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

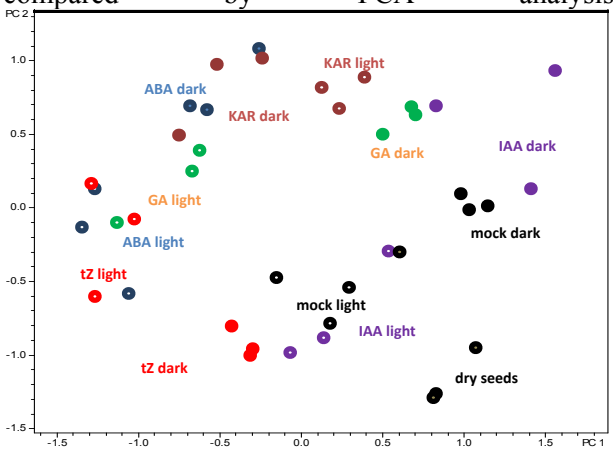
Plant material was prepared and harvested as described in Materials and Methods. Three time points were selected that correspond to dormant seed, phase I and phase II of germination [3, 7]. It is well known that plant growth and development is regulated by substances designated as plant hormones or plant 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

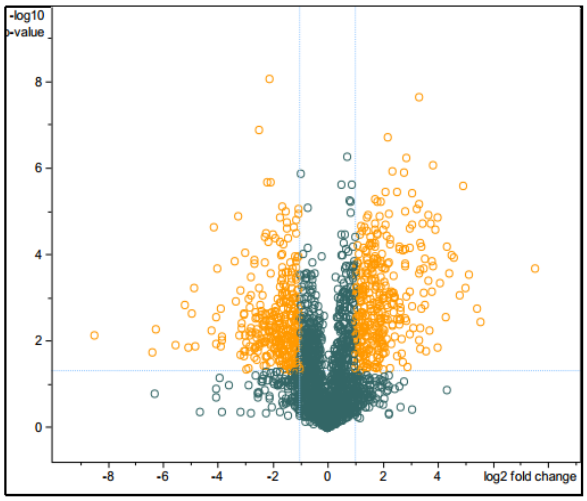


Even though the PCA analysis presented only a very simplified view of complex germination process, it outlines the benefits of proteome analysis and clearly demonstrates the power in proteomics approach to study seed germination.

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.

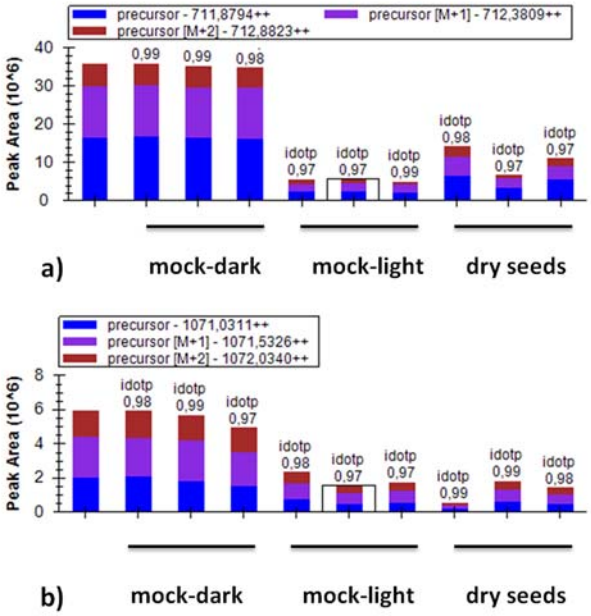


revealed that germination in the absence of light has a significant effect on a large number of buckets (Figure 2). In numbers, 1565 out of 2876 showed significant ($p<0.05$, absolute ratio <2.00) differences. The following automatic assignment of MS/MS spectra identified 2858 peptides that represented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

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