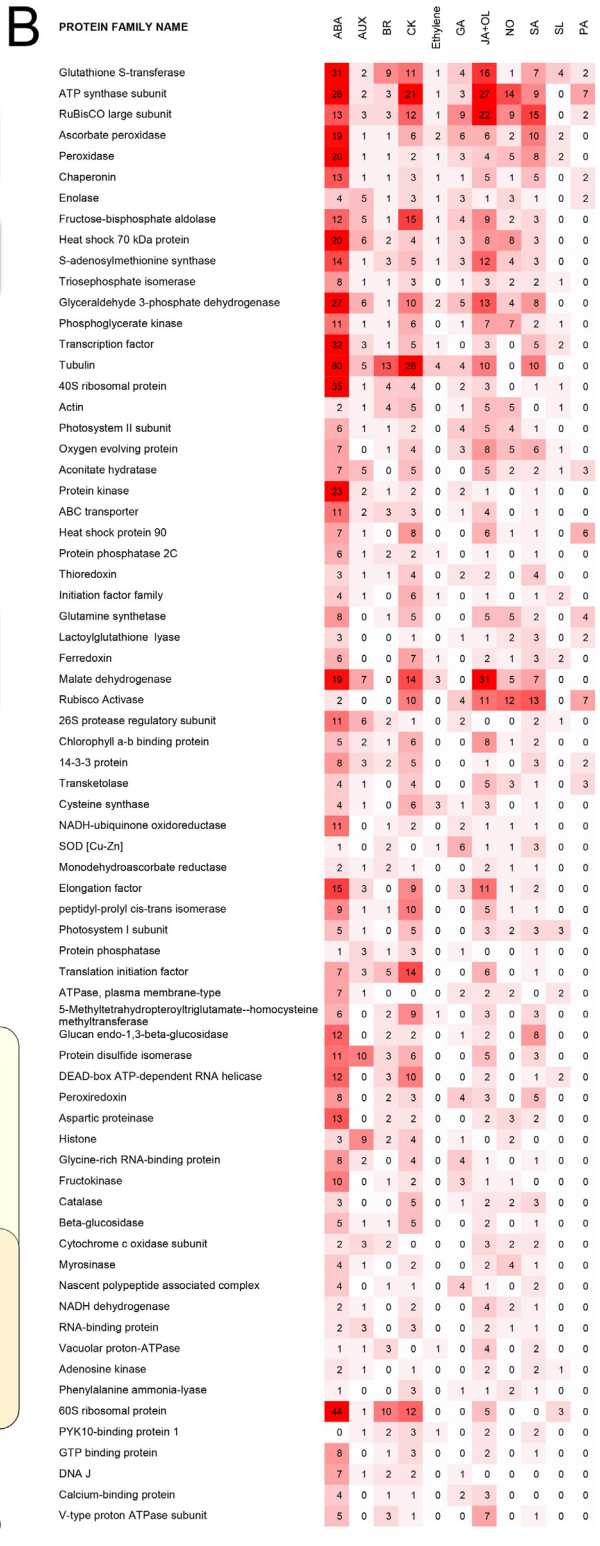

Fig. 2. Phytohormones and similarities in the response at the proteome level. (A) Localization and major functional categories of phytohormone-responsive proteins identified in *Arabidopsis*, and Venn diagram representation of the dataset. The color of each cellular compartment indicates the number of localized proteins; the font size corresponds to the size of the category for the respective hormones. Based on Supplementary tables, the SUBA 3.0 database [5] and MIPS classification (BioMaps) [6]. (B) Protein families found in response to phytohormone treatments. Based on data collected in Supplementary tables; only entries found in response to more than four substances are shown. The phytohormone abbreviations correspond to those in Table 1.

of information about proteoforms and PTM dynamics at the protein level. Furthermore, even the higher level of sensitivity does not make it possible to follow established phytohormone signaling cascades in an untargeted analysis. For instance, there are seventy known cytokinin metabolism genes (39) and cytokinin signaling genes (31) in *Arabidopsis*, but only four of these were found in phytohormone-responsive proteomics and none of them in response to cytokinin (Supplementary tables). The problem probably lies both in the low abundance of these proteins and in the experimental design, which is not optimized to capture such proteins.

3. Phytohormones, their signaling pathways and responses identified at the proteome level

In the following section, we describe the individual phytohormones and refer to the corresponding proteomic analyses.

3.1. Abscisic acid

Abscisic acid is a plant hormone with key roles in abiotic stress responses and dormancy regulation. In plants, it is synthetized from the C_{40} carotenoid zeaxanthin by a series of oxidation steps and it is inactivated by oxidation or conjugation with monosaccharides. There are three major components in abscisic acid signaling in *Arabidopsis*: the PYR/PYL/RCAR receptor, protein phosphatase 2C and protein kinase SnRK2. After binding the hormone, PYR/PYL/RCAR associates with phosphatase 2C and inhibits its activity, thereby allowing the activation of SnRK2 and the phosphorylation of ABF transcription factors (abscisic acid-responsive element binding factors) and activation of downstream signalization (for details see e.g. [7]). Abscisic acid signaling has been of major interest to proteome researchers. In total, there have been more than thirty proteome-wide analyses, including phosphoproteome profiling and redox proteome analysis [8–39]. Phosphoproteome analyses have identified substrates of SnRK2 [33,35] and large scale interactome analyses have indicated more than a hundred protein–protein interactions, illustrating the complexity of the abscisic acid signaling network[40,41]. Of particular interest are experiments describing early signaling events that occur 5–15 min after exogenous treatment with the hormone [22,29,33].

3.2. Auxin

Auxin, the first plant growth hormone to be discovered, influences a wide range of physiological processes, including embryogenesis, vascular differentiation, apical dominance, flower development and tropic responses. It is synthesized from tryptophan via a two-step pathway by transamination (tryptophan aminotransferase) and oxidative decarboxylation (YUCCA); the existence of an alternative, tryptophan-independent, pathway has been disputed [42]. Auxin can be deactivated by oxidation or conjugation, forming esters, amino acid or sugar conjugates. The major auxin signaling cascade requires the proteasome (Fig. 3). Auxin response factors (ARFs) are associated with AUX/IAA proteins that block their transcriptional activity. Once intracellular auxin is perceived by the TIR1/AFB1-3 receptor, AUX/IAAs are targeted for degradation[43]. An integral component of auxin signaling is the PIN protein that facilitates the transport of auxin across the plasma membrane and hence its distribution in the plant. Proteome analyses have focused on the long-term effects of auxin [44–48], but early (within hours) auxin-mediated changes have also been analyzed [49,50]. Probably the most comprehensive analysis employed 1 nM or 1 μM auxin for 6, 12, or 24 h and compared the dynamics observed in wild type plants with those in the auxin receptor-deficient mutant *tir1-1*. Phosphoproteomics singled out 20 proteins [51] differentially phosphorylated in response to auxin, though this number excludes some prominent proteins, such as PIN2, that were not found in two biological replicates.

3.3. Brassinosteroids

Brassinosteroids have been shown to mediate stress responses, and to regulate cell elongation and division, vascular differentiation, flowering, senescence, pollen development and photomorphogenesis. They are polyhydroxylated sterol derivatives, and thus structurally similar to animal steroid hormones. Because of this, most of the enzymes of brassinosteroid metabolism belong to the cytochrome P450 family, including those of both the biosynthesis and the inactivation of brassinosteroids [52]. The signaling cascade has been reviewed recently (see [53]). In essence, like that of cytokinin and ethylene, the brassinosteroid signaling pathway is a phosphorelay, with the receptor (BRI1 in *Arabidopsis*) being localized in the plasma membrane and endosomal membrane. Ligand binding leads to autophosphorylation of the receptor and association with the second kinase (BAK1). The resulting heterodimer then regulates gene expression via a chain of regulatory phosphorylation and targeted degradation steps. Proteomics have contributed to describing the signaling cascade by identifying phosphorylated sites in the BRI1 receptor [54] and revealing its protein–protein interaction with BAK1 [55]. Proteome-wide responses to brassinosteroids have been analyzed in brassinosteroid deficient mutants [56–58] and in wild-type plants treated with 10–10,000 nM brassinosteroids [59–62].

3.4. Cytokinins

Cytokinins are plant hormones involved in the regulation of diverse developmental and physiological processes including cell division, shoot initiation, apical meristem function, leaf senescence, nutrient mobilization, seed germination, vascular formation and root development. Naturally occurring cytokinins are adenine derivatives with an isoprenoid or aromatic side chain. The levels of active cytokinins in a cell are regulated by synthesis (phosphate-isopentenyl transferase), conjugation (glucosylation), or degradation [63]. The canonical signal transduction pathway is a phosphorelay similar to bacterial two-component response systems: a sensor hybrid histidine kinase phosphorylates histidine-containing phosphotransfer proteins which are then translocated into the nucleus, where they transfer the phosphate to type-B response regulators. The latter play roles in mediating transcriptional responses to cytokinin, including the rapid induction of negative regulators (type-A)[64]. Proteome-wide responses to cytokinin have been characterized using two alternative approaches — exogenous cytokinin treatment [16,62,65–70] and transgenic plants with conditionally increased/decreased levels of endogenous cytokinins [68,71–74]. An early response at the proteome- and phosphoproteome-level, occurring within 30 min after the cytokinin treatment, has been analyzed [65,69,70] and the results indicated minimal correlation with observed gene expression dynamics at the same time point. The study also showed a role for calcium ion signaling in cytokinin induced phosphoproteome dynamics and demonstrated that the degrees of involvement of individual cytokinin receptors in mediating proteome changes are consistent with previously reported physiological data. Several studies have found cytokinin binding proteins. The role of this interaction in cytokinin signaling is not clear, but in tobacco, these proteins include endochitinase and osmotin-like protein [75], implying a possible function in plant–pathogen interaction.

3.5. Ethylene

Ethylene, which participates in numerous physiological processes including growth, development and stress responses, can be produced by almost all parts of higher plants from S-adenosylmethionine through the activity of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase. Ethylene perception occurs at the endoplasmic reticulum membrane, mediated by the receptor, which is a negative regulator of ethylene signaling. In the absence of ethylene, the receptor (which is related to two-component histidine protein kinases) activates protein kinase (CTR1 in *Arabidopsis*) which phosphorylates, and thus targets for degradation, EIN2, preventing the stabilization of ethylene transcription factors. There have been several proteome-wide analyses of plant responses to ethylene, employing either gaseous ethylene [76,77] or the ethylene precursor ACC [45,78], and recently, protein–protein interaction analysis has contributed to our understanding of ethylene signaling by identifying an essential interaction between a conserved nuclear-targeting domain of EIN2 and the ethylene receptor [79].

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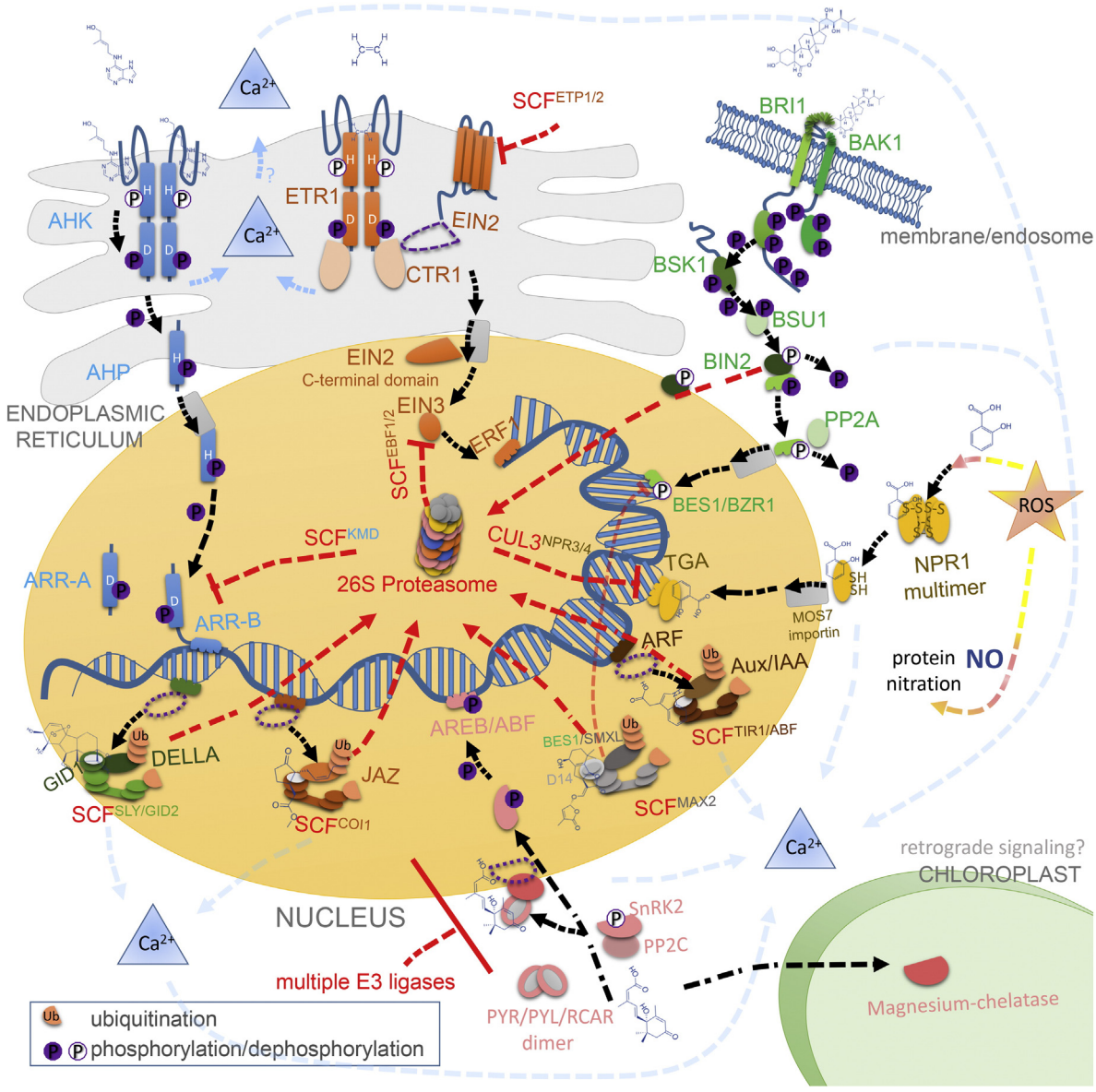

Fig. 3. Simplified overview of phytohormone perception pathways.

3.6. Gibberellins

The phytohormones of the gibberellin group are essential for growth and development, promoting seed germination, and modulating plant defense responses, elongation or reproductive development. The gibberellin signaling cascade includes the GID1 receptor, which upon gibberellin binding interacts with DELLA proteins (signaling repressors) and targets them for degradation via the ubiquitin–proteasome pathway (Fig. 3). Chemically, gibberellins are derived from a basic diterpenoid carboxylic acid skeleton that is converted to the bioactive hormones through a series of oxidation steps. Inactivation is catalyzed by gibberellin oxidation, epoxidation and methylation [80,81]. Gibberellins were first identified as the source of disease symptoms in rice infected by the fungus *Gibberella fujikuroi*, and for this reason, the majority of proteome-response data originate from analyses of *Oryza sativa* [21,82–87]. Gibberellins stimulate germination by counteracting abscisic acid. A number of proteome analyses have therefore characterized seed germination and some have included experiments comparing abscisic acid- and gibberellin-treated seeds [21,30,31]. In *Arabidopsis* proteomics, Gallardo et al. [88] compared wild-type imbibition to that of gibberellin deficient seeds and seeds with gibberellin biosynthesis blocked (by paclobutrazol treatment) [88].

3.7. Jasmonic acid and other oxylipins

Jasmonates are a subgroup of the oxylipins, molecules derived from polyunsaturated fatty acids. The first step in biosynthesis is predominantly catalyzed by lipoxygenase and, in contrast to animals, plant oxylipin precursors are mainly C16 and C18 fatty acids. Jasmonates and their close relatives 12-oxo-phytodienoic acid (OPDA), dinor-OPDA, traumatic acid and traumatin not only are well established wound hormones, but also play roles in, for example, seed maturation, pollen development or stomatal closure [89,90]. The best characterized oxylipin is jasmonic acid and its conjugates methyl jasmonate and jasmonyl isoleucine. Its signal transduction requires proteasome-mediated degradation of JAZ proteins (Jasmonate ZIM-Domain), which are negative regulators of jasmonate-responsive gene transcription (Fig. 3). The assembly of the JAZ protein complex was validated and further clarified by protein–protein interaction analysis employing tandem affinity purification followed by MS [91]. Jasmonic acid

signaling at the proteome level has been intensively studied in rice [92–95] and Brassicaceae [16,39,96–100];, another study included an iTRAQ analysis of proteins in *Brassica napus* guard cells after 120 min treatment with 50 μM methyl jasmonate. Although the gene expression profiles of jasmonic acid treated plants are for the most part different from those of plants treated with OPDA [101], a comparison of available proteome profiles showed that 14 out of 60 OPDA- and A1-phytoprostane-responsive *Arabidopsis* proteins [102], including five members of the 14-3-3 protein family, were also identified in the jasmonic acid response, (Supplementary tables).

3.8. Nitric oxide

Nitric oxide (NO) increases during stress responses, but it also acts during specific plant developmental processes including seed germination, photomorphogenesis, leaf and root growth, fruit maturation and senescence. In spite of its relevance as a growth regulator, our current knowledge about NO in plants is limited. The animal NOS gene family has not been found in the plant genome, but an alternative non-enzymatic pathway exists and several genes utilizing arginine or nitrite for NO production have been described [103,104]. The major feature identified in plant NO signaling is the post-translational modification of proteins by S-nitrosylation (cysteine residues) and nitration (tyrosine residues via peroxynitrite generated by a reaction between NO and oxygen). These modifications can alter enzyme activity or block the sites of regulatory PTMs and thus influence signaling pathways, including those of abscisic acid (nitrosylation of OST1 protein and SnRK2) [105,106], auxin (nitrosylation of TIR1 receptor) [107] and cytokinin (nitrosylation of type-A response regulators)[108]. Analyses of NO-induced proteome alterations can be divided into two groups. The first consists of protein nitrosylation/nitration analyses, including *in vitro* protein nitrosylation assays and identification of potential nitrosylation targets (e.g. [109–113]. These studies are of interest, though the biotin switch method employed in them does not allow the direct MS/MS validation of nitrosylated peptides and thus some previous reports of (potential) nitrosylation would benefit from further validation. The second set of experiments covers proteome-wide response to NO or NO-producing substances including polyamines [114–118].

3.9. Salicylic acid

Salicylic acid is best known as the hormone governing plant–pathogen interactions, but it is also involved in cell growth, senescence, germination and abiotic stress responses. It is produced by two pathways, in which the key enzymes are phenylalanine ammonia lyase and isochorismate synthase respectively. The majority of the salicylic acid that is synthesized is conjugated to an inactive sugar derivative. Alternatively, it can be converted to a methyl ester, which is possibly a long distance signaling molecule [119]. The salicylic acid signaling cascade triggers changes in cell redox potential that induce monomerization of the protein NPR1 and its translocation to the nucleus where it activates a transcriptional response. NPR1, its paralogs, and an increasing number of diverse proteins including enzymes in redox signaling and carbohydrate metabolism, have been found to bind salicylic acid [120]. Proteome-wide responses to salicylic acid have been studied in the model plant *Arabidopsis* [99,121–123], maize [124], rice [125], wheat [126,127], and a number of other species [128–133]. In response to infection, an increase in salicylic acid concentration causes programmed cell death. It should be noted that though there are some exceptions, most published studies employed a prolonged treatment with a relatively high salicylic acid concentration. This is likely to have resulted in a general stress response and at least some of the proteins reported as being salicylic acid-responsive could therefore be false positives.

3.10. Other growth regulators and phytohormones

Plant growth and developmental processes are controlled by numerous signaling molecules, including some that have only been recognized recently and whose functions and signaling mechanisms are poorly understood. These include strigolactone, terpenoid lactones derived from carotenoids, and the structurally related growth regulators the karrikins. There are two available proteomic analyses of strigolactone responses, but the number of identified strigolactone-responsive proteins is low in both cases: 38 *Arabidopsis* proteins identified by LC-MS [134] and 16 rice (phospho)proteins identified by 2-DE [135]. Similarly, there is only a single proteome-wide analysis of the response to karrikin [136]. The available data indicate roles for protein metabolism and proteasome mediated degradation, findings which are in agreement with the proposed signaling cascade. Recently, there has been progress in the analysis of peptides with functions in plant growth regulation and it has been shown that plants possess more than 1,000 genes encoding putative peptide ligands and over 600 putative receptor-like proteins. Peptide hormones play roles in a wide spectrum of processes (for details, see [137] and references therein) and though the proteomic approach is essential for peptide-hormone analysis, it has been mostly limited to identification of peptides and mapping of their putative receptors [138–141]. The true potential of the approach is illustrated by a recent phosphoproteome analysis of *Arabidopsis* seedlings treated for 5 min with a 1 μM concentration of the recombinant peptide RALF, which identified the RALF receptor and four downstream signaling components[142].

4. Central nodes in plant hormone signaling

4.1. Proteasome mediated degradation and the hunt for the ubiquitinome

Protein homeostasis in a cell is maintained by transcriptional and translational control, and also at the level of targeted protein degradation. The most frequent means of protein degradation in eukaryotic cells requires the ubiquitin-26S-proteasome system (UPS). Proteins are identified for targeted degradation via the attachment of a highly conserved 76-amino acid protein called ubiquitin. Modification of substrate proteins by ubiquitin is one of the major regulatory events in eukaryotic cells, and plants use this modification to react to exogenous and endogenous cues. More than 6% of the *Arabidopsis* genome encodes components of the ubiquitin conjugation system [143]. In effect, most plant regulatory circuitry, and many steps in the execution of processes, depend on ubiquitin modification. Ubiquitination is catalyzed through the sequential action of three discrete enzymes: the ubiquitin activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). The E3 ligase is responsible for substrate recognition and there is therefore a large group – more than 1000 proteins – of this family in *Arabidopsis*. These ligases can be divided into four groups according to the interaction domain they contain, with the cullin-RING domain family being the most important in plant hormone signaling pathways [144]. In the auxin signaling pathway, it is the SCF^{TIR1} complex which serves as E3 ubiquitin ligase to degrade AUX/IAA; $SCF^{SLY/GID2}$ recognizes DELLA proteins in the presence of gibberellins; the SCF^{COI1} complex mediates JAZ protein degradation in response to jasmonyl isoleucine; $SCF^{EBF1/EBF2}$ targets EIN3 and EIL1 for degradation in the absence of ethylene; SCF^{KMD} controls cytokinin signaling by regulating the degradation of type-B response regulators; SCF^{MAX2} is involved in strigolactone signaling, $CUL3^{NPR3/4}$ in that by salicylic acid, and abscisic acid signaling includes, among others, CRL^{BPM} [145,146]. Furthermore, many enzymes in pathways of phytohormone biosynthesis are targets of the UPS.

From this perspective, it should not be surprising that protein ubiquitination is of major interest in plant proteomics. In *Arabidopsis*, there have been several attempts to characterize the ubiquitinome [147–153]. Over 2800 proteins have been identified employing an

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anti-ubiquitin antibody, a ubiquitin-binding domain matrix or His-tagged ubiquitin. This number is relatively high, but it should be noted that fewer than half these proteins were found in multiple analyses and a large proportion of the reported proteins do not have MS/MS validation of the ubiquitination site, so they could be false positives or protein–protein interactors with ubiquitinated proteins. A comparison of hormone-responsive proteins (Supplementary tables) with the ubiquitinome set shows that 1014 (47%) of the former are potential targets of ubiquitination, with the highest numbers of candidates representing responses to abscisic acid (445 of 955; 47%), cytokinin (410; 59%), jasmonic acid (222; 74%) and brassinosteroids (174; 46%).

4.2. Phosphorylation and the phosphoproteome in response to plant hormone treatment

Transient phosphorylation is the key mechanism in cytokinin signaling, and regulatory phosphorylation is also required for the brassinosteroid and abscisic acid signaling pathways. Moreover, phosphorylation often precedes protein ubiquitination in the ubiquitin-dependent degradation process. It is estimated that the number of potential phosphorylation sites in plant proteins is approximately three times the number of protein-coding genes and one-third of expressed proteins are assumed to be phosphorylated at any given time. This requires about 1100 protein kinases and 100 to 200 protein phosphatases in *Arabidopsis*. There are seven amino acids which can undergo phosphorylation (Ser, Thr, Tyr, His, Lys, Arg, Asp) but the stability of acyl-phosphorylation and of the phosphate-nitrogen bond is low and thus phosphorylations involved in the cytokinin or ethylene signaling phosphorelays are not detected in proteome-wide analyses. Phosphoproteome profiling has identified only 540 (9%) of the hormone-responsive proteins [16,18,22,29,33,35,37,57,65,69,76,135]. Although hormone signaling is not necessarily the only cause of phosphorylation, it should be noted that the majority of reported phytohormone responsive proteins are targets of phosphorylation. In total, 1180 out of 2157 *Arabidopsis* proteins found to be responsive to hormonal treatment (Supplementary tables) are listed in the phosphoproteome database (PhosPhAt 4.0; [154]).

4.3. Calcium ion signaling

Vacuoles and the lumen of the endoplasmic reticulum are major Ca^{2+} stores in plant cells, and the cytosolic calcium concentration is maintained in the nanomolar range by ATP-dependent pumps and calcium/proton antiporters. A transient flux of calcium ions into the cytoplasm represents a rapid regulatory mechanism and one which is reportedly involved in the transduction of diverse abiotic, biotic and developmental stimuli including temperature, light, pathogen attacks, drought and high salinity [155]. It activates a number of regulatory proteins in the cytoplasm by binding to Ca^{2+}-sensing proteins such as calmodulin, kinases, or phosphatases, but it also transfers signals between organelles. For example, it has long been established that chloroplast-localized physiological processes are subject to regulation by Ca^{2+}, and a Ca^{2+}-sensing receptor has been localized to the chloroplast and found to modulate cytoplasmic Ca^{2+} concentrations [156,157]. In the nucleus, transient nuclear Ca^{2+} events (whether autonomous or of cytoplasmic origin) stimulate gene expression and are required for, for example, sphingolipid-induced programmed cell death and the perception of symbiotic signals [158]. Like proteasome-targeted degradation, calcium ion signaling seems to be a shared node in phytohormone signaling. In brief, perception of brassinosteroids and of auxin causes an elevation in cytosolic Ca^{2+} concentration and initiation of a signaling cascade [159,160]. Several calcium signaling proteins are also involved in abscisic acid signaling via SnRK2s and abscisic acid-responsive element-binding proteins/factors [7], and calcium-dependent protein kinase functions in abscisic acid signaling under drought stress [161]. Cytokinin-mediated protein phosphorylation was abolished in *Arabidopsis* seedlings pretreated with calcium channel blockers [69]. Further, jasmonic acid induces transient Ca^{2+} signals in both the cytosol and the nucleus of a stimulated transgenic tobacco cell culture [90]. Calcium is also required for a variety of ethylene-dependent processes and ethylene seems to mediate cross-talk between calcium-dependent protein kinase and MAPK signaling [162,163]. Gibberellins induce an increase in cytosolic calcium and several calcium-dependent protein kinases have been found to act downstream of gibberellin signaling [164,165]. Last but not least, salicylic acid induces extracellular superoxide generation followed by an increase in cytosolic calcium ion concentration in tobacco suspension culture; and calcium/calmodulin regulation plays a role in salicylic acid-mediated plant immunity responses [166,167]. The importance of calcium signaling in phytohormone signaling is also reflected in the *Arabidopsis* phytohormone-responsive protein set. This set contains 34 calcium signaling proteins and more than 50 calcium-binding proteins, with the highest number being found in abscisic acid (39), cytokinin (29) and jasmonic acid (20) responses (Supplementary tables). Of these proteins, the only one found in response to more than three different phytohormones is PCaP1 (ABA, BR, CK, SA), a protein that interacts strongly with phosphatidylinositol 3,5-bisphosphate and calmodulin in a calcium-dependent manner.

4.4. Redox homeostasis in phytohormone signaling

The maintenance of redox homeostasis is an important factor in cellular metabolism and its disruption plays a role in signal transduction. The key components are reactive oxygen and nitrogen species (ROS/NOS), which were earlier considered to be only harmful waste products of aerobic metabolism. These radicals, in synergy with antioxidant enzymes and low molecular weight antioxidants such as ascorbic acid, tocopherols or glutathione, function in the cellular signaling hub that integrates internal and environmental information to control and modulate plant growth, development and responses to stress. For example, NADPH oxidase-dependent ROS production induced by abscisic acid plays an important signaling role in the process of stomatal closure under drought conditions [168], and the monomerization of the NPR1 salicylic acid receptor requires a change in redox homeostasis (Fig. 3). It is not only the stress-related hormone pathways involving salicylic, abscisic and jasmonic acids or ethylene that are interconnected with the production of, and/or crosstalk with, reactive species. It is known that gibberellin induces ROS-mediated programmed cell death in barley aleurone [169]; ROS were shown to mediate auxin-regulated root gravitropism [170]; and the oxidation state of root cells, regulated by auxin distribution, is thought to arrest the cell cycle in the quiescent center [171]. From the proteome-wide point of view, ascorbate peroxidase and glutathione S-transferase and peroxidase were found at least once in all reported hormone-responsive proteomes (Table 2) and catalases, peroxiredoxins, disulfide isomerases and thioredoxins also occur at high frequencies. The last of these, thioredoxins (Trx; EC 1.8.1.8), are ubiquitous small regulatory disulfide proteins, which catalyze the reduction of disulfide bonds. In plants, Trxs constitute six distinct families with subcellular localizations in plastids, mitochondria, nucleus and cytosol [172]. Trxs have been recognized as regulators of the day/night switch in metabolism and especially in the light-dependent regulation of the Calvin–Benson cycle. However, they also seem to participate in protein targeting, cell-to-cell trafficking and gene regulation [173]. Trx-encoding genes and Trx proteins have been recognized as responding to the plant hormone cytokinin [74,174], but they were also found in auxin-, abscisic acid-, brassinosteroids-, salicylic acid- and jasmonic acid-responsive proteomes (Supplementary tables). In addition to these enzymes, there is the family of NADPH oxidases, RBOH (respiratory burst oxidase homolog), whose activity is regulated by small GTPases (auxin response mediators), calcium ions [175] and the hormones ethylene and the brassinosteroids [176,177]. Besides their roles in phytohormone signaling, ROS are also involved in the

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Table 2
Phytohormone concentration in physiological and proteome analyses.

Hormone	Physiological effect	Phenotypic change	Species	Proteome analyses
Auxins (IAA)	10–100 nM	Root length	*Arabidopsis* [185]	0.25–100 μM
Abscisic acid	10 nM	Transpiration rate	Maize [186]	1–100 μM
Brassinosteroids (brassinolide)	10–100 nM	Root length	*Arabidopsis* [187]	0.1–10 μM
Cytokinins (tZ)	10 nM	Root length	*Arabidopsis* [188]	1–100 μM
Ethylene	100 nL/L	Root length	*Arabidopsis* [185]	36–100 μL/L
Gibberellins (GA4)	10 nM	Hypocotyl length	*Arabidopsis* [189]	0.1–100 μM

regulation of phytohormone metabolism and translocation (reviewed in [178,179]).

5. Why is it so hard to decipher phytohormone signaling mechanisms?

For the last two decades, "omics" have been extensively utilized in phytohormone research. Despite this, though the central elements in signal transduction pathways have been identified, the mechanisms controlling their outputs remain to be elucidated. There are two major reasons for this. The first is the sheer complexity of phytohormone signaling. As illustrated in Fig. 4, a phytohormone effect is specific to a particular developmental stage and tissues. For practical reasons, analyses, including the majority of published phytohormone proteomics, focus on the whole seedling/plantlet, or on only the upper/lower part of the plant (Fig. 4, Supplementary tables). The detail of spatial distribution is thus completely neglected and the resulting proteome/transcriptome snapshot cannot easily be used to reconstruct the signaling pathway. The second reason is the extent of variability and system perturbations that result in low reproducibility and the detection of false positives/negatives. For example, plant responses to cytokinin have been extensively studied at the transcriptional level and the number of unique cytokinin-regulated genes identified in just a few recent studies is nearly 6000 [174,180,181]. In the last ten years, similar experimental set-ups have reported around 10,000 unique *Arabidopsis* cytokinin-responsive genes. This is clearly a huge overestimate, and the size of the overlaps indicate that these experimental designs do not allow us to distinguish between signaling-related events and any response that originates solely as the consequence of altered growth or perhaps even due to experimental error. In this respect, it should be noted that the two proteins found in the largest number of phytohormone-response proteome analyses are an ATP synthase subunit and ribulose bisphosphate carboxylase. These proteins are highly abundant in plant total-protein extracts, usually being represented among the top ten most abundant proteins. Though they may serve as signaling hubs and scaffolds for protein–protein interactions, it may also be possible that their presence indicates potential bias in the proteomic data.

5.1. The most problematic aspect of phytohormone-responsive omics — Is there a skeleton in the closet?

The major problem with omics data is the validation of reported datasets. Gene expression data can be validated by western blotting or targeted proteomics, but this does not necessarily work vice-versa. For example, PTM regulation results in a significant change in the pattern of a protein spot in 2-DE without there being any change in the profile of the corresponding transcript. If the protein of interest is a metabolic enzyme, its phytohormone response can be validated from changes in corresponding metabolites. Mutant and transgenic lines can be constructed, but for an analysis of tens of candidates this is not easily done. Moreover, the role of PTM is usually not clear and thus even a null mutation may not always produce the expected response. Overall, phytohormone-(prote)omic analyses have highlighted pathways of interest but validated only a small fraction of the data reported.

The second potential problem may lie in the experimental set-ups used. *In planta*, most hormones act at low nanomolar concentrations to maximize the efficiency of signaling cascades. Even though the uptake of an exogenously supplied hormone may be limited, visible macroscopic changes are detectable following a prolonged exogenous treatment within a similar concentration range (Table 2). Surprisingly, proteomic analyses have mostly employed concentrations three to four orders of magnitude higher (median 100 μM), even for long-term phytohormone treatments extending over periods of days. It is well-known that supraoptimal phytohormone concentrations have negative impacts on plant cells. For example, highly increased levels of endogenous cytokinin resulting from ectopic expression of bacterial *ipt* were reported to cause oxidative stress followed by total collapse of whole tobacco plants [182]. The lethal concentration of cytokinin in a cell culture is in the micromolar range: 30 μM cytokinin caused death of almost 30% of cells of the tobacco BY-2 culture within 24 h [183]. A similar dose–response pattern of induced cell death was also documented in the case of exogenous application of gibberellin to barley aleurone (5 μM) [169] and a 150 μM concentration of the ethylene precursor ethephon caused death of rice epidermal cells within hours [184]. Moreover, auxin analogues are commercially used herbicides, including the infamous defoliant Agent Orange. In conclusion, it is possible that some reported similarities between individual phytohormones at the proteome, phosphoproteome and even the transcriptome level are hormone-independent signs of a general response to toxicity and/or programmed cell death.

6. Summary and future perspectives

The completion of many genome sequencing projects and the availability of genome arrays have led to an increase in interest in transcriptome profiles. However, it has rapidly become obvious that the correlation between the amount of a mRNA and the amount of its protein product is not always clear. In fact, comparative analyses indicate that probably only changes in the amounts of proteins with high turn-over rates can be estimated from transcript levels. Difficulties associated with proteome analysis have not hindered its application in phytohormonal research. Since the first proteome-wide experiments in the early 2000s, the number of analyses has been steadily rising hand-in-hand with improvements in proteomic platforms. Indeed, more than 70% of all proteome-wide studies have been published in just the last five years. The fact is that proteome analysis has provided valid data to complement that from transcriptomics, be it thanks to data about PTMs or about regulation of protein turnover. For example, besides PTM-based signaling pathways like that of nitric oxide, the established model of phytohormone signaling neglects the paramount role of plastids in the life of the plant cell. With the exception of abscisic acid, signal transduction to chloroplasts has not been described. In this context, proteomic data clearly highlight the role of chloroplasts, as some 20% of phytohormone-responsive proteins identified in *Arabidopsis* are chloroplastic, including those found in the first minutes

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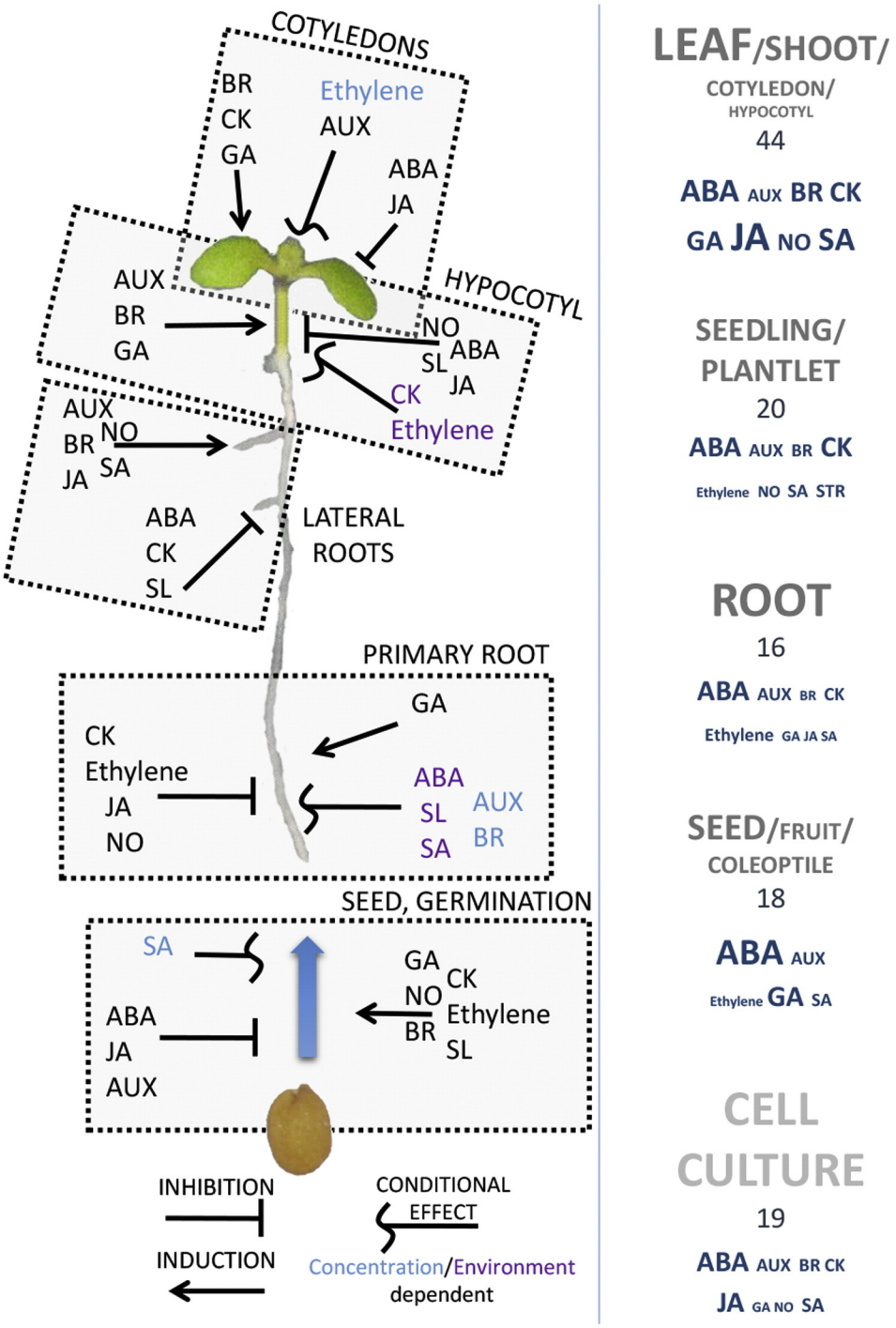

Fig. 4. Spatial effect of phytohormones and overview of tissue-specific proteome analyses. Numbers indicate the number of analyses; the font size corresponds to the contribution made by a given tissue and phytohormone.

after hormonal stimuli, and thus the data present evidence that this signaling could correlate with ROS and calcium signaling (Fig. 2, Supplementary tables). The future of phytohormone-responsive proteome analysis lies both in a more targeted approach including an increased focus on cellular compartmentalization, and in advanced PTM analysis. Modifications such as glycosylation or acetylation have not yet been analyzed on a large scale, but they are very likely to play roles in phytohormone responses and/or in the regulation of hormone biosynthetic pathways. Protein–protein interaction analysis is also a highly promising technique that has already proved useful in resolving several key points in signaling cascades. In addition to the results detailed in Section 3, recent evidence shows that it can reveal signaling hubs connecting different hormones [190]. This technique, if further optimized to capture even the weaker interactions, will be a valuable asset in the next generation of phytohormonal proteomics. Similarly, mass spectrometric imaging has huge potential (for reference see e.g. [191]). Its present application is mostly limited to small molecules but, if further developed, it could provide the spatial resolution that is so badly needed in plant proteome research.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbapap.2015.12.008.

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Transparency document

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

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SEED PROTEOME ANALYSIS AND PROTEOME DYNAMICS DURING SEED GERMINATION

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Abstract: Despite the huge progress that has been made in the last decade, the molecular mechanisms regulating seed germination and early seed development are far from being resolved. Induction of metabolic genes involved in germination starts around 12 hours after imbibition. Thus, most of the early events are mediated by molecules stored in the seed during maturation and are not accessible to transcriptomic analyses. Proteome analysis has been extensively employed in the past but the coverage of observed seed proteome is relatively low even in present-day high-impact studies. Here, we analysed proteome of two model species, *Arabidopsis thaliana* and barley (*Hordeum vulgare*). We employed several complementary approaches to increase the proteome coverage and build a library suitable for targeted protein quantitation. The combination of fractionations and an alternative MS/MS data processing significantly improved our detection limits. Our results indicate that the seed proteome coverage is limited not only by extraction efficiency or depletion of abundant proteins, but also by an inadequate spectral data interpretation.

Key Words: seed proteomics, mass spectrometry, proteome fractionation

INTRODUCTION

Germination is a crucial process which affects viability and productivity of plants. In terms of physiology, germination is quite well described. However, in terms of molecular biology it still remains unclear. Germination is defined as a three-phase process which begins with intensive water intake and which is ended by testa and endosperm rupture. The first phase of germination of non dormant seeds is characterized by metabolism activation after achieving approximately 60% of hydration. Induction of metabolic genes involved in germination starts around 8 hours after imbibition (Rajjou et al. 2012). Thus, most of the early events are mediated by molecules stored in the seed during maturation and are not accessible to transcriptomic analyses. Therefore, proteomic analysis should be the method of choice to understand molecular mechanism regulating seed germination and early seed development.

Proteome analysis has been extensively employed in the past but the coverage of observed seed proteome is relatively low even in present-day high-impact studies. Moreover, genome of majority of agriculturally important crops has not yet been fully sequenced and thus the databases for proteome annotation contain only small sets of well validated proteins. The techniques to increase the proteome coverage are available, but usually not optimized. Here, we tested two different approaches to reach a higher proteome coverage: (i) proteome fractionation and (ii) in silico reprocessing of HRMS (high resolution mass spectrometry) data.

MATERIAL AND METHODS

Plant material, cultivation and total protein extraction

Seeds of *Arabidopsis thaliana* (Col-0) and *Hordeum vulgare* (variety Sebastian) were imbibed with distilled water and harvested after 0-24 h of imbibition, frozen in liquid nitrogen and homogenized using a Retsch Mill MM400. The total protein was extracted by acetone/TCA/phenol extraction as described previously (Černý et al. 2013). In brief, homogenized tissue was extracted overnight with 10% (w/v) TCA in acetone (2 ml, -20°C), washed with 10% (w/v) TCA in distilled water then 80% (v/v) acetone, resuspended in 0.8 ml SDS buffer [2% (w/v) SDS, 30% (w/v) sucrose, 5% (v/v)

β-mecraptoethanol, 5 mM EDTA, 100 mM Tris, pH 8.0], and protein was extracted by 0.4 ml buffer-saturated phenol. Phenolic phase was collected and protein was precipitated overnight in 1.6 ml ice-cold 100 mM ammonium acetate in methanol (-20°C). Protein pellets were washed with 1.0 ml 80% (v/v) acetone in distilled water, dried and stored at -80°C until used.

Off-Gel Fractionation

Barley total protein extracts prepared as described above were dissolved in OFFGEL Stock Solution (thiourea 2 mM, DTT 60 mM, 10% (v/v) glycerol, ampholytes pH 3-10), loaded into wells with 24 cm IPG strips (pH 3-10, nonlinear) and processed according to the manufacturer's instructions (Offgel Fractionator 3100, Agilent). The resulting fractions were collected and then digested in solution with trypsin.

LC-MS analysis

Arabidopsis samples were prepared as described previously (Baldrianová et al. 2015). In brief, dried protein pellets were dissolved in 100 mM NH_4HCO_3, 8 M urea (400 µl). The protein concentration was estimated by the Bradford assay (Sigma-Aldrich), samples were diluted with acetonitrile in 100 mM NH_4HCO_3 to the final concentration 5% acetonitrile, 2M urea, 50 mM NH4HCO3 and subjected to in-solution digestion with immobilized trypsin beads (Promega; 3 µl beads per 100 µg of protein) at 37°C overnight. The resulting peptides were desalted (SPEC plate C18, Agilent), dried and dissolved in 0.5% (v/v) formic acid in 5% (v/v) acetonitrile, then analysed online by nanoflow C18 reverse-phase liquid chromatography using a 15 cm Ascentis Express Column (0.1 mm inner diameter; Sigma-Aldrich) and a Dionex Ultimate 3000 RSLC nano UPLC system (Thermo) directly coupled to a nanoESI source CaptiveSpray and an UHR maXis impact q-TOF mass spectrometer (Bruker). Peptides were eluted with a 120-min, 4% to 35% acetonitrile gradient (Novák et al. 2015).

Data processing

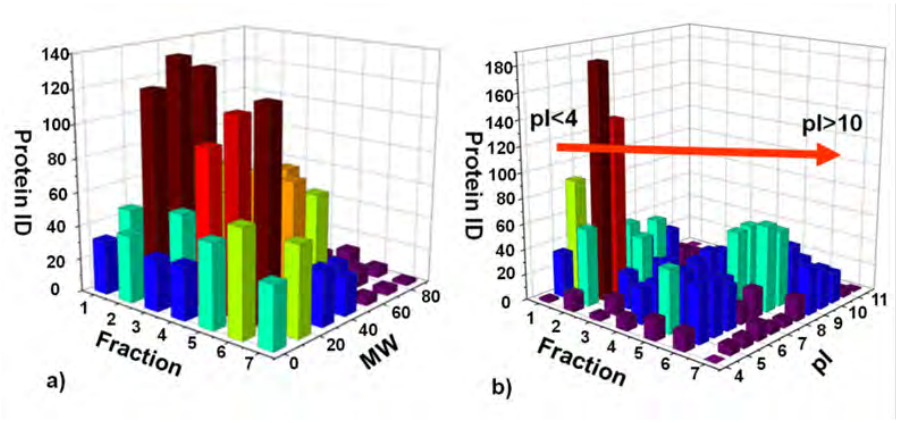
Peptide spectra were preprocessed with DataAnalysis (Bruker) and searched against barley and *Arabidopsis* TAIR10 protein databases using the Mascot algorithm and Bruker's ProteinScape inbuilt percolator algorithm (target FDR<1%). Skyline, Search GUI (1.30.1) and Peptide Shaker (0.41) (Vaudel et al. 2011) were used for a further spectra analyses and processing.

RESULTS AND DISCUSSION

Off-Gel separation of barley grain proteins

There are several suitable methods of proteome fractionation. Seeds are formed to provide nutrition for the embryo, thus the storage compounds (including storage proteins) represent the greatest portion of a seed's mass. The abundant storage proteins complicate analysis and interfere with the detection of lower abundant proteins. The fractionation on tissue-level (e.g. microdissection) is possible, but very demanding. Further, for some species, including *Arabidopsis*, the methodology does not allow rapid harvest of a sufficient amount of material for protein extraction. A more accessible is the fractionation on the protein level. The most common is the use of liquid chromatography or protein electrophoresis. The Off-gel fractionation method is an electrophoretic method based on isoelectric focusing and enables separation of proteins in a solution. Here, 1 mg of barley seed protein was fractionated into 12 fractions, but only seven fractions had a sufficient amount of protein for a further analysis (>100 µg). Fractions were analysed via LC-MS and MS/MS spectra were searched against barley database. The analysis of the theoretical molecular weight (MW) showed that the majority of proteins in all fractions have MW between 10 to 50 kDa (Figure 1a). The distribution of theoretical isoelectric points illustrates the efficiency of the separation and indicates a presence of proteoforms and/or post-translational modifications (Figure 1b). In accordance, there was a significant overlap between proteins identified in individual fractions. In total, 951 (a high confidence gene model version) and 561 (a low confidence gene models) were identified. This represented some 30% increase compared to the standard shot-gun approach. Though the peptide-based fractionation methods like strong cation exchange chromatography (SCX) would have better fractionation efficiency, the Off-gel separation retains the information about different proteoforms in the sample.

Figure 1 Distribution of proteins according to molecular weight (a) and isoelectric point (b) after Off-Gel separation



Advanced processing of mass spectrometry data

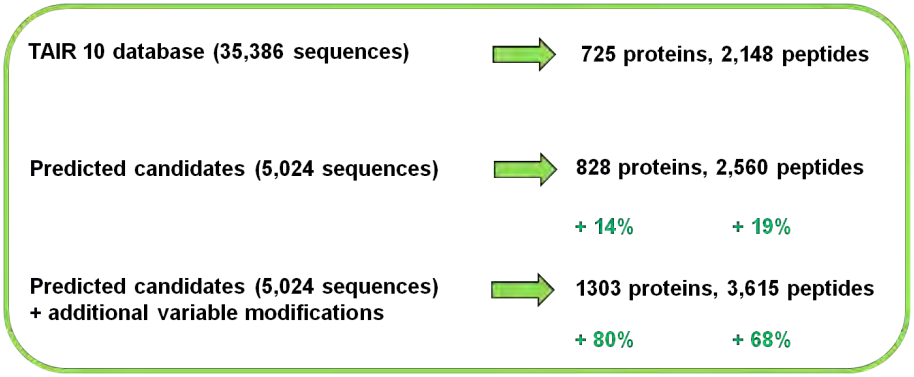
Proteomic analysis of barley seeds rely on still poorly annotated genome. Therefore barley is not the perfect candidate for digging in MS/MS spectra, increasing variable modifications and pin-pointing new peptide spectral matches. However, in the case of model plant *Arabidopsis thaliana*, we can use the available bioinformatics to increase the number of identifications in complex sample. *A. thaliana* seeds were prepared as is described in Material and Methods. Classical shotgun analysis followed by a gold-standard Mascot search engine resulted in identification of 1,450 proteins (the summary from several analyses). However, the Skyline analysis indicated that the 4,899 peptides used for protein identification by Mascot can be assigned to more than 7,000 known proteoforms in *Arabidopsis* proteome. These proteoforms correspond to 5,752 unique genes and 5,024 of them contain unique proteotypic peptides (Table 1). In theory, all these proteins could be present in the sample and they should be excluded only if there is no evidence of their proteotypic peptides.

Table 1 MS data post-processing

Number of detected proteins (peptides)	Search Algorithm
1.450 (4.899)	MASCOT
7.038	proteoforms
5.752	unique genes
5.024	proteins with proteotypic peptides

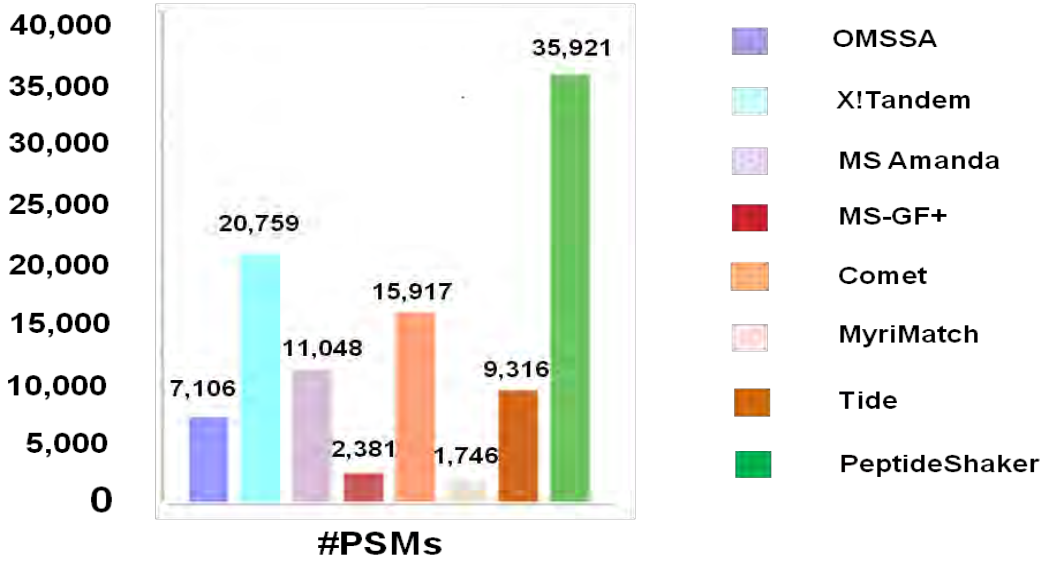
To test this theory, we used the sequences of these proteins as the Mascot database. As the number of detectable proteins is limited by the size of the reference database, by decreasing its size by more than 80% we were able to detect 828 proteins in a single analysis (14% increase). Moreover, we increased this number even further when we included additional variable modifications into the search parameters (1303 proteins identified) (Figure 2).

Figure 2 Backward analysis with database consisting of predicted candidate proteins as a useful approach to increase number of detectable proteins



As the next step, we complemented Mascot results with that of seven alternative search engines and the resulting data were combined in PeptideShaker. In total, we reached almost 36,000 peptide spectral matches (PSMs) (Figure 3).

Figure 3 Combination of data resulting from different search algorithms provides increasing number of peptide spectral matches (PSMs)



CONCLUSION

Seed proteomic analysis is a promising tool to study the molecular mechanism regulating seed germination and early seed development. However, seed proteome analysis is still difficult and limited by many obstacles. Here, we show the benefits of fractionation and bioinformatics in analysis of barley and *Arabidopsis* seed proteome. Our data will serve as the protein library and will be used for a targeted proteomic analysis of seed germination.

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Effects of plant growth regulators on proteome dynamics during seed germination

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Abstract: Seed germination is a complex process during which an embryonic plant contained within a seed transforms into a seedling. The majority of species, including model plant *Arabidopsis thaliana* produce dry seeds that will fully re-establish metabolism after imbibition. This early phase of a plant's life is highly important for its survival and its progress depends on both internal and external stimuli. This thesis entitled „Effects of plant growth regulators on proteome dynamics during seed germination" is focused on effects of light and plant growth regulators on germination. To get an insight into molecular mechanism of germination, proteome dynamics in germinating *Arabidopsis* were investigated. Seeds were imbibed in presence of plant growth regulators in continuous light or dark and material for LC-MS proteome profiling was harvested after 24 and 48 hours. Altogether, abundances of more than 1100 proteins were followed. PCA analysis and a detailed pair-wise comparison of mock-treated seeds provided evidence that the modern proteome-wide analysis is a promising tool for plant germination research.

Key-Words: - seeds, growth regulators, proteomics, mass spectrometry

Introduction

Germination is a crucial phase of plant life which is influenced by many external and internal factors. These stimuli may affect a seedling's vitality and even have an impact on the adult plant yield. Among others, external environmental stimuli include the light intensity, water quantity, temperature or pH. The internal stimuli are more complex and include DNA integrity, damage to cellular structures or levels of individual plant hormones. Internal and external factors interact with each other during germination and create highly complicated signaling networks. It is crucial to understand particular processes and their links during germination not only for the basic research but also for the optimization of sustainable agricultural and horticultural practice [1].

At least part of the seed germination is regulated on protein level. For example, DELLA proteins must be ubiquitinated and degraded to promote the germination onset. There are many obstacles in seed proteome analysis. For example, seed contains a large portion of storage proteins that interfere with the detection of lower abundant proteins and limit the total number of detectable proteoforms. Still, proteome analysis is an important source of new data. Here, we show that the state-of-the-art modern proteomics offer new possibilities to comprehend the molecular mechanism of plant germination.

Material and Methods

Cultivation and hormonal treatment

Seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) were surface-sterilized (using 75% ethanol) and sown on a Whatman filter paper rinsed with distilled water supplemented with DMSO (0.01% v/v) or selected growth regulator (abscisic acid, ABA; gibberellin GA3; cytokinin trans-zeatin, tZ; auxin indole-3-acetic acid, IAA; karrikin KAR1) to the final concentration of 1 μM .

Table 1 Scheme of the experiment

	light	darkness
24h	IAA, tZ, ABA, GA3, KAR, DMSO	IAA, tZ, ABA, GA3, KAR, DMSO
48h	IAA, tZ, ABA, GA3, KAR, DMSO	IAA, tZ, ABA, GA3, KAR, DMSO
dry seeds	-	-

The seeds were cultivated at 20°C for 48 hours at continuous light (80 μmol m^{-2} s^{-1}) or covered with aluminium foil to simulate germination in the

absence of light. Germinating seeds were sampled in two aliquots after 24 and 48 hours. In total we prepared 26 samples including control aliquot of dry seeds (Table 1).

LC-MS proteome profiling

Proteomic analyses were performed using a gel-free shotgun protocol based on nano-HPLC and MS/MS [2]. Briefly, proteins were extracted by a combination of acetone/TCA and phenol extraction then digested in solution with endoproteinase Lys-C and immobilized trypsin beads (Promega). The resulting peptides were desalted, dried and dissolved in 0.5% (v/v) formic acid in 5% (v/v) acetonitrile, then analyzed online by nanoflow C18 reverse-phase liquid chromatography using a 15 cm Ascentis Express Column (0.1 mm inner diameter; Sigma-Aldrich) and a Dionex Ultimate 3000 RSLC nano UPLC system (Thermo) directly coupled to a nanoESI source CaptiveSpray (Bruker) and an UHR maXis impact q-TOF mass spectrometer (Bruker). Peptides were eluted with a 60-min, 4% to 35% acetonitrile gradient. Raw files obtained from the MS analysis were analyzed by Profile Analysis 2.1 (Bruker) and MS precursors with significant differences (absolute ratio ≥1.5, with t-test p-values <0.05) were targeted and identified in consecutive MS/MS analyses. Peptide spectra were searched against the TAIR10 Arabidopsis database using the Mascot algorithm and Bruker's ProteinScape inbuilt percolator algorithm (target FDR<1%). Only high confidence peptides (p<0.05) with better than 10 ppm precursor mass accuracy and at least one distinct proteotypic peptide per protein met identification criteria. Quantitative differences were further manually validated by comparing respective peptide ion signal peak areas in Skyline 1.4 (MacCossLab Software; available on https://skyline.gs.washington.edu).

Data analysis

Information about protein/metabolite function(s) was collected from the UniProt database, (http://www.uniprot.org/) UniGene database (http://www.ncbi.nlm.nih.gov/unigene), TAIR database (http://www.arabidopsis.org), a conserved domains search (http://www.ncbi.nlm.nih.gov/Structure/index.shtml), a homology search (http://blast.ncbi.nlm.nih.gov/Blast.cgi), Kyoto Encyclopedia of Genes and Genomes (http://www.kegg.jp/kegg/), GeneVestigator (NEBION AG, http://www.genevestigator.com; Hruz et al., 2008), and literature.

Results and Discussion

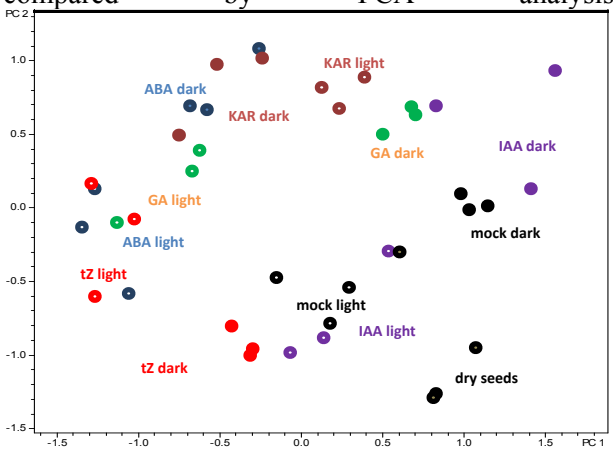
Plant material was prepared and harvested as described in Materials and Methods. Three time points were selected that correspond to dormant seed, phase I and phase II of germination [3, 7]. It is well known that plant growth and development is regulated by substances designated as plant hormones or plant growth regulators [4]. The interplay of these substances is also crucial for timing and progress of germination. Here, we followed effects of four major hormones and one recently discovered growth regulator originating from smoke of the burning vegetation.

Comparison of proteome-wide changes by Principal Component Analysis

Principal Component Analysis (PCA) is a statistical method that can be used to cluster samples according to the distribution of their characteristics. In this case, the raw data from LC-MS analyses are processed and intensity values for distinct m/z within a specific retention time window (buckets) are calculated. These values are than used for PCA analysis. Most of the buckets originate from protein digestion, but even those that are of non-protein origin can be used to characterize sample. The visualization of PCA results is presented in Figure 1.

The biplot in Figure 1 explains more than 40% of system variance and (with certain level of confidence) can be used to draw some preliminary conclusions about similarities and differences between individual treatments. For example, we can conclude that the sample treated with cytokinin and cultivated in dark has similar characteristics to the mock-treated sample cultivated in light. This would indicate that cytokinin treatment at least partially compensates the absence of light in germinating seeds.

Fig. 1 Proteome of germinating Arabidopsis seeds compared by PCA analysis

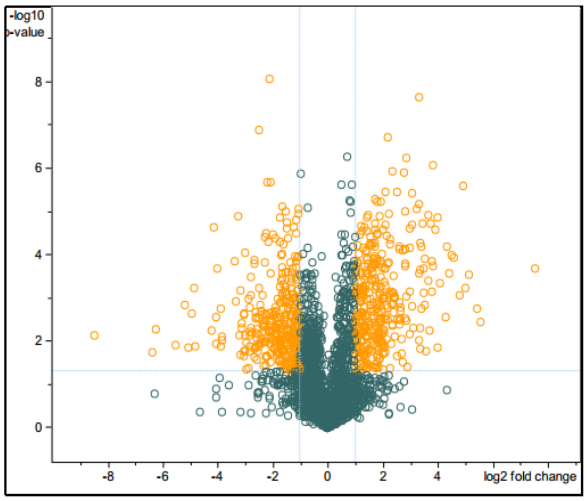


Even though the PCA analysis presented only a very simplified view of complex germination process, it outlines the benefits of proteome analysis and clearly demonstrates the power in proteomics approach to study seed germination.

Pair-wise comparison

To get more in-depth results, samples were compared in a pair-wise comparison. Here, mock treated samples will be discussed. T-test analysis

Fig. 2 Volcano plot representing differentially abundant buckets found in proteome of mock treated seeds germinating in light or dark.

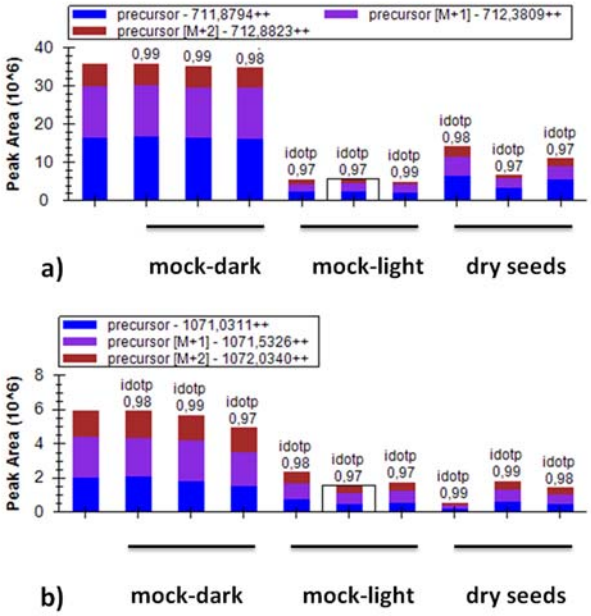


revealed that germination in the absence of light has a significant effect on a large number of buckets (Figure 2). In numbers, 1565 out of 2876 showed significant ($p<0.05$, absolute ratio <2.00) differences. The following automatic assignment of MS/MS spectra identified 2858 peptides that represented more than 1100 identified and quantified proteins.

Dark induced accumulation of Aspartate protease APA1 during seed germination

Proteome analysis revealed (among others) accumulation of protease APA1 in dark-grown *Arabidopsis seeds.* This protease is apparently involved in the breakdown of propeptides of storage proteins in protein-storage vacuoles and is supposedly activated during senescence and in response to light [5, 6]. In our experiments, APA1 levels in light grown seeds are similar to that of dry seed (Figure 3). Germination in the absence of light induces accumulation of APA1 and thus it is likely that this enzyme could be important in processes preserving seed viability prior the onset of autotrophic metabolism.

Fig. 3 Graphs shoving peak areas of two regulated peptides of APA1 protein. The first one (a) shows the intensity of peptide with the amino acid sequence K.VFDLAPEEYVLK. The second graph (b) shows the peak area intensity of peptide with the amino acid sequence K.NYLDAQYYGEIAIGTPPQK.F.



Conclusion

In conclusion, we have used LC-MS proteome profiling to analyze *Arabidopsis* germination. Our results illustrate that proteomics can provide insight into plant germination and indicate some novel aspects of hormonal regulation in this process.

Acknowledgement

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Proteomics and Metabolomics of Plant Systems
Lecture 2

SEED PROTEOME ANALYSIS AND PROTEOME DYNAMICS DURING SEED GERMINATION

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Despite the huge progress that has been made in the last decade, the molecular mechanisms regulating seed germination and early seed development are far from being resolved. Induction of metabolic genes involved in germination starts around 12 hours after imbibition. Thus, most of the early events are mediated by molecules stored in the seed during maturation and are not accessible to transcriptomic analyses. Proteome analysis has been extensively employed in the past but the coverage of observed seed proteome is relatively low even in present-day high-impact studies. Here, we analysed proteome of two model species –*Arabidopsis thaliana* and barley (*Hordeum vulgare*). We employed several complementary approaches to increase proteome coverage and build a library suitable for targeted protein quantitation. The combination of strong cation exchange fractionation (SCX), off-gel isoelectric focusing and PEG fractionation allowed confident detection of over 3,500 proteins in dry seed. Similarly, an alternative MS/MS data processing significantly improved our detection limits. Our results indicate that the seed proteome coverage is limited not only by extraction efficiency or depletion of abundant proteins, but also by an inadequate spectral data interpretation.

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CÍLENÁ ANALÝZA PROTEOMU JAKO ALTERNATIVA FRAKCIONAČNÍCH METOD

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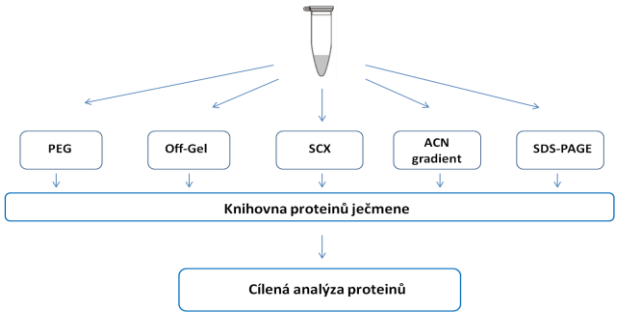
V minulosti bylo vyvinuto mnoho metod, které lze využít pro frakcionaci proteomu a zvýšit tak šance identifikovat i méně abundantní proteiny. Jejich velkou nevýhodou je jak vyšší časová náročnost, tak i významné navýšení nároků na množství vstupního materiálu, který je pro analýzu použit[1]. Množství frakcí pak s ohledem na limitace následné LC-MS analýzy také limituje počet vzorků, které je možné souběžně analyzovat.



Obr. 1. **Využití pěti komplementárních frakcionačních metod k přípravě proteinové knihovny pro cílenou analýzu.**

Pomocí cílené proteomické analýzy lze dosáhnout až o několik řádů vyšší citlivosti v porovnání s běžnou necílenou metodou. Současná instrumentace již umožňuje kvantifikovat více jak 1000 proteinů v hodinové analýze a je tak otázkou, zda by nemohla být dostatečnou náhradou frakcionačních technik. Jako model pro testování jsme použili proteom obilky ječmene, která má sice vysoký obsah proteinu, nicméně většinu tvoří rodina zásobních proteinů omezujících detekci dalších komponent. Využili jsme pěti odlišných technik frakcionace a celkově identifikovali přes 4 000 proteinů, což představuje zhruba trojnásobek proti běžné analýze (Obr. 1). Na základě získaných dat jsme sestavili metodu pro cílenou analýzu a využili ji pro kvantifikaci identifikovaných proteinů v běžné extrakci. Naše výsledky ukazují, že cílená analýza může efektivně nahradit frakcionace a má tak velký potenciál stát se proteomickou obdobou DNA čipu.

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