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Plant proteomics as a tool for elucidating the molecular mechanisms underlying signaling and plant interactions with the environment

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Contents

1. Introduction

The discovery of protein as a chemical entity is attributed to the 19th-century Dutch chemist Gerardus Johannes Mulder. Jöns Jakob Berzelius was the one who suggested this name, which originated from the Greek word πρῶτος (first), representing the importance of this biomolecule. Despite Mulder's mistake in stating that the protein formula $C_{40}H_{31}N_5O_{12}$ is established beyond question, and the erroneous speculation that the protein is formed in plants and then introduced to animals through their food, Mulder's description of the protein cannot be disputed:

"*It is one of the most complicated substances, is very changeable in composition under various circumstances, and hence is a source of chemical transformations, especially within the animal body. It is unquestionably the most important of all known substances in the organic kingdom. Without it no life appears possible on our planet. Through its means the chief phenomena of life are produced.*" G.J. Mulder (English translation by P.F.H. Fromberg, 1849).

It took almost a century to obtain evidence that proteins are more than the nutrition or structural components of the cell. In 1926, James B. Sumner published a preliminary paper summarizing nearly a decade of his research on the enzyme urease. Sumner successfully crystallized protein from the jack bean extract and demonstrated that his protein crystals contained urease activity (Sumner, 1926). Although the theory that enzymes are derived from proteins had been postulated before (Fischer, 1907), Sumner's report was controversial at the time and disputed by renowned scientists, including Nobel laureate Richard M. Willstätter (Willstätter, 1927).

The following decades were marked by rapid development in protein separation, visualization, and identification (Figure 1). Arne Tiselius laid the foundation of protein mixture separation by electrophoresis in 1937. This method gradually evolved, employing
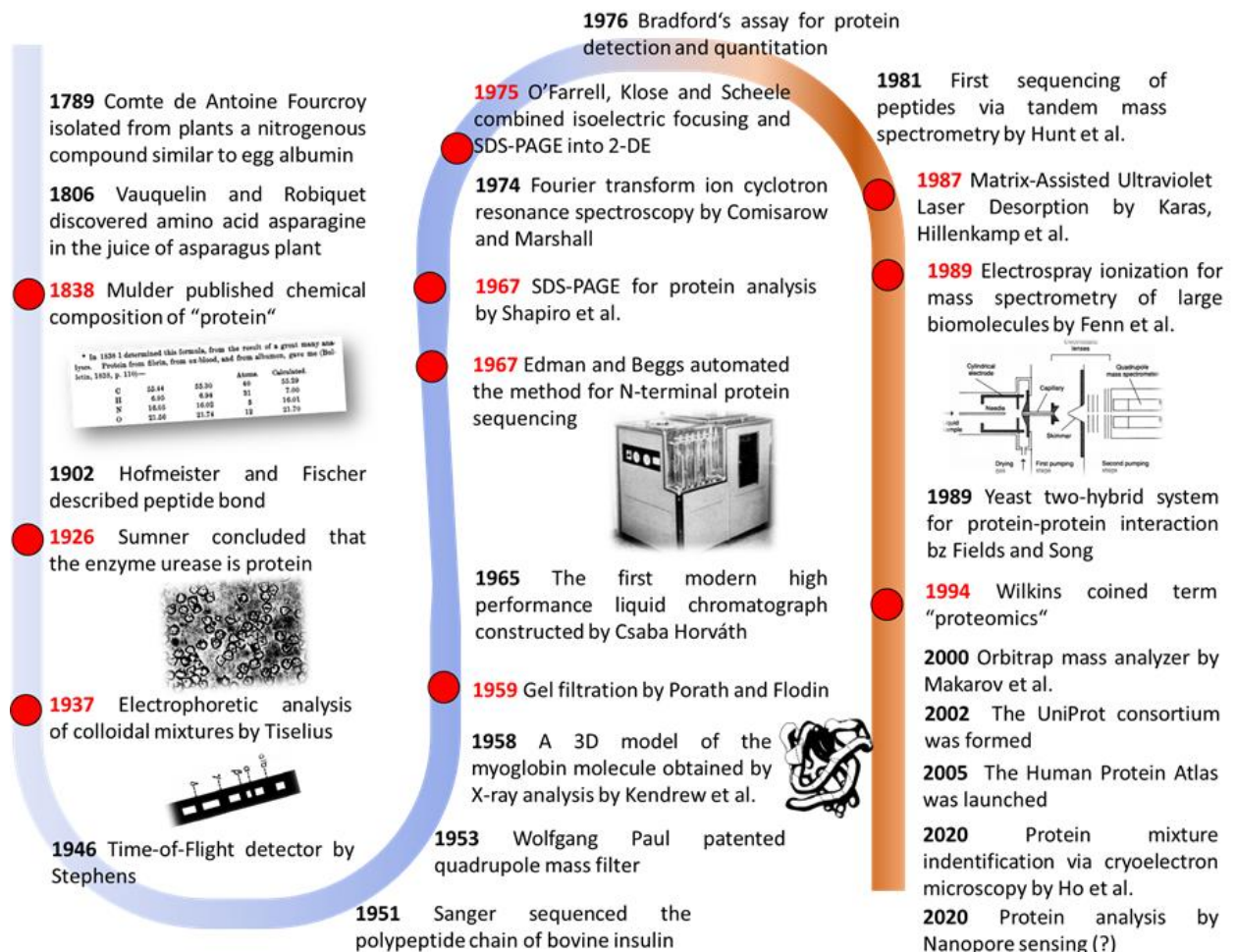
Figure 1. Milestones in proteome research (The official website of the Nobel Prize - NobelPrize.org; Edman and St Vincent, 1967; Tanford and Reynolds, 2003; Manchester, 2005; Perrett, 2007; Asandei et al., 2020; Ho et al., 2020).

different matrices for separation, finally reaching the form of polyacrylamide gel electrophoresis (PAGE). The combination of sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) with isoelectric focusing has been indispensable for at least four decades of proteome research. In parallel, chromatography originally invented by Mikhail Tsvet was fully adapted in the 1960s for protein research, and the reverse-phase version of liquid chromatography (LC) has been an integral part of protein identification first via N-terminal sequencing and later in combination with mass spectrometry (MS).

The N-terminal sequencing was the first successful genome-independent method of protein sequence analysis. Pehr V. Edman published his version of the technique in 1950, a year before Frederic Sanger completed the insulin chain sequencing. Edman did not receive a Nobel Prize for his work, yet his method was more robust than Sanger's, and its automated version was surpassed only in the late 1990s by MS. By the 1950s, MS was a well-established technology for the analysis of volatile compounds. However, there were two obstacles limiting its application for proteomics. First, proteins are too large for gas chromatography and not volatile. Second, the original ionization methods were too harsh for fragile biomolecules, and the resulting fragmentation patterns were not useful for large-scale sequencing. The situation changed in the 1990s with the invention of soft ionization techniques. The resulting development rapidly transformed proteomics, steadily increasing the speed and sensitivity of the analysis. It is not likely that this development would be over. However, there are new emerging techniques that could eventually challenge MS dominance in proteomics. These include methods that can capture a single protein molecule, namely, cryo-electron microscopy and nanopore sensing.

This thesis summarizes the current state of proteome research, discusses the benefits of proteome fractionation, and presents the application of plant proteomics in the analysis of germination, response to stimuli, pathogen detection, and biotic interactions. These examples are based on the author's selected published and ongoing research and research of his students.

2. Fractionation techniques to increase plant proteome coverage

Proteins are the dominant components of a cell, representing on average 15 and 18% of *Escherichia coli* and mammalian cells, respectively (Alberts et al., 2002). The exact proportion is development-dependent and may significantly vary for specialized tissues, but rough estimates indicate that this corresponds to a range of two to four million proteins per
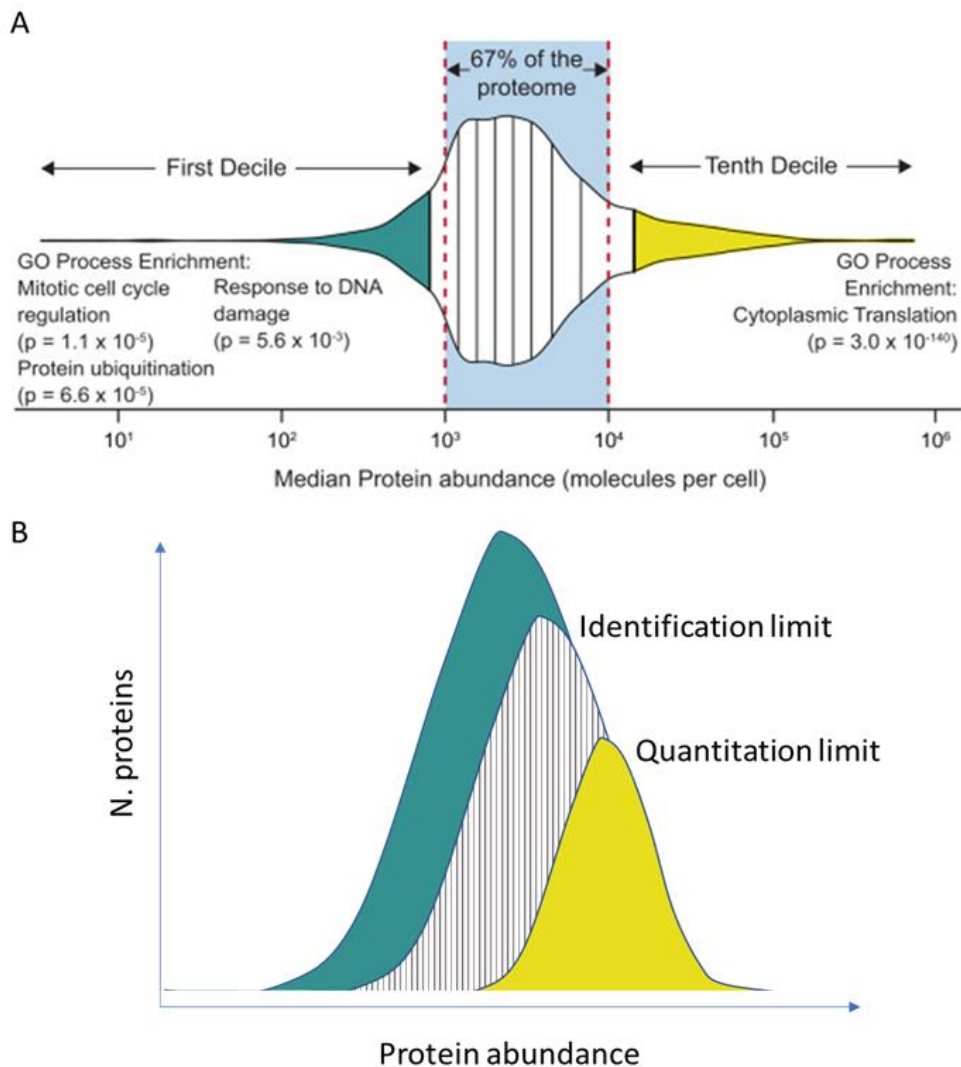

Figure 2. Composition of proteome (A) according to Ho et al. (2018) and simplified visualization of detection and quantitation limits in proteomics (B).

cubic micron (Milo, 2013). As illustrated in Figure 2A, most of these proteins usually belong to only a few highly abundant protein families. Ho et al. (2018) estimated that the median protein abundance in the yeast proteome is 2,622 molecules per cell and found that the difference in concentration between a low-abundance protein and a highly abundant protein can easily be five to six orders of magnitude. The dynamic concentration range is further expanded in multicellular organisms. The identity of the most abundant proteins is organism and tissue-specific. For instance, collagen is the most abundant protein in mammals, and RuBisCO may represent more than 30% of the soluble protein in photosynthetic organisms. Highly abundant proteins are also immunoglobulins, proteins of the translational machinery, energy metabolism enzymes, and histones. Despite the increase in sensitivity of proteomics tools, the total amount of protein that can be analyzed is finite and usually in the range of micrograms. The presence of highly abundant proteins that occupy this space thus affects the detection and quantitation limits and decreases the proteome coverage (Figure 2B).

Proteome complexity is further increased by enzymatic and nonenzymatic posttranslational modifications (PTMs). This overall complexity represents a significant obstacle, and a high proteome coverage is achieved only by extensive fractionation on protein or peptide levels. This is best illustrated by two examples: Sun et al. demonstrated that 10 ng of protein (the equivalent of 20-200 cells) is sufficient for a proteome profile, yet the output was less than 1,000 identified proteins (Sun et al., 2017). In contrast, Bekker-Jensen et al. employed several milligrams of protein, complementary digestion techniques, multi-step fractionation, and found over 14,000 proteoforms (Bekker-Jensen et al., 2017). For this reason, proteome fractionation is the best approach if a reasonable level of proteome coverage is to be achieved and a relatively large amount of starting material is available.

2.1. Tissue separation

A large-scale analysis of *Arabidopsis thaliana* tissue proteome found evidence for more than 18,000 proteins and demonstrated tissue-specific protein localization (Mergner et al., 2020). Separation of plant organs is an effective tool for proteome fractionation and may provide an increase in proteome coverage, as well as novel insights into the organ-specific response. For instance, we have demonstrated the role of the shoot and root-specific response to

temperature (Dobrá et al., 2015; Skalák et al., 2016), observed light-dependent changes in the hypocotyl proteome (Luklová, 2018; Hloušková et al., 2019), and identified proteins involved in cytokinin-mediated leaf morphogenesis (Skalák et al., 2019).
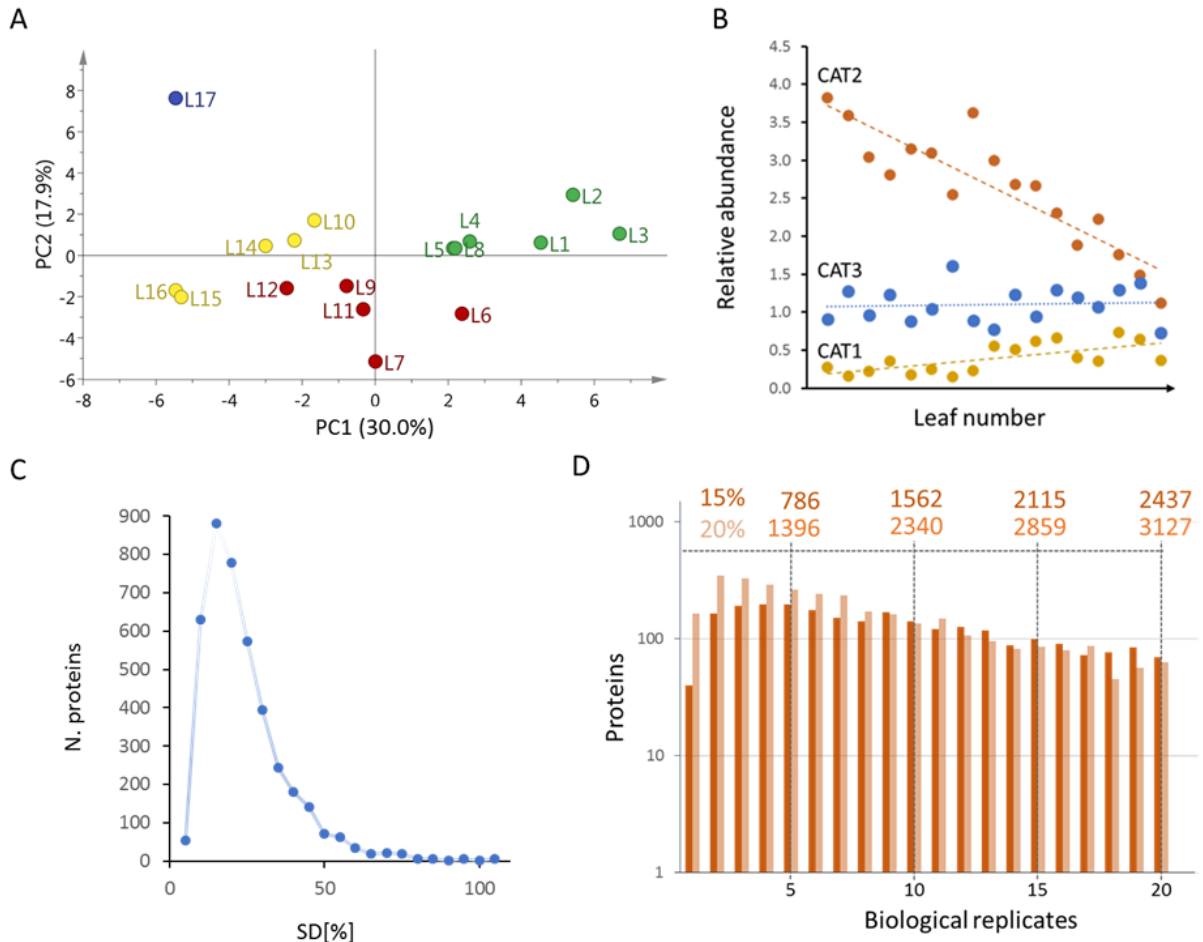
Figure 3. Leaf proteome analysis. (A) Principal component analysis of leaf proteome profiles of a four-week-old *Arabidopsis* plant and (B) Correlation of catalase isoforms abundance with leaf stage. Based on the estimated protein abundances of 2,591 proteins that were identified with at least two unique peptides; (C-D) Reproducibility of protein quantitation based on ten biological replicates. Adapted from (Liberdová, 2020) and unpublished results.

Arabidopsis rosette presents a collection of different stages of leaf development, morphogenesis, and senescence. Consecutively, these leaves may have a different response to stimuli. To capture the underlying proteome composition, we dissected and analyzed all developed leaves of a four-week-old *Arabidopsis* plant. In total, we identified 4,347 proteins,

which was about two-fold more than what we found with the same method in the total shoot extracts of a similar plant (Prerostova et al., 2021). Proteome profiles of individual leaves showed a distinct age-dependent clustering (Figure 3A), and as exemplified by catalase 3, we observed interesting correlations of protein abundance with leaf stage (Figure 3B).

Finally, we were interested in the biological variability in proteome composition and reproducibility of our method. We optimized the protein extraction step to minimize the sample loss, dissected a single cotyledon from seven-day-old seedlings, and analyzed its proteome. In total, over 3,500 proteins were found in all ten biological samples. The evaluation of standard deviation revealed that ten biological replicates are sufficient for revealing 1.5-fold differences in the abundances of 2,340 proteins (20% precision at 95% confidence level). For higher coverage, the ratio threshold or the number of biological replicates would have to be increased (Figure 3C-D).

2.2. Subcellular plant proteome

Not all proteins are functional at the site of their synthesis. Some are stored in an inactive form (e.g., proenzymes), some are being secreted, and some have to shuttle between organelles (e.g., signaling components). In essence, the subcellular location of a protein may be an integral part of its function. Plants show a high degree of cellular compartmentalization due to the presence of compartments like vacuoles and plastids, and it is estimated that the typical plant cell compartment contains 500–4,000 proteins (Millar and Taylor, 2014). Several approaches for subcellular proteome fractionation have been described, but not all organelles are easily accessible such as plastids, mitochondria, peroxisomes, and nuclei. Classical methods of gradient centrifugation are still desirable for the higher loading capacity yet have a lower resolving power and have to be optimized for the given tissue (Sikorskaite et al., 2013). Furthermore, the whole separation protocol usually takes over several hours, requires native conditions, and the protein composition may suffer. We compared the histone content in protein extracts obtained by total protein precipitation to that of the nuclei-enriched fraction (Svetláková, 2017). The total amount of histones was significantly higher in the enriched fraction. Conversely, at least some members of the H1 family and H3 family were more abundant in the total protein extracts, indicating that the native extraction and

percoll gradient fractionation are prone to nuclear protein loss. The spin column-based methods for separating nuclei are faster, yet the efficiency for plant material does not seem to be optimal. In our hands, the NE-PER extraction kit (Thermo) depleted cytosolic proteins but did not provide significant enrichment of nuclear proteins compared with the total protein extract (Svetláková, 2017).

The proportion of subcellular proteins is tissue-specific. As can be seen in Figure 4, the enrichment of chloroplastic proteins in photosynthetic tissues will not provide significant benefits compared to the total protein precipitate, which is well in line with our results (Breineková, 2014).
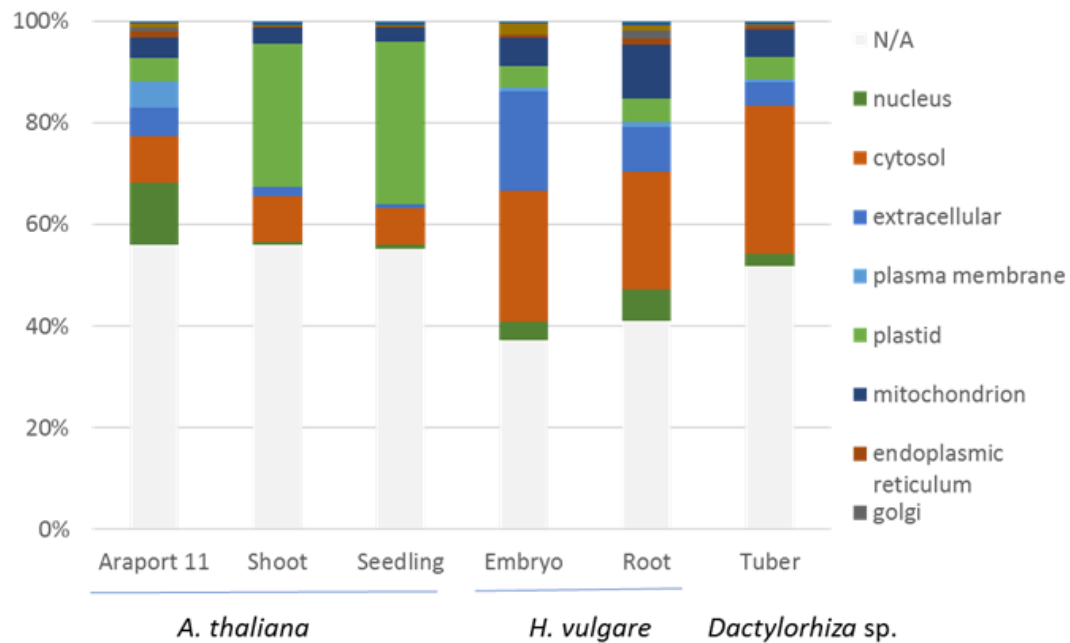
Figure 4. Subcellular plant proteome. The expected localization of all *Arabidopsis* proteins, proteins found in four-week-old *Arabidopsis* shoots (PXD020480), *Arabidopsis* seedlings (Berková et al., 2020; Included Publication 8), barley root (Berka et al., 2020b; PXD020627, Included Publication 7), barley embryo (PXD025075), and orchid tuber (PXD025095). Localization of *Arabidopsis* proteins and *Arabidopsis* protein orthologues predicted by SUBAcon algorithm (Hooper et al., 2017).

As illustrated in Figure 4, the barley (*Hordeum vulgare* L.) embryo contains a substantial amount of putative extracellular proteins, which are predominantly utilized for nutrition acquisition from the seed aleurone layer. Extracellular proteins are an important part of the

plant proteome. These proteins encompass components of the apoplastic fluid, cell wall, as well as the plant secretome. The secretome plays crucial roles in anchoring the plant in its environment (Vincent et al., 2020), yet its analysis is more demanding and these proteins are usually underrepresented in a standard proteomics experiment.

The fastest method for subcellular proteomics is a non-aqueous fractionation (Fürtauer et al., 2019), but the boundaries between the organellar fractions are less defined and the method selectivity is not comparable to that of gradient centrifugation. The laser-assisted microdissection and flow sorting are promising but time-intensive techniques that may provide higher selectivity and spatial resolution (Day et al., 2005; Petrovská et al., 2014). However, these techniques are presently more suitable for transcriptomics analyses.

2.3. Separations at the protein level

Proteome fractionation at the protein level is probably the oldest technique in protein research. In 1888, Franz Hofmeister, a professor of pharmacology at the First Faculty of Medicine, Charles University in Prague, published his study about the effect of salts. Hofmeister studied the protein solubility in aqueous salt solutions and classified the ions in order of their ability to precipitate (salt out) the proteins. The exact mechanism of the so-called Hofmeister effect is still under debate (Kang et al., 2020), but the salting-out technique was indispensable for early protein research, and the ammonium sulfate precipitation became a common purification step. Conversely, the presence of salts interferes with proteomics techniques and salts have to be removed by dialysis or filtering. Alternatively, proteins may be precipitated by nonionic water-soluble polymers, in particular polyethylene glycol (PEG). That technique was introduced in the 1960s (Polson et al., 1964) and can be employed for proteome fractionation and selective depletion of highly abundant proteins such as RuBisCO (Sehrawat et al., 2013). In our experiments, the PEG fractionation was one of the most effective protein fractionation techniques and was surpassed only by SDS-PAGE (Habánová, 2016).
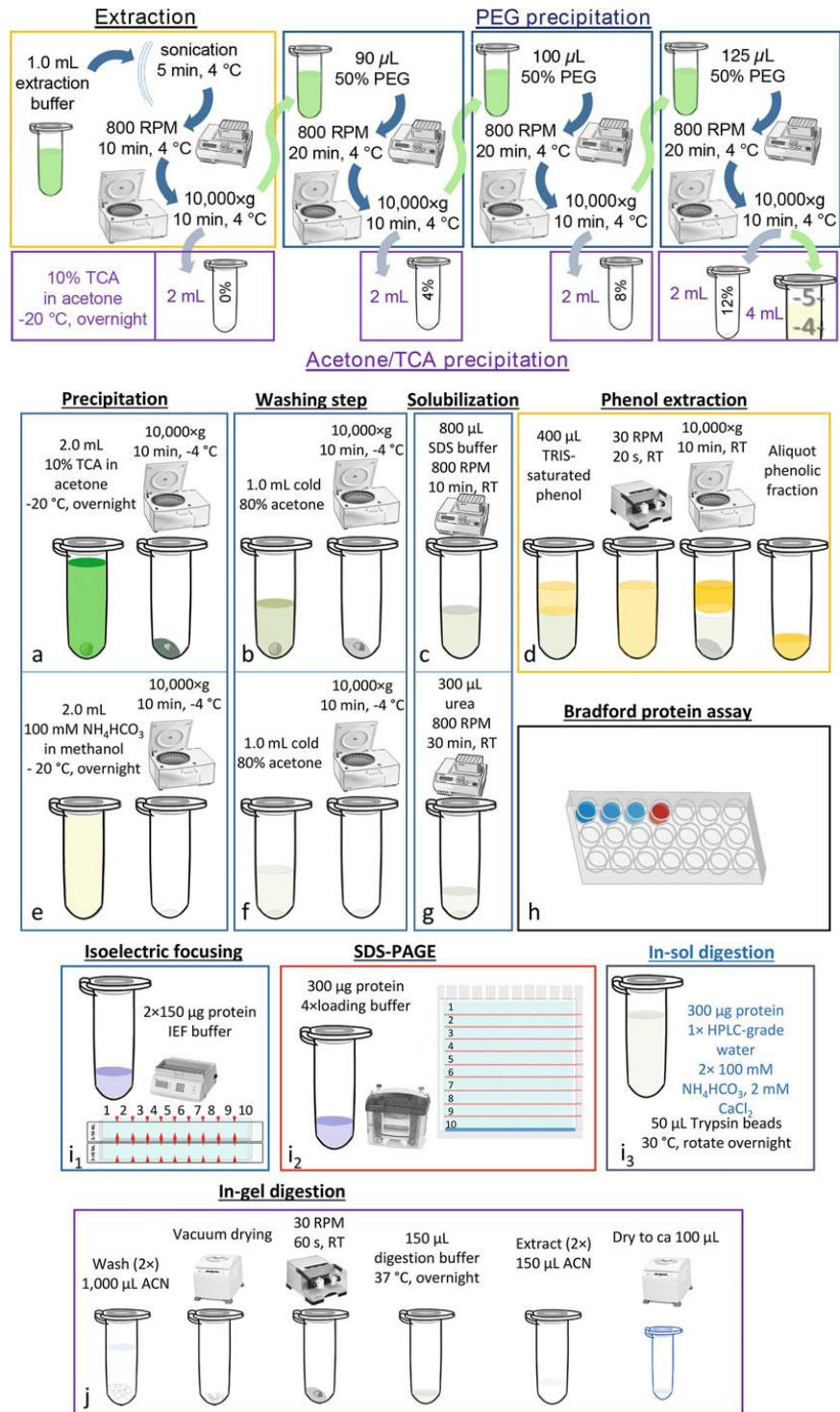
Figure 5. Separation at the protein level. (A) Native extraction and PEG fractionation; (B) Denaturing protein extraction and protein digestion. (a–g) Protein extraction and purification. (h) Determination of protein concentration, (i1–2) protein separation, and (i3, j) digestion (Adapted from Černý et al., 2019; Included Publication 5).

There are many other dedicated techniques for protein separation, and most are derived from LC, PAGE, and affinity/immunoaffinity separations. The broad overview is summarized in our review about the separation of posttranslationally modified proteins (Černý et al., 2013b), and techniques suitable for general proteome analysis are outlined in Figure 5 and Included Publication 5.

2.4. Separation at the peptide level

There have been tremendous advances in the LC-MS analysis of intact proteins during the last decade. The combination of different fragmentation strategies, higher precision and resolution, and ion mobility spectrometry has extended the detection limits of high-throughput top-down analysis of whole proteomes (Cleland et al., 2017; Griffiths et al., 2020). Despite the increased efficiency and success of top-down analyses in the detection of previously uncharacterized biologically relevant modifications, the peptide level bottom-up proteomics analysis is still a superior technique for maximizing the proteome coverage. Furthermore, with high orthogonality to the protein level separation, peptide fractionation steps can be added to any protein separation workflow that has sufficient protein input (Wilhelm et al., 2014; Mergner et al., 2020). The reversed-phase C18 LC is the most common separation method for complex peptide mixtures. Several factors influence its resolution (peak capacity), including temperature, column length, and gradient slope. In general, the column length and gradient slope are proportional and inversely proportional to the resolution, respectively (Breineková, 2014). The length of commercially available columns has reached 200 cm (e.g., PharmaFluidics Micro Pillar Array Column), but the column cost and measuring time per sample are high. Alternatively, the peak capacity is increased by coupling multiple separation methods, and the effectiveness is determined by the degree of separation orthogonality. Strong-cation-exchange chromatography (SCX) is the most common method used in the first dimension of multidimensional peptide LC-MS (Manadas et al., 2010). However, the online setup has limited capacity for chemicals that are incompatible with LC-MS, and the so-called off-line fractionation techniques using stand-alone LC or solid-phase extraction columns are more convenient. A simple workflow is illustrated in Figure 6 and Included Publication 5.
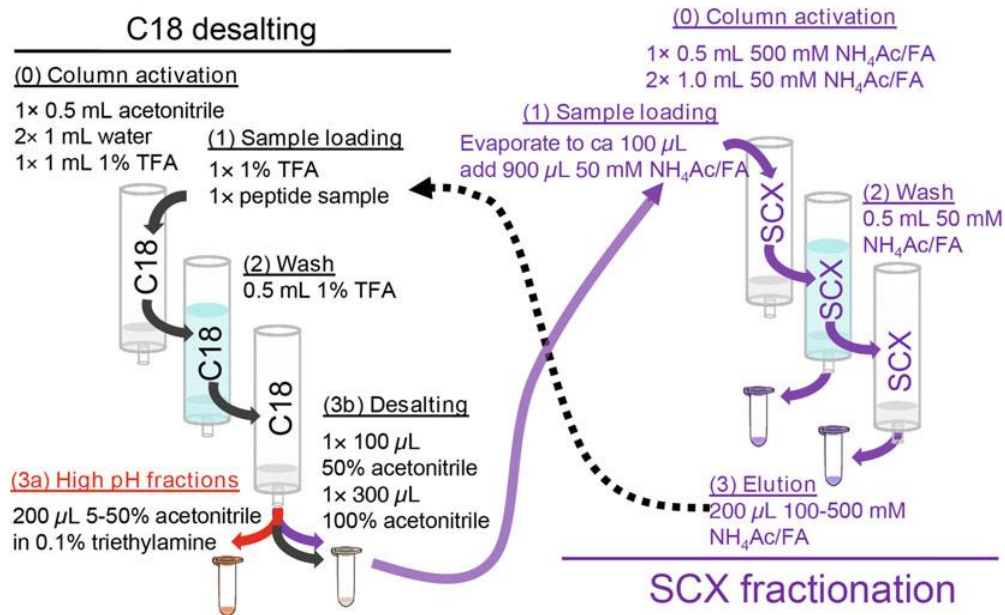
Figure 6. Separation at the peptide level. C18 desalting, high pH fractionation, and SCX fractionation. Adapted from Černý et al. (2019), Included Publication 5.

The major disadvantage of fractionation techniques is the inherent increase in the number of samples that have to be analyzed. For the fractionation at the peptide level, concatenated methods have been developed (e.g., Kulak et al., 2017). Concatenating multiple contrasting fractions that have little overlap improves analysis coverage while maintaining reasonably high throughput. That is especially important for quantitative analyses of discontinuous fractionations for which identical peptides may be found in multiple fractions, and the precise quantitation without an internal standard may be difficult.

2.5. Comparison of fractionation techniques

The success of a proteomic analysis depends on the ability to detect most of the possible number of proteins or protein families in the sample, including the low-abundant ones. Furthermore, a high protein sequence coverage is necessary for confident identification and quantitation. To date, there is no single separation technique with sufficient capacity to resolve complex biological samples. However, fractionation requires a relatively large amount of starting material, and the methods are time-consuming. This problem can be circumvented by targeted methods that improve detection limits but require the availability of a reference peptide spectral library (Černý et al., 2019; Included Publication 5).
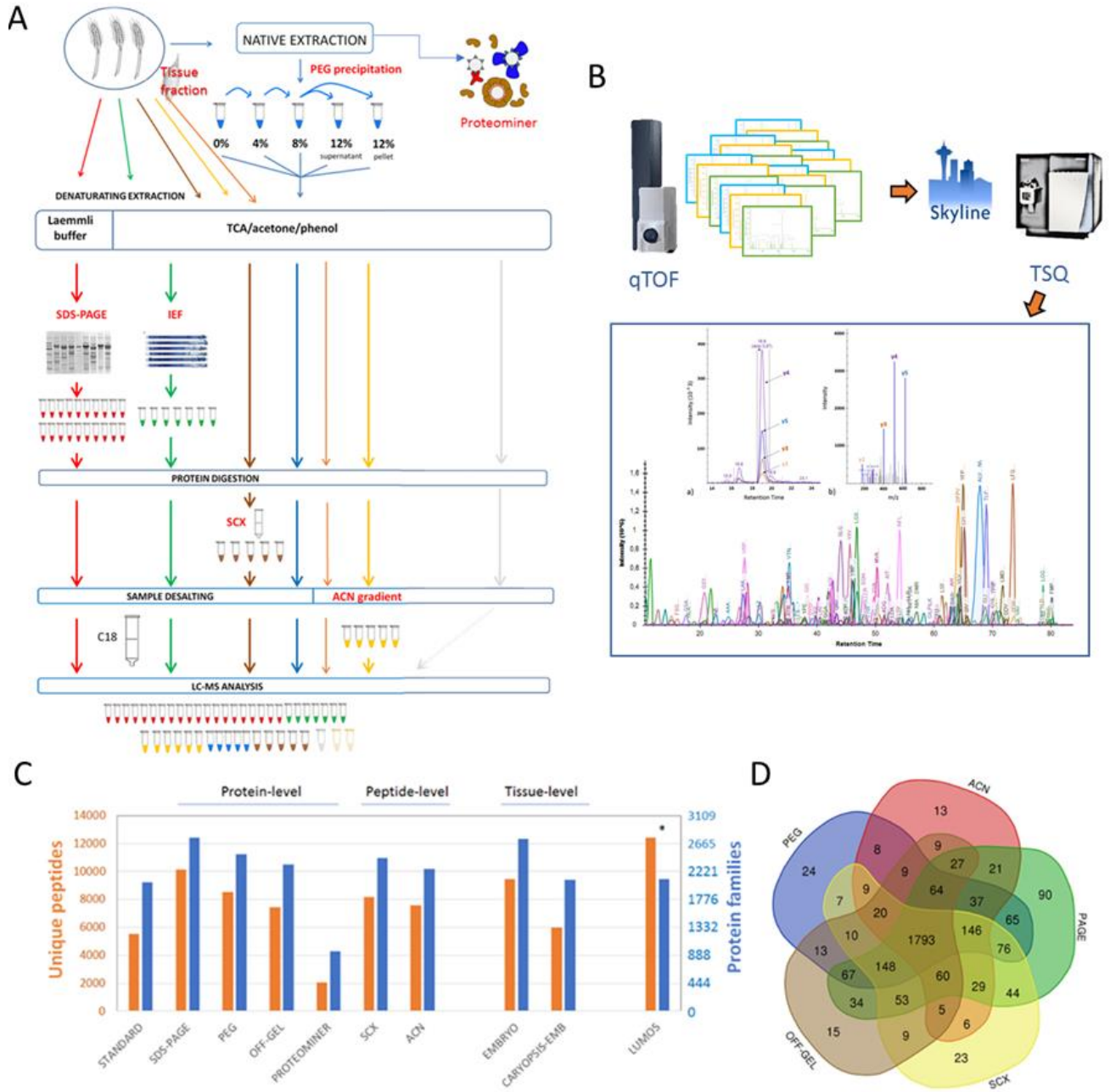
Figure 7. Barley seed proteome fractionation. (A) Visualization of tissue, protein and peptide fractionation (not including experimental replicates); (B) Overview of the Skyline library construction; (C) Number of identified proteins and peptides and (D) an overlap in identified proteins between the five selected datasets; PEG, polyethylene glycol; IEF, isoelectric focusing (Off-gel); SDS-PAGE, polyacrylamide gel electrophoresis; SCX, strong cation exchange chromatography; ACN, acetonitrile; LUMOS, independent analysis with a high-end MS (Adapted from PXD020324 and Habánová, 2016).

Experimental libraries are available, but plant samples are seriously underrepresented (PeptideAtlas). Hence, we decided to build our spectral library for barley seed proteome. The seed proteome includes several highly abundant grain storage proteins, complicating the analysis of less abundant ones. First, a barley peptide spectral library was constructed by analyzing more than 50 fractions of the barley grain proteome obtained by complementary fractionation techniques, including PEG precipitation, SCX, Off-Gel separation, SDS-PAGE, acetonitrile elution gradient, and ProteoMiner protein enrichment. In total, 4,303 proteins were identified: three- to four-fold more than we obtained by standard proteome profiling, using the same LC-MS instrumentation and a 120-minute ACN gradient (Figure 7, Habánová, 2016). Next, the collected spectra were used to develop a targeted assay for large-scale targeted multiple reaction monitoring-based protein analysis. Finally, the targeted method was validated using 72 different seed stocks. We were able to detect at least 70% of the library proteins, but the quantitative data were suitable only for 1,544 and 785 grain peptides and proteins, respectively. Notably, that is still a superior result compared to the quantitation limits of our qTOF instrument that was used for the library construction.

3. Proteomics of seed, germination and early development

3.1. Germination

Plants are sessile organisms and have evolved a broad spectrum of mechanisms to cope with unfavorable environmental conditions. Probably the most amazing is the ability to postpone growth, minimize metabolic activity, and wait for conditions to improve. The survival and persistence of numerous plants strongly depend on successful reproduction by seed. The so-called orthodox seeds sustain intense desiccation at the end of their maturation and retain embryonic cell viability in the dry state, which can extend over centuries (Rajjou et al., 2012). The mature seeds of most species require at least a short period of dormancy. That is a physiological state in which seeds will not germinate even under optimal conditions. However, the metabolic activity under suitable environmental conditions is usually restarted following a simple uptake of water (imbibition). Behind this apparent simplicity lies a complex mechanism integrating internal and external stimuli that coordinates cellular events to allow the embryo to emerge and regulates subsequent seedling growth. The process can be separated into three distinct stages that correlate with water uptake by germinating seeds. Initially, the rapid water uptake is observed, and cells within the seed become hydrated, and energy metabolism is initiated. During the second phase, the seed water content is relatively constant, and the germination of a seed is completed with the emergence of the embryo from its enclosing covering (visible germination). In the third phase, the water uptake is increased as the emerging seedling becomes established and utilizes stored reserves (Nonogaki et al., 2010; Rosental et al., 2014).

The energy and building blocks required to support the fully heterotrophic process of seed germination are predominantly stored in lipids, starch, and proteins in the form of lipid bodies, amyloplasts and protein bodies, respectively (Bewley et al., 2013). Proportions of these reserves in seeds are taxon specific and reflect natural adaptation and evolution, and in seeds of crop species the effects of selective breeding. For instance, more than 70% of energy reserves in seeds of crops such as wheat (*Triticum aestivum* L.) and barley are in the form

of starch, while triacylglycerols and proteins are the main reserves in *Arabidopsis thaliana* seeds, which have less than 0.05% starch contents (Baud et al., 2002). Conversely, orchids, which represent one of the largest families in the plant kingdom, form deficient and dust-like seeds that fully rely on mycorrhizal fungi for organic carbon and other nutrients.

3.1. External stimuli regulate germination and early development

Many known external factors determine the rate and extent of germination, and nondormant seeds exposed for some time to unfavorable germination conditions may even enter a state of dormancy again. The most essential factors are water availability, temperature, light, and nutrients (Bentsink and Koornneef, 2008; Bewley et al., 2013). In most plant species, seeds can remain desiccation-tolerant even after initial imbibition, but a shortage of water after radicle emergence would retard growth and decrease viability. Some consider temperature to be the most critical environmental factor in regulating seed germination. Consequently, there is an optimal temperature range for seed germination, below and above which seed germination of the given species will be inhibited (Wang et al., 2015; Zou et al., 2015). Temperature-sensing mechanisms in plants are sensitive, capable of registering changes of 1 °C (Penfield, 2008). However, despite recent advances, the primary temperature sensor(s) and molecular mechanisms underlying temperature perception in plants are far from being resolved. It seems that nucleosomes, photoreceptors, sequences of messenger RNAs, a transcription factor part of a circadian clock, the physical state, and lipid composition of cellular membranes, as well as plant hormones, may serve as thermosensors (Černý et al., 2014; Jung et al., 2016, 2020; Shen et al., 2019; Lin et al., 2020).

Plants use many types of photoreceptors that are sensitive to light of various wavelengths. The most prominent are four classes, namely, phototropins, phytochromes, cryptochromes, and UVR8 (Kong and Okajima, 2016). Among these, phytochromes were identified as receptors involved in seed germination, with PhyB being the most important in the process (Bewley et al., 2013; Liscum et al., 2014). Light is a stimulant of germination for small seeds with limited nutrient reserves. On the other hand, high fluence rates of light inhibit the germination of many species, presumably to prevent seedling damage from intensive solar radiation.

Finally, seeds of many species will promote germination in the presence of ample nutrient supply that could signal the absence of a competitor plant. For example, nitrate ions in the millimolar range stimulate germination (Bewley et al., 2013).

3.2. Internal stimuli regulate germination and early development

Phytohormones gibberellins and abscisic acid govern the checkpoints in the metabolic transition from dormancy to germination, promoting and inhibiting the process, respectively. Besides, other known phytohormones and growth-promoting substances are needed (at least to some extent). For instance, brassinosteroids and ethylene suppress abscisic acid and stimulate germination (Corbineau et al., 2014; Hu and Yu, 2014). Strigolactones stimulate germination of parasitic plant species and alleviate seed thermoinhibition (Toh et al., 2012). Nitric oxide stimulates seed germination (Beligni and Lamattina, 2000), as do karrikins, substances identified in smoke from burning vegetation (Nelson et al., 2012). Salicylic acid may promote germination under salt stress, but it inhibits germination via reduction of amylase expression (Rajjou et al., 2006; Xie et al., 2007). The cytokinin pool increases after imbibition and may trigger abscisic acid-insensitive seed germination (Wang et al., 2011; Stirk et al., 2012). In contrast, jasmonic acid and oxylipins inhibit germination (Dave et al., 2011; Linkies and Leubner-Metzger, 2012), and an increase in auxin signaling or biosynthesis greatly enhances seed dormancy (Liu et al., 2013). Not surprisingly, all these signals interact and share some common mechanisms, including proteasome signaling and redox homeostasis.

A major redox metabolite is hydrogen peroxide, and at high concentrations induces oxidative damage to biomolecules, which can culminate in cell death. However, at concentrations in the low nanomolar range, H_2O_2 acts as a signaling molecule and in many aspects, resembles phytohormones. For instance, it has dedicated catabolic and anabolic pathways, and it is perceived by several receptors, including Leucine-rich repeat receptor kinase (Wu et al., 2020). Its role in plant growth and development is summarized in Figure 8 and Included Publication 4.
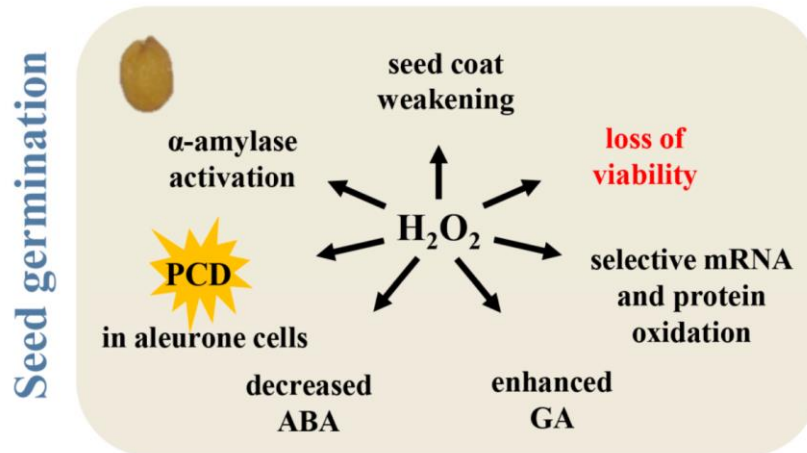
Figure 8. The role of hydrogen peroxide in germination is similar to that of a growth regulator. H_2O_2 promotes endosperm weakening, triggers an increase in gibberellin biosynthesis, and a decrease in abscisic acid levels. It mediates selective oxidation of mRNA and proteins, activation of α-amylase, and the promotion of programmed cell death (PCD) in the aleurone layer. Conversely, an accumulation of reactive oxygen species in quiescent seeds is a sign of aging and may cause significant damage to storage molecules and loss of viability. For details, see Included Publication 4 and references therein (Černý et al., 2018).

3.3. Monitoring of seed germination

A common scoring method for monitoring seed germination is to evaluate its final point, the visible germination manifested by the emergence of the radicle tip. Efficient seed germination is an important trait for agriculture, and automated seed imaging systems have been developed to circumvent laborious subjective methods that are often prone to experimental error (Colmer et al., 2020). However, germination has three distinct phases, and the early events following water uptake are much harder to evaluate. Imbibition increases the seed's water content and volume, yet these processes occur even in those seeds that will not germinate. Direct imaging of the hidden phase of germination is possible with X-ray computed tomography (Ahmed et al., 2018) and magnetic resonance imaging (Munz et al., 2017). These techniques can monitor the radicle growth of individual seeds and evaluate the progress of germination. Equally, germination progress is correlated with the mobilization of storage proteins, and monitoring of these can provide an estimate for a single seed or the whole population. As illustrated in Figure 9A, the mobilization of storage proteins corresponds to the observed radicle length. We have also found that the relative conductivity of steep water is negatively proportional to the radicle length and that this simple technique

can be utilized for a rapid determination of the germination progress of the seed population (Figure 9B).
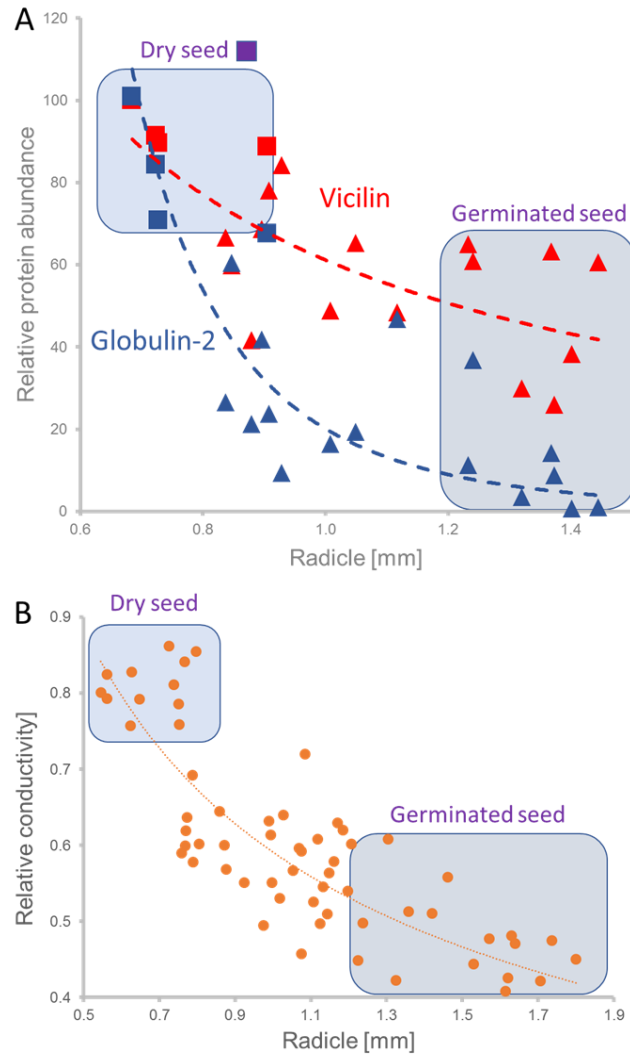
Figure 9. Monitoring of barley seed germination. (A) The relative abundance of the two major storage proteins and (B) the relative conductivity of steep water are negatively proportional to radicle growth (Adapted from Berka, 2019, and patent application n. PV 2019-411).

We found that the dependency can be simplified to the following Equation 1:

$$[\text{Radicle length}] = a \times [\text{Relative conductivity}]^{b}$$

Equation 1. Parameters a and b are constants that have to be determined for the given set of seeds.
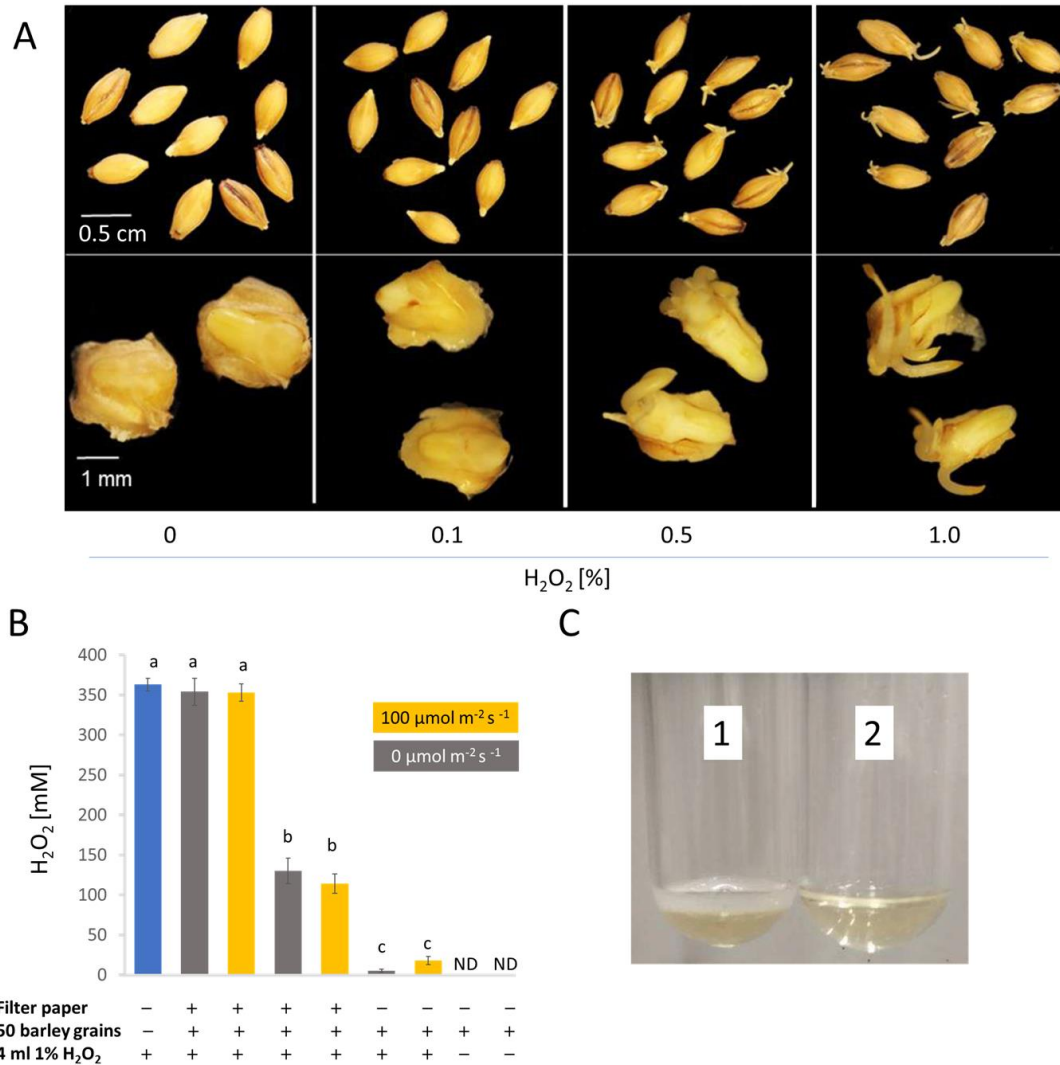
Figure 10. Hydrogen peroxide stimulates barley germination and is actively decomposed by secreted peroxidases. (A) Germinating barley grains 24 h after imbibition with hydrogen peroxide; (B) Concentration of hydrogen peroxide in the imbibing solution. Results represent means and standard deviation of three biological replicates; Statistical significance ANOVA $p < 0.05$; (C) Peroxidase activity in the steep water collected 24 h after the initial imbibition. Reaction mixtures contained 100 µl of steep water, 0.33% (v/v) Triton X-100, and 10% H_2O_2. Activity was fully abolished in the presence of 1 mM sodium azide (2). For details, see (Berka, 2017, 2019).

In our experiment with germinating barley, the conductivity of more than sixty individual seeds was measured. Next, seeds were dissected, and the radicle lengths were determined. Parameters *a* and *b* were found to be 0.54 and -1.21, respectively. The method was validated by measuring ten sets of seeds, and the average ratio between the estimated radicle length

and the experimental data from radicle measurements was 0.93±0.13, indicating that the method is reliable. The results were summarized in the patent application n. PV 2019-411.

3.4. Hydrogen peroxide is decomposed by enzymes secreted from barley grain

Hydrogen peroxide is a potent stimulator of barley germination (e.g., Ishibashi et al., 2017), which was confirmed in our experiments (Figure 10A, Berka, 2017). Surprisingly, we also observed its rapid decomposition in the imbibing solution (Figure 10B). We confirmed that this process is catalyzed by enzymes secreted from the germinating grain and found the activity inhibited by sodium azide (Figure 10C). Finally, proteomics analysis of the steep water content associated the activity with the enzyme peroxidase HORVU2Hr1G044360, the seventh most abundant protein in the list (Berka, 2019). The set of secreted proteins included proteins with putative roles in plant defense, including chitinases, cysteine-rich proteins, thaumatin-like proteins, and proteases. That would indicate that the secreted peroxidase may have a role in plant biotic interactions.

3.5. Eggplant germination is promoted by hydrogen peroxide and temperature in an independent but overlapping manner

Eggplant (*Solanum melongena*) is believed to have originated in tropical regions. Its seeds require a warm temperature for germination and are characterized by a relatively slow germination rate (Figure 11A-C, Included Publication 6). We observed that hydrogen peroxide may promote eggplant germination in a way not dissimilar to that of increased temperature stimuli (Figure 11C). However, our analysis of the total protein extracts found only a very low overlap between hydrogen peroxide and temperature-responsive proteins. Only five of the differentially abundant proteins showed a similar response to temperature increase and to the hydrogen peroxide treatment, including four ribosomal proteins and a putative persulfide dioxygenase (Included Publication 6).

We believed that the hydrogen peroxide treatment would have a stimulating impact on the eggplant seed's endogenous hydrogen peroxide metabolism, but the only significant change
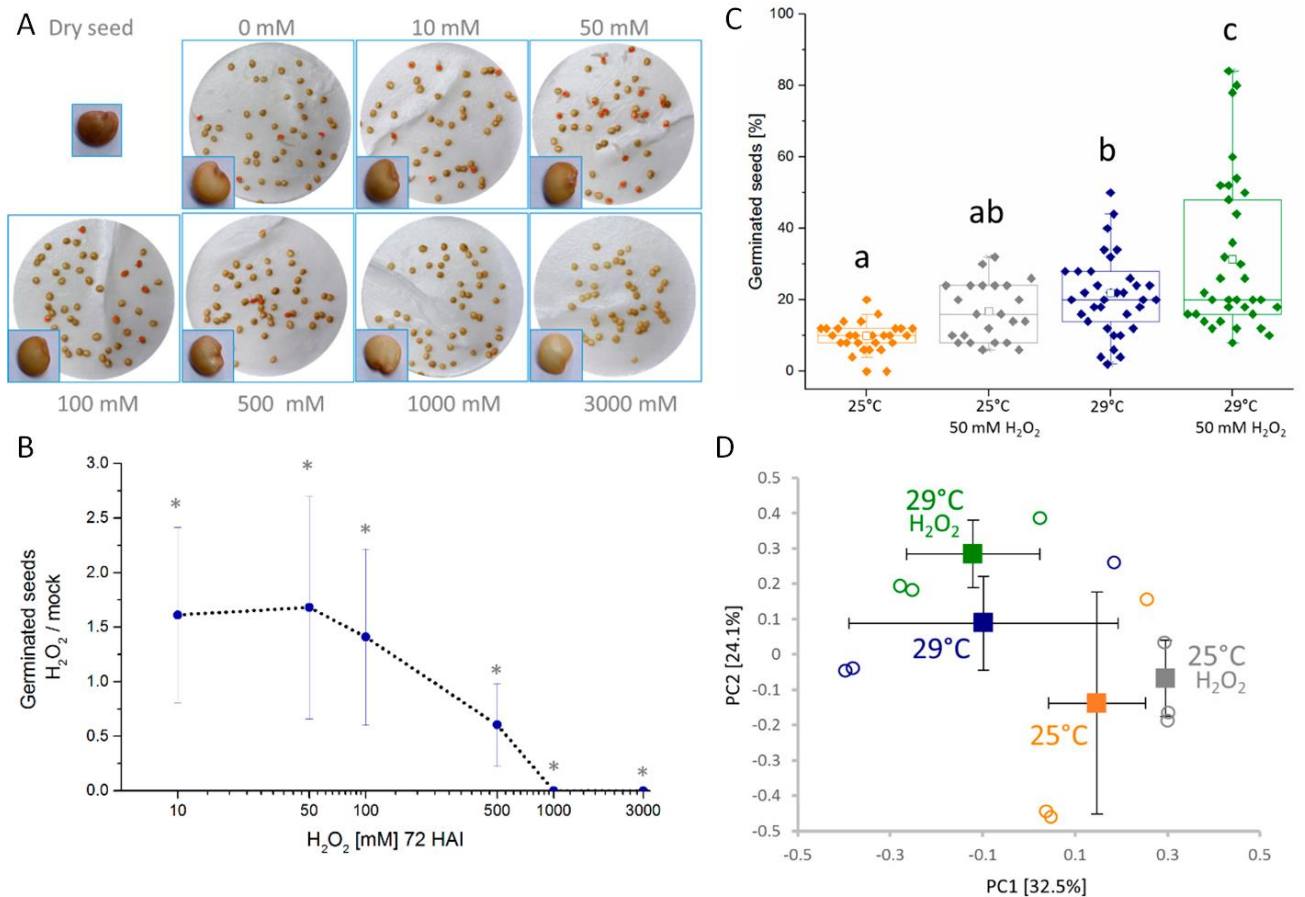
Figure 11. Eggplant germination is promoted by hydrogen peroxide and temperature in an independent but overlapping manner. (A, B) Hydrogen peroxide significantly promotes eggplant seed germination at low mM concentration. Representative images of germination assays at 72 h after imbibition; (C) Both temperature and hydrogen peroxide promote eggplant germination; (D) Comparison of germinating seed proteomes at 72 h after imbibition. The separation of replicate averages (± SD) highlights a distinct clustering of seeds germinated at 25 and 29 °C (separated in PC1) and a less significant separation of the hydrogen peroxide treatment in PC2; The experiments were conducted in up to eight biological replicates, each consisting of three independent sets of 50 seeds. Letters and asterisks indicate statistically significant differences determined by Kruskal-Wallis and Student's t-test, respectively. For details, see Included Publication 6.

that was determined was the abundance of catalases that were depleted in response to hydrogen peroxide. Similarly, multiple orthologues of known hydrogen peroxide-responsive proteins were depleted in eggplant seeds germinated in the presence of hydrogen peroxide. This led us to the hypothesis that the hydrogen peroxide treatment boosted the seed′s scavenging mechanisms, resulting not only in the elimination of the supplied hydrogen peroxide but also in the depletion of its endogenous pool. We have observed a similar effect

on the enzyme superoxide dismutase in barley (Berka, 2017). However, given the significant differences in the development, that possibility was dismissed (Figure 10A). Eggplant germination is much slower, and we were able to preselect seeds with a seemingly similar development stage for the proteome analysis. The decrease in hydrogen peroxide metabolism and response was found at both temperature regimes, and it is thus unlikely that this would reflect a developmental effect. We could speculate that the germination that promoted the effect of hydrogen peroxide was the result of hydrogen peroxide scavenging metabolism mobilization.

3.7. Environmental impacts on barley grain composition

Seeds exhibit memory of the parental environment (Fernández-Pascual et al., 2019). To observe the effects of variations in environmental factors on grain composition, we selected 12 barley breeding stations, with altitudes ranging from 190 to 647 m, and widely differing average monthly temperatures and rainfall (up to 2.3-fold), and yield (up to 2-fold in grain mass per unit area during our sampling year) (Figure 1A-C). Three high-quality malting varieties of *Hordeum vulgare* L. *sensu lato* (spring barley Sebastian, Bojos and Kangoo) were selected, and each of them was cultivated in two independent fields per breeding station.

To estimate the optimal sample size for the experiment, we used the results of germination assay described in Chapter 3.3 and determined that at least seven grains should be analyzed to compensate for the expected biological variability in the population. Accordingly, in the following proteomics analyses we used 10 randomly selected grains representing each combination of variety and field per locality for large-scale profiling and 20 from plants in one of the fields per variety in each locality for in-depth analyses.

Rough estimates based on calculated peptide and protein abundances indicate that the quantifiable proteins accounted for ca. 80% of the total grain protein content, with quantitation limits ranging between 0.004 and 430 µg of protein per grain. The most abundant proteins were carbohydrate-active enzymes (CAZymes), storage proteins, proteins involved in proteosynthesis, protein folding and protein degradation, and protein inhibitors.

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Cross-location comparison revealed that the composition of these categories was not identical, but the total average amount was mostly comparable (Figure 12D-E). The only striking differences, relative to average abundances, were in samples from location 2 (significantly higher contents of LEA and reactive oxygen species-related proteins), 5 (lower contents of proteins involved in folding, storage and LEA proteins) and 11 (significantly higher contents of CAZymes, storage proteins and proteins involved in proteosynthesis).
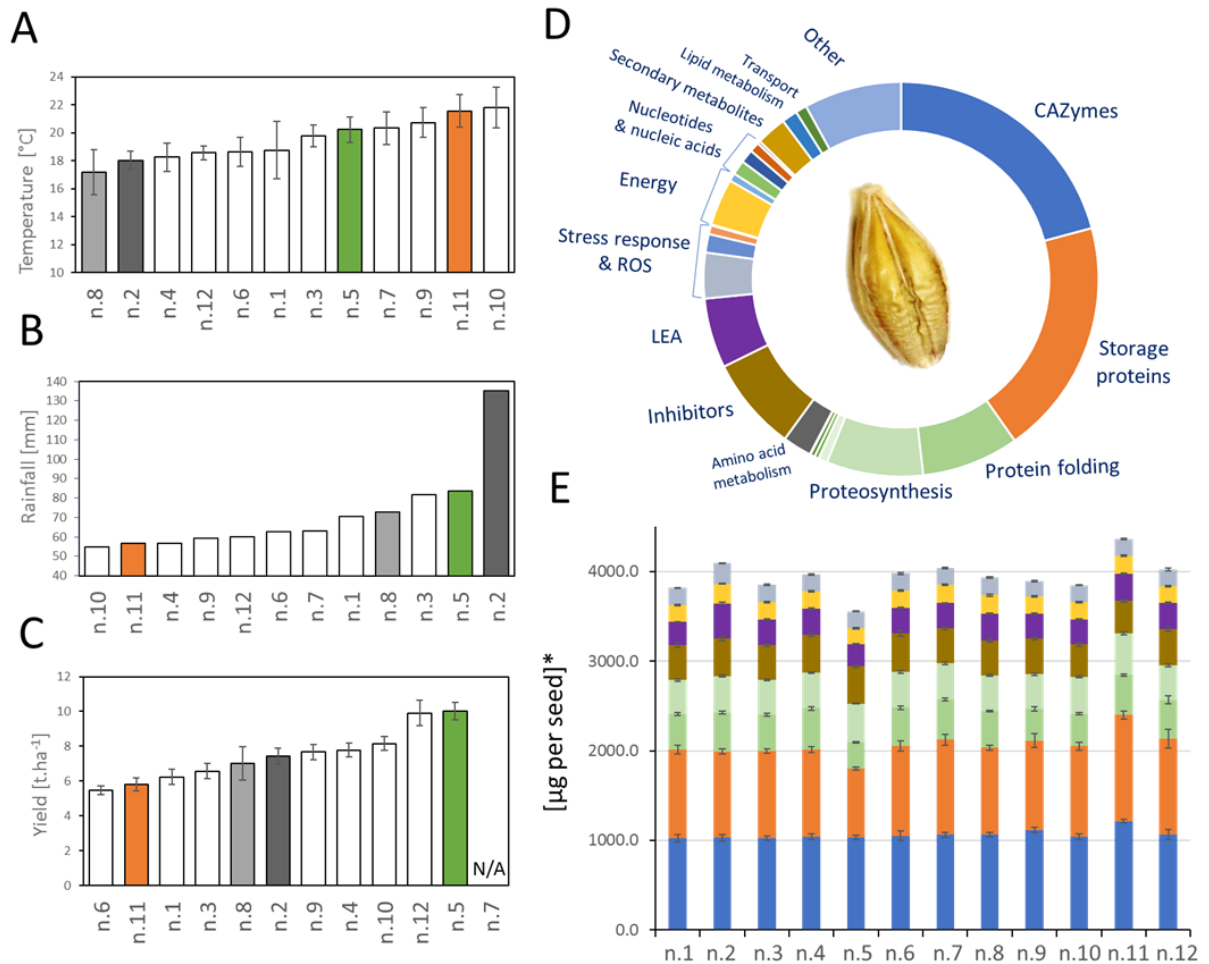
Figure 12. Proteome composition of seeds from different locations. (A, B) Average temperature and rainfall in the 30 days preceding harvest at each location; (C) Documented yield; (D) Average protein composition of seeds from all locations (wheel diagram) and (E) abundance of proteins of indicated major categories of seeds from each location (estimated from the mean peptide and protein abundances; bar chart). Adapted from Habánová, 2016 and Dufková et al. (Manuscript in preparation).

The key environmental factors affecting seed production, at least in agricultural areas where there are no severe nutrient limitations or other stressors, are temperature and water

availability. Thus, correlations with these factors were analyzed in detail. We compared profiles of quantified peptides to average temperature and rainfall data during the 30 days preceding the harvest. The material representing each combination of field and variety was considered as an independent biological replicate, providing six data points per location. Partial Least Squares modelling revealed that profiles of peptides originating from 150 proteins were significantly correlated with average temperature (VIP score >1.4, correlation coefficient >0.6): 84 positively and 56 negatively. This represents more than 12% of the targeted proteins and at least a quarter of the total grain protein content. Several distinct clusters were revealed, indicating positive correlations between temperature and ribosomal proteins, proteins involved in translation and lipid droplet formation, CAZymes, and (unsurprisingly) heat-shock proteins (HSPs). We also observed temperature-related accumulation of GF14 (HORVU4Hr1G043300), a 14-3-3 orthologue of a protein associated with heat stress transcription factors. Positive correlations were also found for histone family protein, farnesyl pyrophosphate synthase, and glucosidase. Surprisingly, we detected negative modulation of protein inhibitors (ca. 28%) and multiple proteins reportedly involved in seed development, including Translationally Controlled Tumor Protein and adenosylhomocysteinase. Rainfall did not seem to have strong linear effects on grain protein composition. The cumulative seasonal data correlated with the highest number of proteins (10), in many cases in an inverse fashion to their correlation with temperature. These relations included negative correlations with the abundance of the storage protein germin and two enzymes: farnesyl pyrophosphate synthase and pyrophosphate-fructose 6-phosphate 1-phosphotransferase.

3.6. Heat shock proteins in seed viability and germination

HSPs are ubiquitous and widely spread proteins across all taxonomic kingdoms and are divided into five groups according to their size (HSP100, HSP90, HSP70, HSP60, and small sHSP) (e.g., ul Haq et al., 2019). HSPs were first discovered in response to an increase in temperature, but accumulated evidence indicates that these proteins are involved in diverse processes. Plants contain a wide spectrum of HSPs that interact with pleiotropic factors involved in the signaling pathways of multiple abiotic and biotic stress responses. Besides their chaperon functions, HSPs participate in proteasomal degradation, protein-protein

interactions and may also play a role in signaling cascades (e.g., McLoughlin et al., 2019; Khan and Shahwar, 2020; Tichá et al., 2020). HSPs interact with key components of the circadian clock and a depletion of HSPs lengthens the circadian period (Davis et al., 2018). Furthermore, HSP90 and HSP70 play an essential role in the plant defense signal transduction pathway (Chapter 5.3). Despite the name, HSPs are not always induced by temperature. For instance, our proteomic analyses of germinating eggplant did not find any effect of temperature, but all three detected sHSPs showed a significant decrease in abundance in response to hydrogen peroxide treatment, and two HSP70s were depleted in the presence of hydrogen peroxide at 29 °C (Habánová et al., 2019; Included Publication 6). The observed decrease of sHSP could coincide with decreasing abscisic acid sensitivity because the overexpression of its *Arabidopsis* orthologue showed hypersensitive response to abscisic acid (Kim et al., 2013a). In accordance, our analysis revealed the presence of putative abscisic acid-responsive *cis*-regulatory elements for at least five genes encoding hydrogen-peroxide-depleted proteins.

HSPs may also play a role in seed longevity (Kaur et al., 2016). Our analysis of the environmental impact on barley grain proteome composition revealed two outliers associated with locations 5 and 11. These locations represent highly contrasting conditions (low yield, high temperature, and low rainfall at location 11; high yield, above average temperature, and high rainfall at location 5; Figure 12A-C). We selected representatives of two of the other clusters - location 2 (average yield, low temperature, very high rainfall) and 8 (average yield, low temperature, average rainfall) - and evaluated the seed viability and longevity (by measuring ion leakage and germination percentage of artificially aged grains, respectively) of grains collected from these locations (Figure 13). Our accelerated ageing experiments revealed that grains from locations with relatively high temperature and low rainfall had significantly higher resilience than grains from other locations, and the consecutive proteomics analysis found that this trait could coincide with a significantly higher abundance of proteins involved in abiotic stress adaptation, including LEA proteins, HSP-family proteins (HSP90, four HSP70s and 14 small HSPs), HSP organizing protein and chaperones (Figure 13B-C).
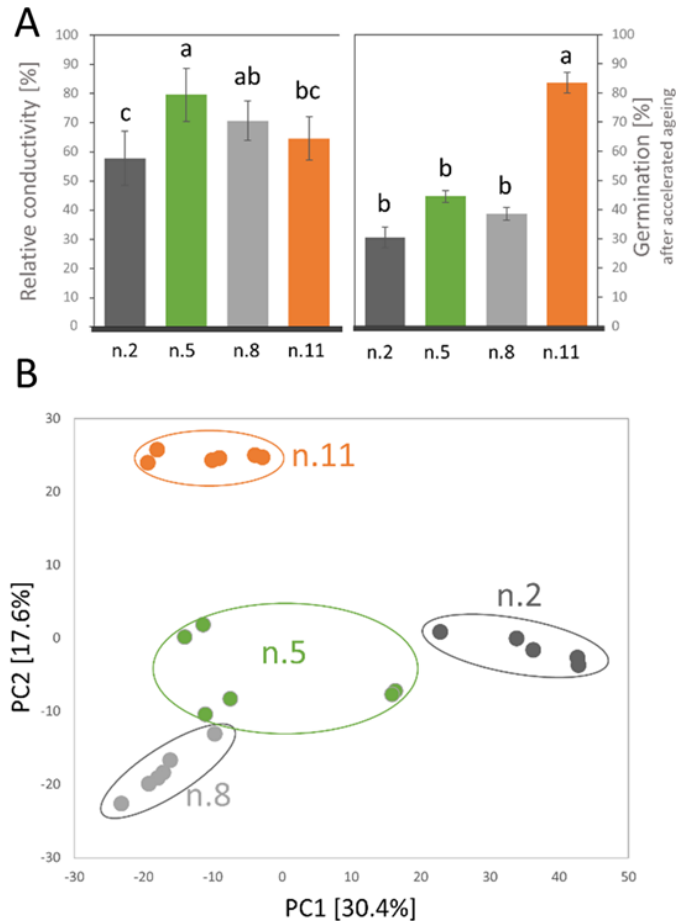
Figure 13. Putative positive markers of seed resilience. (A) Relative conductivity (left) and seed germination after accelerated ageing (right); (B) The separation of seed proteomes from contrasting locations. Colors indicate locations 2 (dark grey), 5 (green), 8 (light grey), and 11 (orange). Different letters indicate significant differences (ANOVA, p<0.05). Adapted from PXD020324 and Dufková et al. (Manuscript in preparation).

3.7. Modification of storage proteins may represent a form of abiotic stress memory

Proteins are major targets of oxidative stress (Černý et al., 2013b), and as storage proteins serve dual nutritive and protective roles, it is likely that the environmental conditions could result in unique PTM patterns. We have tested the possibility of using peptide quantitation
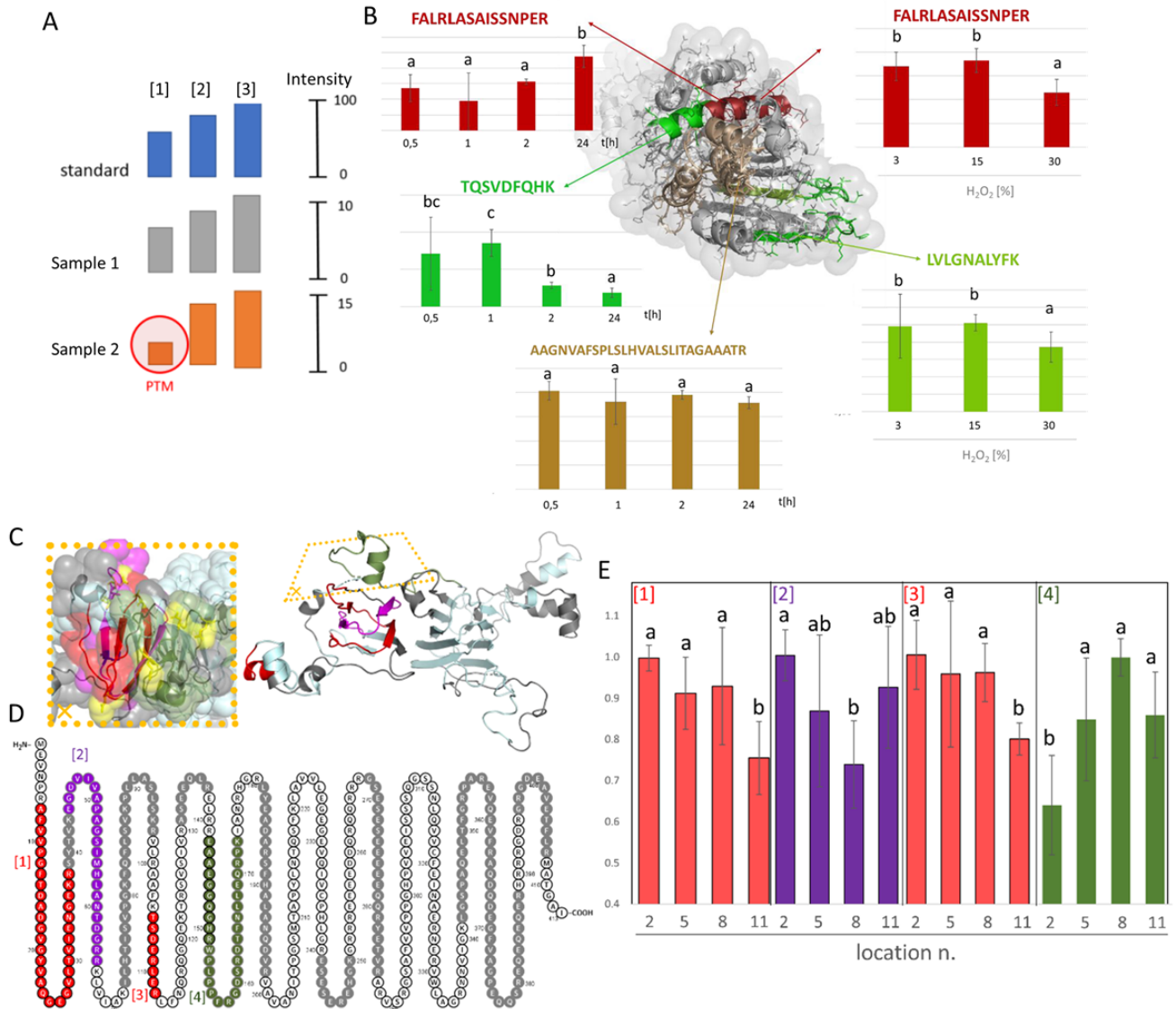
Figure 14. Putative environmentally-dependent PTMs of the major storage proteins in barley grain storage proteins. (A) Simplified diagram illustrating the detection of modified sequences; (B) Targeted quantitative analysis of tryptic peptides originating from Serpine Z4, and identification of modified sequences in response to extreme temperature stimuli (left part, 99 °C for up to 24 h), and hydrogen peroxide (right); (C) Predicted 3D model of globulin-2 with highlighted identified peptides (red, magenta, green - significant differences in at least one of four locations; grey - no significant difference in abundance; yellow - accessible residues prone to deamidation); (D,E) Corresponding sequence visualization by Protter (Omasits et al., 2014) and quantified relative abundances. Bar plots representing means of three biological replicates and standard deviations; Different letters indicate significant differences (Kruskal-Wallis test, p<0.05); 3D models constructed with Phyre 2 (Kelley et al., 2015). Adapted from Dufková et al., (Manuscript in preparation) and (Blaženiaková, 2017).

for monitoring the occurrence of these PTMs in storage proteins. As illustrated in Figure 14A, the presence of unknown PTMs should result in a decrease in the corresponding PTM-free peptides' abundances. First, we probed Serpin-Z4, which is a highly abundant barley grain protein. We optimized the targeted method for analysis of 14 of its tryptic peptides, representing more than 50% of the protein sequence coverage. Next, we incubated barley grain powder at 99°C for 0.5-24 h or with hydrogen peroxide (0-30%), extracted proteins and analyzed the abundances using the developed targeted method. Abundances of seven and four peptides were significantly decreased by heat and oxidative damage, respectively (Figure 14B, Blaženiaková, 2017). We have employed a similar strategy to observe changes that could correlate with seed longevity and analyzed the most abundant grain protein globulin-2 (HORVU4Hr1G002800). Abundances of peptides covering most of the protein sequence were similar, but only samples from location 5 showed no significant depletion of the observed PTM-free peptides. In total, four regions were found to be underrepresented in peptides in samples from locations 2, 8, or 11 (Fig. 14C-E). Structural analysis, based on orthologue modelling, indicated that these regions are on the protein surface and thus accessible for PTM (Fig. 14C). These results provide a significant first step towards understanding the complex role of storage proteins in seed protection and longevity.

4. Analysis of signaling and phytohormone responsive proteins

4.1 Role of proteome in signaling

The signaling cascade comprises the perception, transduction, and response processes. The fastest responses mediated via allosteric control occur within milliseconds (Figure 15). Allosteric modulators are small molecules that bind to a protein, induce conformational changes in its structure, and modify its activity. It is well known that a calcium ion flux can act as a regulator in this way, and there are over 82,000 different known allosteric modulators listed in the AlloSteric Database (Liu et al., 2020), including 60 ions, 614 polypeptides, and 81,396 other compounds. The second level of regulation is governed by protein PTMs and occurs typically in minutes. The most common PTMs in plant signaling are phosphorylation, ubiquitination, and redox modifications (Černý et al., 2016; Included Publication 1). The final level of regulation is the slowest process, with an effective time span of hours. It is mediated by gene expression and the transcription-translation machinery. It is the best-understood part of plant signaling but has its limits. The presence of an mRNA does not guarantee that the corresponding protein is being translated. On the other hand, the absence of mRNA expression does not exclude its protein's presence. There are many reasons for that. First, translation elongation rates differ between mRNAs. That is primarily due to codon usage and the availability of the corresponding tRNAs (Riba et al., 2019). It has also been shown that post-transcriptional regulations may significantly impact the translation rate (Arango et al., 2018) and maybe even change the resulting protein sequence (Eyler et al., 2019). The additional factor is protein life span. The experiments showed that the half-lives vary greatly, ranging from hours to a day (Chen et al., 2016). Optimistic estimates based on available results indicate that differences in protein concentrations are only 30–40% attributable to mRNA levels (Vogel and Marcotte, 2012). Furthermore, the comparison of related species showed that genes with significant expression differences between species at the mRNA level had little or no difference in protein abundance (Khan et al., 2013). That would imply that protein abundances evolve under a higher evolutionary constraint than

mRNA expression levels. To conclude, proteome analysis would be far superior to transcriptomics in reflecting the molecular mechanisms of a cell if the present-day sensitivity limits were to be overcome.
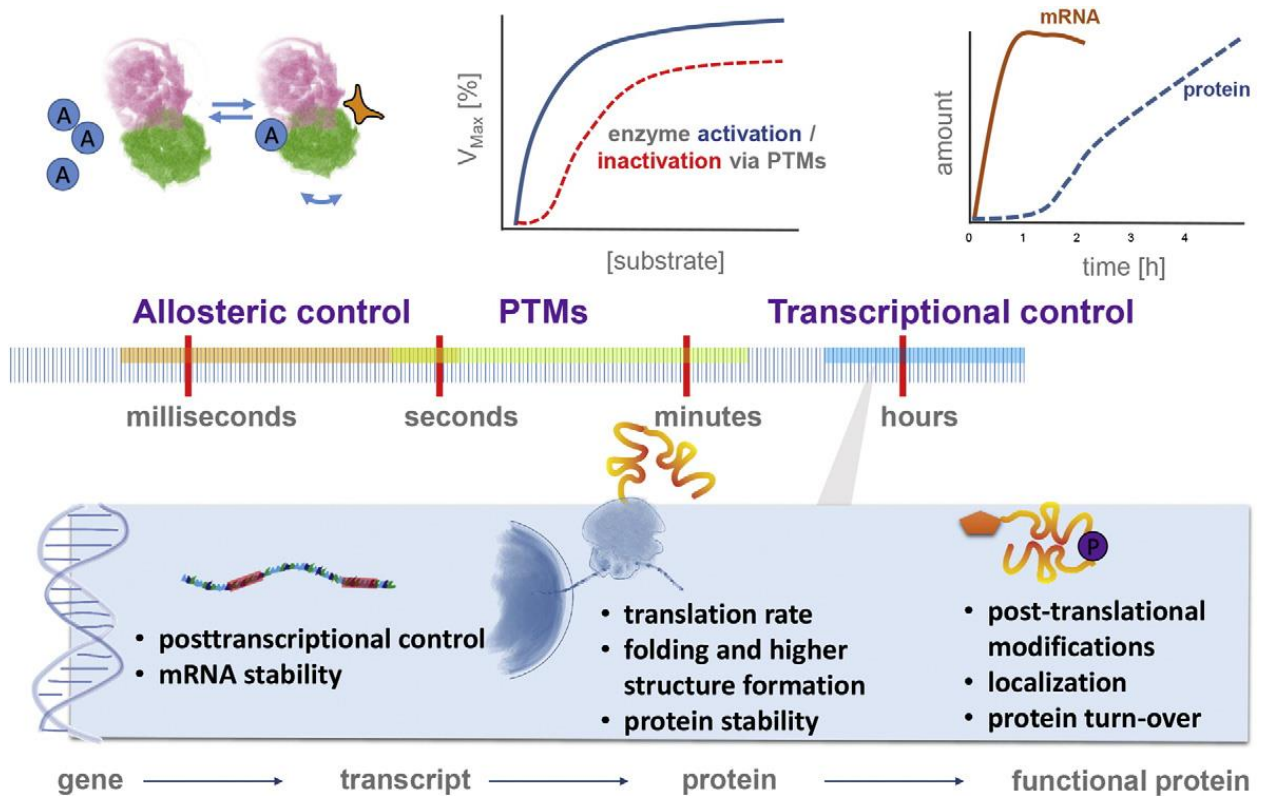
Figure 15. Signaling mechanisms (Černý et al., 2016; Included Publication 1).

4.2 Early response proteins

The regulatory circuits that induce changes in transcription patterns and (hence) protein biosynthesis occur within a few minutes of the perception of stimuli, and thus proteomics experiments describing early signaling events that occur 5–15 min after exogenous treatment are of particular interest. However, these experiments are inherently more demanding and prone to experimental bias. For instance, the treatment may unintentionally trigger touch and wound signaling responses, and it has been demonstrated that a long-distance wound signal mediated by a glutamate receptor is being transmitted throughout the plant body within 120 s (Toyota et al., 2018). A similar rapid response has been found for light-induced

reactive oxygen species production (Yokawa et al., 2011). The major disadvantage of early response proteomics is the limited interpretability of the obtained data. Knowledge of the role of PTMs in protein activity is scarce, and independent validations via transcriptomics analysis are usually not possible. That probably explains that analyses of early-responsive proteins represent less than 10% of phytohormone proteomics (Černý et al., 2016; Included Publication 1). Indeed, most of the putative signaling mechanisms found in our proteomics analyses have not yet been validated. On the other hand, our proteome analysis indicated a role of calcium ion signaling in cytokinin-induced phosphoproteome dynamics, and we proved that by cotreatment with calcium signaling inhibitors (Černý et al., 2011). Similarly, we have confirmed with mutant genotypes the putative link between cytokinin and the perception of alleviated temperature (Černý et al., 2014) and its role in heat stress (Dobrá et al., 2015; Skalák et al., 2016).

An additional obstacle in early response protein analysis is the methodology because the standard high-throughput shotgun LC-MS proteomic approach is not optimal for monitoring PTMs. That can be illustrated in our study of early response to the growth regulator karrikin. Out of all 113 karrikin-responsive proteins, 49 and 74 were found by LC-MS and 2-DE, respectively (Baldrianová et al., 2015). That is a strikingly different ratio to that observed in our proteome analysis of transgenic *Arabidopsis* seedlings *CaMV35S>GR>HvCKX2*. In that study, 24 h after dexamethasone-induced expression, 107 and 19 differentially abundant proteins were revealed by LC-MS and 2-DE, respectively (Černý et al., 2013a).

Despite the fact that PTMs are probably the major regulatory circuit of early response, the total protein content is altered too. Some genes have very high transcription rates, reaching more than 100 mRNAs per hour (Hausser et al., 2019). The rate of proteosynthesis is usually within the range of 1-20 amino acids per second (Riba et al., 2019), but polysomes may produce more than 1000 proteins per mRNA per hour. Similarly, protein degradation is rapid. The catalytic efficiency of some proteases is reaching that of enzymes limited by diffusion (up to $10^7\,M^{-1}s^{-1}$; Dickey et al., 2013), and the 26S proteasome, an integral part of all known phytohormone signaling pathways, may cleave proteins with an average speed of 40 amino acids per second (Peth et al., 2013). Our analysis of cytokinin and temperature-

shock response proteins found the ratio between decreased and increased protein spots to be in favor of repression (~2-fold), indicating that degradation could be the dominant factor in the regulation of early response proteome (Černý et al., 2011, 2014).
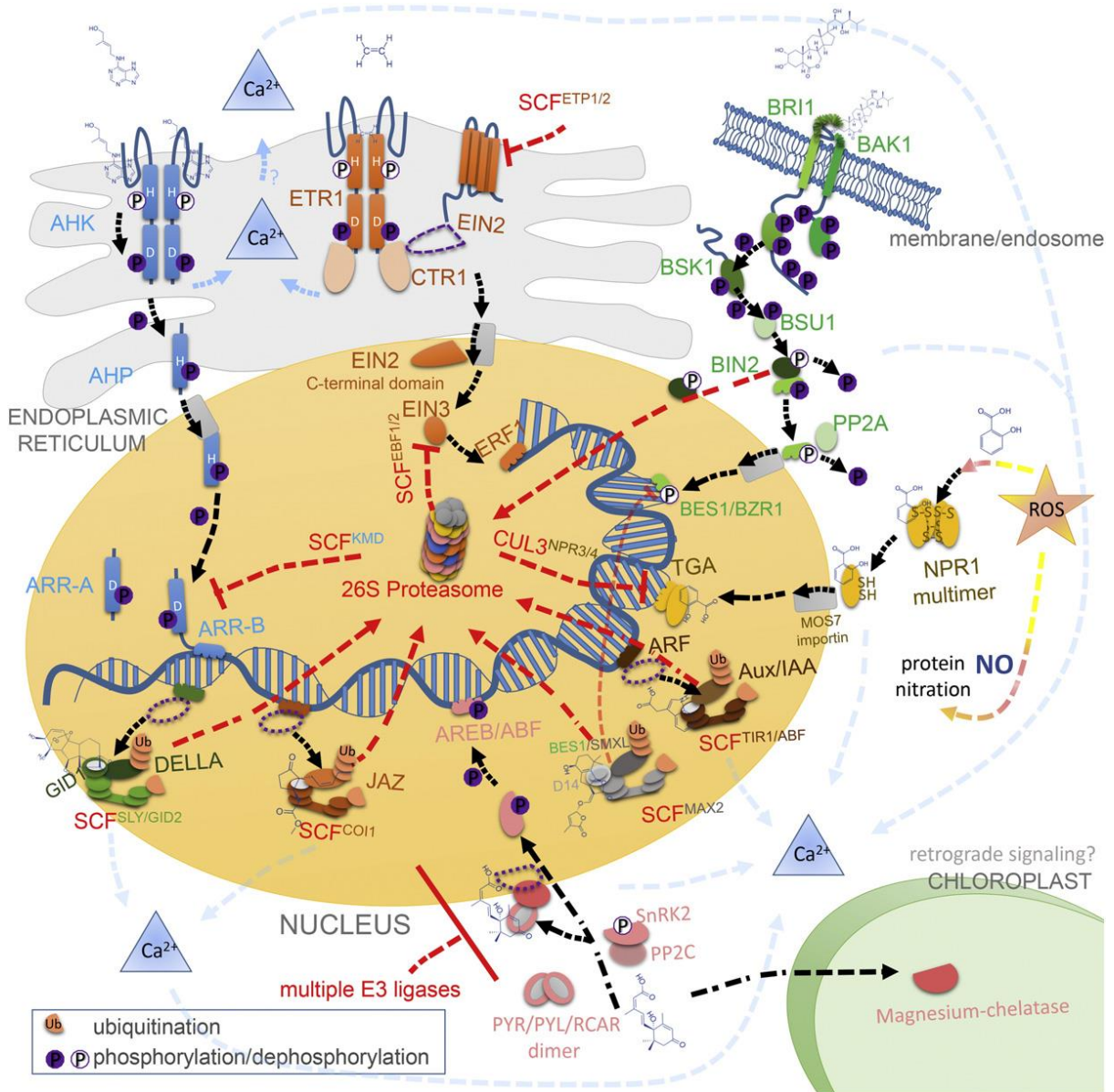
Figure 16. Proteasome mediated degradation - the point of convergence for phytohormone signaling (Černý et al., 2016; Included Publication 1).

4.3 Proteasome mediated regulation of plant proteome

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 (Vierstra, 2009). In effect, most plant regulatory circuitry and many steps of process execution depend on ubiquitin modification. Substrates modified by conjugation to ubiquitin are usually degraded via the proteasome, and this protein turnover-dependent regulation is common for phytohormone signaling pathways (Figure 16).

4.3.1 Effects of proteasome inhibition on plant growth and response to cytokinin

In 2013, a family of F-box proteins KMD was found in the control of cytokinin signaling by regulating the proteasome-mediated degradation of type-B response regulators (Kim et al., 2013b). We were interested in this response and its effect on cytokinin response proteins (Dufek, 2014, 2016; Luklová, 2016). Two alternative approaches were employed, namely, a proteasome inhibitor MG-132 and the transgenic *Arabidopsis* line harboring modified ubiquitin ubR48 under the control of the dexamethasone-inducible promoter (Schlögelhofer et al., 2006). This modified ubiquitin is used like endogenous wild-type ubiquitin for conjugation to substrates but does not support the formation of ubiquitin–ubiquitin linkages via Lys 48 that are necessary for proteasome-mediated degradation. First, we analyzed root growth and found that proteasome inactivation inhibited root growth. The application of exogenous cytokinin had an additive effect, but only for low and saturating concentrations of dexamethasone (Figure 17). Similarly, 1 µM MG-132 did partially alleviate cytokinin-induced root inhibition (Dufek, 2016). In theory, that could be explained by a higher turn-over rate of type-B (positive) than type-A (negative) response regulators, but we have not been able to validate that. Next, we analyzed the early response to cytokinin in plants with fully inhibited proteasomes (50 µM MG-132, 120 min). We found that proteasome inhibition interfered with the cytokinin response in at least 60 % of cytokinin response proteins (Dufek, 2016).
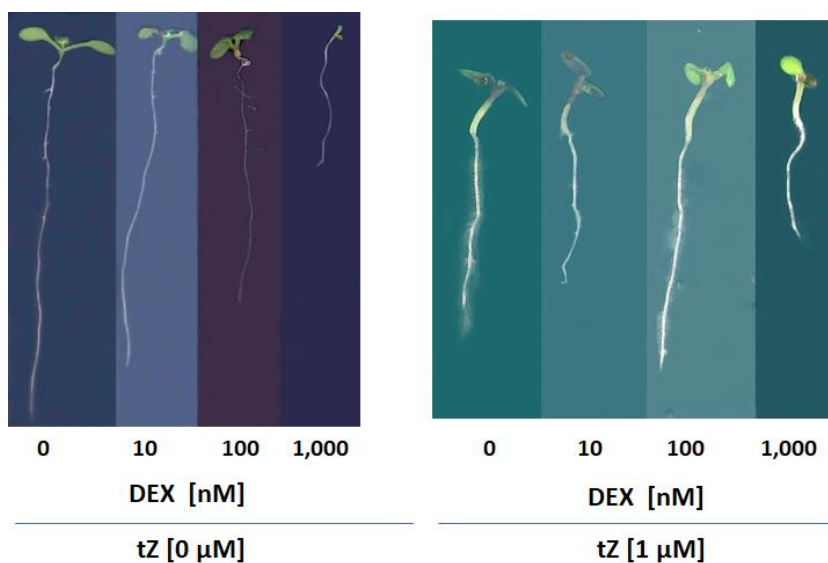
Figure 17. Proteasome inhibition and cytokinin signaling interplay in root elongation (Adapted from Luklová, 2015).

4.3.2 Modifications of DELLA protein RGA

DELLA proteins are plant-specific transcriptional regulators acting as signaling hubs and mediating transcriptional control. We have proved that besides the canonical pathway via the GID receptor, DELLA can be targeted for degradation by an alternative gibberellin-independent pathway via an E3 ubiquitin ligase COP1 (Blanco-Touriñán et al., 2020). However, the exact position of the ubiquitination has not been elucidated. We prepared the recombinant RGA protein in *E. coli* and compared its tryptic digest with that of native RGA captured by immunoaffinity chromatography from transgenic seedlings harboring *GFP-RGA* under the native promoter (Figure 18A-C). Next, we searched for the candidate proteins that were significantly more abundant in the digest of recombinant RGA (missing PTMs) in a proteome fraction enriched by TUBE (Tandem Ubiquitin Binding Entities). The most promising candidate was the peptide SSEMAEVALK, which is localized next to the consensus DELLA sequence and the predicted fragmentation products of its ubiquitinated form were found in the TUBE-enriched fraction (Figure 18D).
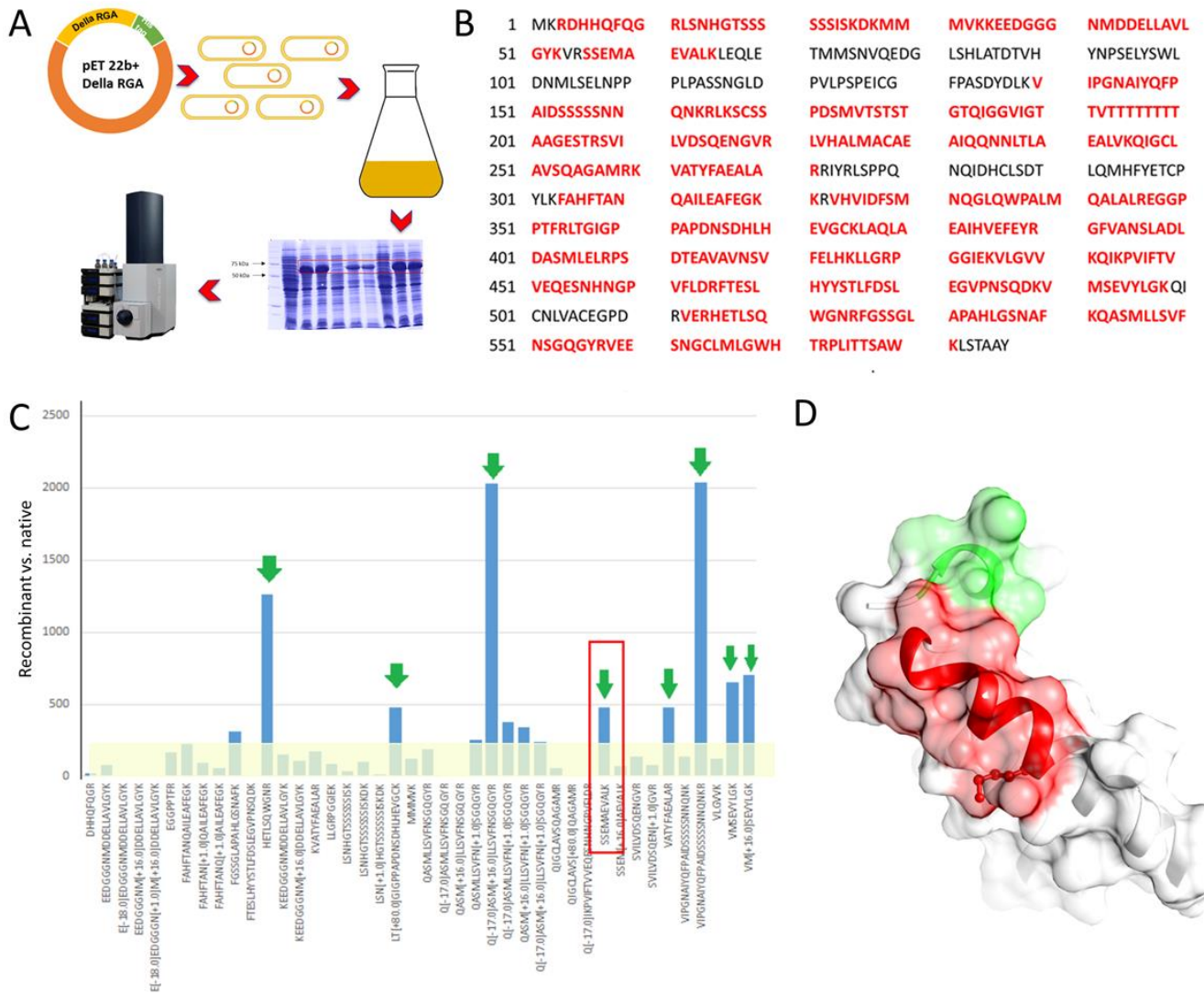
Figure 18. Detection of putative PTMs in the DELLA family protein RGA. (A) The overview of recombinant production of RGA and (B) sequence coverage of the obtained tryptic peptides; (C) Comparison of relative peptide abundances between recombinant standard and native RGA (Adapted from Breineková, 2016); (D) The position of putative ubiquitination site (red) and DELLA sequence (green). The 3D model was constructed with Phyre 2 (Kelley et al., 2015).

4.4 Cytokinin and abiotic stress response in plants
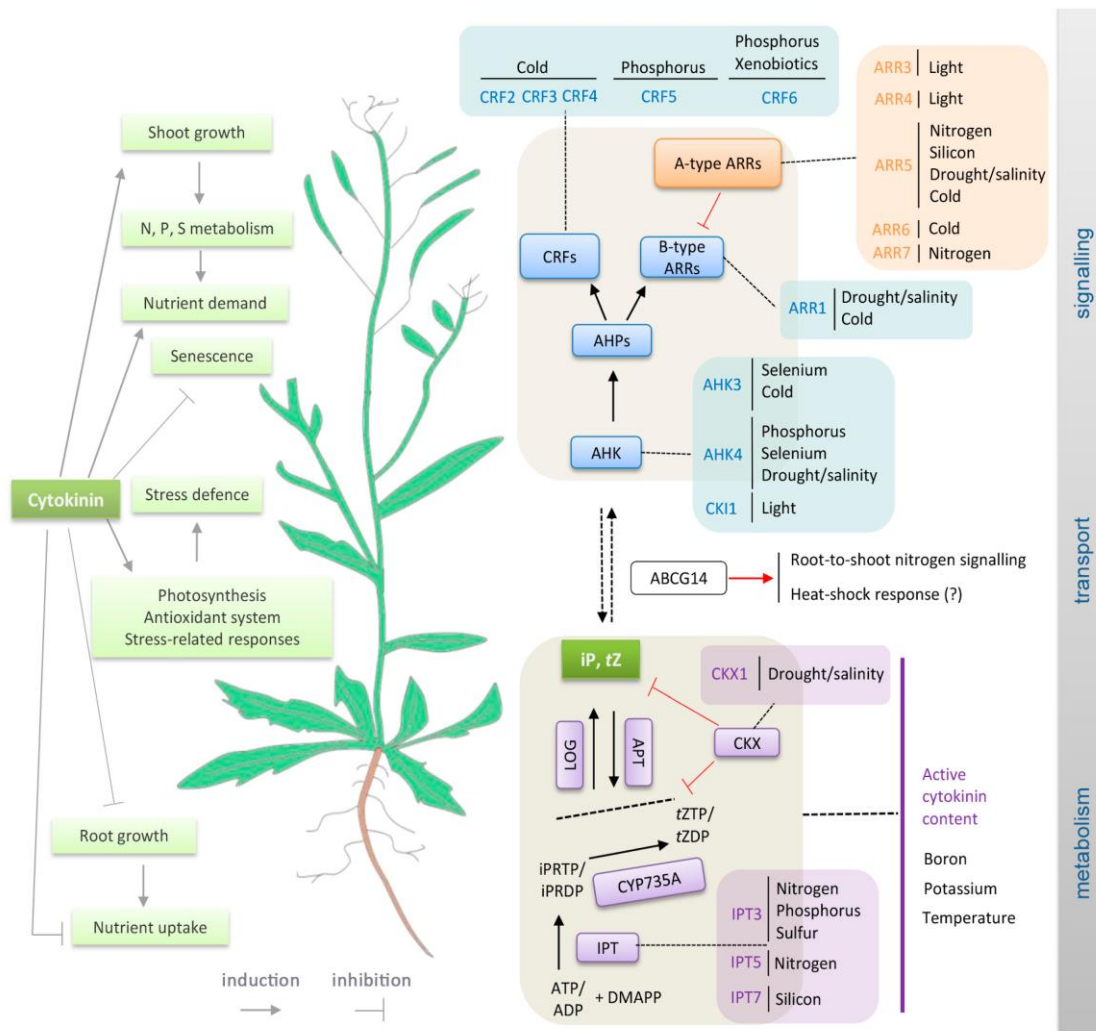
Figure 19. Cytokinin at the crossroads of abiotic stress signalling pathways (Pavlů et al., 2018; Included Publication 3).

Cytokinin is a multifaceted plant hormone that plays major roles in plant interaction with abiotic stimuli. We have summarized these avenues of cytokinin signaling in a recent review (Pavlů et al., 2018, Included Publication 3) and contributed to its elucidation with our own research. Besides the cytokinin role in the early response to temperature shock and thermomorphogenesis (Chapter 4.2), we have analyzed its effect in *Arabidopsis* response to different abiotic factors, including drought (Prerostova et al., 2018), cold (Prerostova et al., 2021) and light (Novák et al., 2015).

4.4.1 Barley root proteome in response to cytokinin and abiotic stimuli

We found that barley seedling root is an excellent model for proteomics analysis of cytokinin's role in early plant development. Three-day-old seedlings were treated with 1 µM *trans*-zeatin for 24 h. The consecutive proteomics analysis revealed a clear separation between cytokinin- and mock-treated samples (Figure 20A). In total, we identified 178 differentially abundant cytokinin-responsive proteins, representing more than 12% of the estimated total root proteome (Berka et al., 2020b; Included Publication 7). The list of cytokinin-responsive proteins indicated the expected crosstalk with abiotic stress perception, including response to salinity, temperature, and reactive oxygen species (Figure 20B). Furthermore, about one-third of the cytokinin-repressed proteins was associated with a stress response, indicating a putative connection between cytokinin-induced alleviation or attenuation of stress.
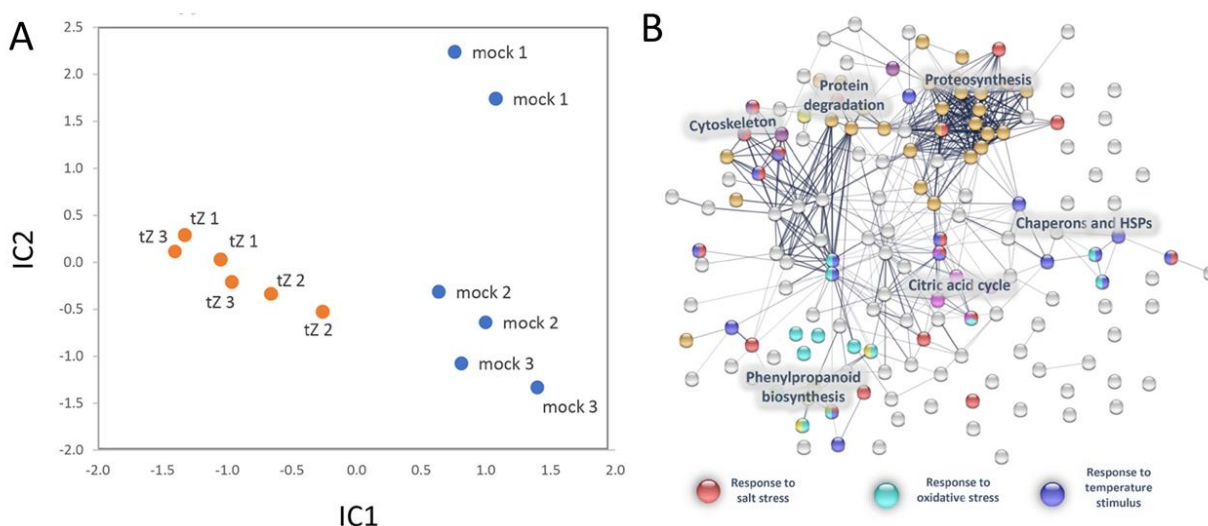
Figure 20. Barley root proteome in response to cytokinin. (A) Proteome profile separation of samples treated with 1 µM *trans*-zeatin for 24 h; (B) Interactions and functional clusters of cytokinin-responsive proteins highlighted by STRING (Szklarczyk et al., 2019). The selected highlighted categories represent the response to abiotic stimuli (Berka et al., 2020b; Included Publication 7).

Next, we designed a set of experiments to explore this putative crosstalk. Sets of 10 germinated seedlings were exposed to abiotic stress. The temperature stress was stimulated by exposure to 30°C or 4°C temperature for 2 h, followed by a 22 h recovery period at 20°C, salinity response by medium supplemented with 80 mM NaCl (final concentration), and

drought by transfer to a dry Magenta box. We analyzed root proteomes and found 308 stress-responsive proteins. The overlap between abiotic stress and cytokinin response was high. Only 76 cytokinin-responsive proteins were not considered differentially abundant in response to any of the abiotic stimuli (Figure 21A). In total, out of 178 cytokinin-responsive proteins, 81 were found in the set of temperature-stress-responsive proteins and most shared a similar response between temperature stress and cytokinin treatment. Furthermore, abundances of 48 cytokinin-responsive root proteins were found with a similar response under salinity stress or water deprivation. The proteome of cytokinin-treated roots was clearly separated from abiotic stress in the ICA (Figure 21B). However, the observed similarities between abiotic stimuli and cytokinin lead us to the speculation that this priming could be responsible for an enhanced resilience found in plants with a modulated cytokinin pool (e.g., Přerostová et al., 2018).

Figure 21. Response to abiotic stress and cytokinin. (A) Venn diagram summarizing all identified differentially abundant proteins in response to cytokinin or abiotic stimuli; (B) Clusters of treatments obtained from independent component analysis of protein profiles. Dashed circles represent statistically significant separation (Kruskal-Wallis test, $p < 0.05$). Adapted from Included Publication 7.

Finally, we validated the expected changes in the reactive oxygen species metabolism. We found more than 90 enzymes of reactive oxygen species metabolism in our dataset, and at least 11 were significantly differentially abundant in response to cytokinin, including all detected catalase isoforms. The measurement of aqueous hydrogen peroxide showed that the

cytokinin treatment significantly reduced hydrogen peroxide content in barley roots by more than 25% (Figure 22A), and histochemical staining revealed that the reduction in hydrogen peroxide and superoxide radicals in the root tips was even higher (Figure 22B-E).

Figure 22. Cytokinin impact on the reactive oxygen species production. The estimated mean content of hydrogen peroxide in barley roots determined by (A) PeroxiDetect Kit and (B) 3,3'-diaminobenzidine (DAB) staining; (C) Cytokinin impact on superoxide radical production as estimated by histochemical staining with nitroblue tetrazolium (NBT); Representative images of cytokinin- and mock-treated roots stained with DAB (D) and NBT (E). Presented data are means and standard deviation of at least three biological replicates; Statistically significant differences (Student's t-test) are indicated (Berka et al., 2020b; Included Publication 7).

4.4.2 *Arabidopsis* response to Inhibitor of Cytokinin Degradation INCYDE

The analysis of barley cytokinin response proteins (4.4.1) demonstrated the intensive crosstalk between cytokinin and abiotic stimuli. Indeed, modulations in cytokinin metabolism and signaling have been successfully used for elevating plant tolerance to abiotic stressors (Iqbal et al., 2006; Merewitz et al., 2012; Reguera et al., 2013). The cytokinin synthesis driven by stress-induced or senescence-activated promoters is a useful tool, but the present-day anti-GMO regulations limit its application. However, plant cytokinin levels can

be modulated by the application of exogenous hormones, and several studies have found a positive effect of cytokinin treatment on crop production (e.g., Iqbal et al., 2006; Kariali and Mohapatra, 2007). Alternatively, a pharmacological treatment can inhibit cytokinin dehydrogenase (CKX), the main enzyme that catalyzes the inactivation by irreversible degradation of cytokinins. INCYDE [2-chloro-6-(3-methoxyphenyl)aminopurine] is one of the compounds with a high affinity for the CKX enzyme, and its application reportedly improved plant resistance to diverse abiotic stresses, including salinity, heat stress recovery, and heavy metal toxicity (Gemrotová et al., 2013; Aremu et al., 2014; Prerostova et al., 2020). It is believed that this positive effect on plant resilience is predominantly the result of cytokinin accumulation, but the exact molecular mechanisms are far from being understood. We analyzed the impact of INCYDE treatment on the model plant *Arabidopsis thaliana* and compared its effect to that of a major active cytokinin base *trans*-zeatin (Berková et al., 2020; Included Publication 8).

We did not find any striking differences in root growth inhibition assays, but the analysis of the cytokinin signaling reporter revealed that the growth of seedlings in the presence of INCYDE led to a higher increase in the *ARR5* promoter activity in cotyledons (100–500 nM) but a lower cytokinin signaling in the roots (10–100 nM) (Figure 23A-B). CKX is encoded by seven genes with different substrate specificity, spatial and temporal expressions, and subcellular targeting into the cytosol (CKX7), vacuole (CKX1, CKX3) and endoplasmic reticulum or apoplast (CKX2, CKX4-6). The observed cytokinin signaling response was similar to the expected profiles of apoplastic isoforms CKX4 and CKX5. The apoplastic CKX enzymes found in *Arabidopsis* act mainly on cytokinin free bases and ribosides (Frebort et al., 2011), and these are also the root-to-shoot long-distance signaling forms of cytokinin. It is thus possible that at a given developmental stage, the majority of the INCYDE effect is due to the interference with the transport of active cytokinin in plants.

To provide an insight into the molecular mechanisms behind the observed contrasting response, we analyzed the proteomes of seven-day-old seedlings treated for 24 h with 500 nM *trans*-zeatin, INCYDE, or dimethylsulphoxide (mock). In total, 3,273 *Arabidopsis* proteins were identified with reliable quantitative data for more than 2,100 of these. As

illustrated in Figure 23C, a statistically significant ($p < 0.05$) separation between *trans*-zeatin, INCYDE and mock-treated samples was apparent. A detailed analysis revealed 89 and 99 *trans*-zeatin and INCYDE early-response proteins compared to mock-treatment, respectively, and 69 proteins that showed statistically significant and reproducible differences between INCYDE- and zeatin-treated samples (Figure 23D-E). Early INCYDE response proteins encompassed diverse processes of both primary and secondary metabolism. The comparison with zeatin-responsive proteins showed only 48 shared proteins, but all with a similar responsiveness. Significantly accumulated proteins included those required for chloroplast biogenesis and development, enzymes involved in cell wall formation, chloroplastic and cytosolic isoforms of glutamine synthetase, and an extracellular protein with a putative role in circadian rhythm GER3. Incidentally, our previous work demonstrated an intensive modulation of *Arabidopsis* proteome diurnal rhythmicity in response to *trans*-zeatin (Luklová, 2018).

Next, we evaluated a dose-dependent response after 168 h. Seven-day-old seedlings cultivated on textile meshes were transferred onto new medium supplemented with 10-1,000 nM INCYDE, and after seven days, shoots were collected for proteome analyses. We found 517 INCYDE responsive proteins, representing an estimated 29% of the total protein extract. A detailed comparison of differentially abundant proteins confirmed the expected overlap between the INCYDE treatments. This set of 167 proteins included proteins involved in RNA metabolism, chromatin remodeling, proteosynthesis, ribosome biogenesis, tRNA metabolism, or protein folding. Interestingly, the strongest response was present at 100 nM INCYDE treatment (230 unique INCYDE-responsive proteins), and the GO enrichment revealed that this response elicited the highest similarity to the annotated response to cytokinin. We also searched for proteins that abundance would correlate with the INCYDE dosage and identified 25 and 11 statistically significant positive and negative correlations, respectively. These INCYDE-responsive proteins are involved in signaling, primary and secondary metabolism, including γ-aminobutyric acid biosynthesis, glucosinolate degradation, and processes of chloroplast biogenesis and development (Berková et al., 2020; Included Publication 8).
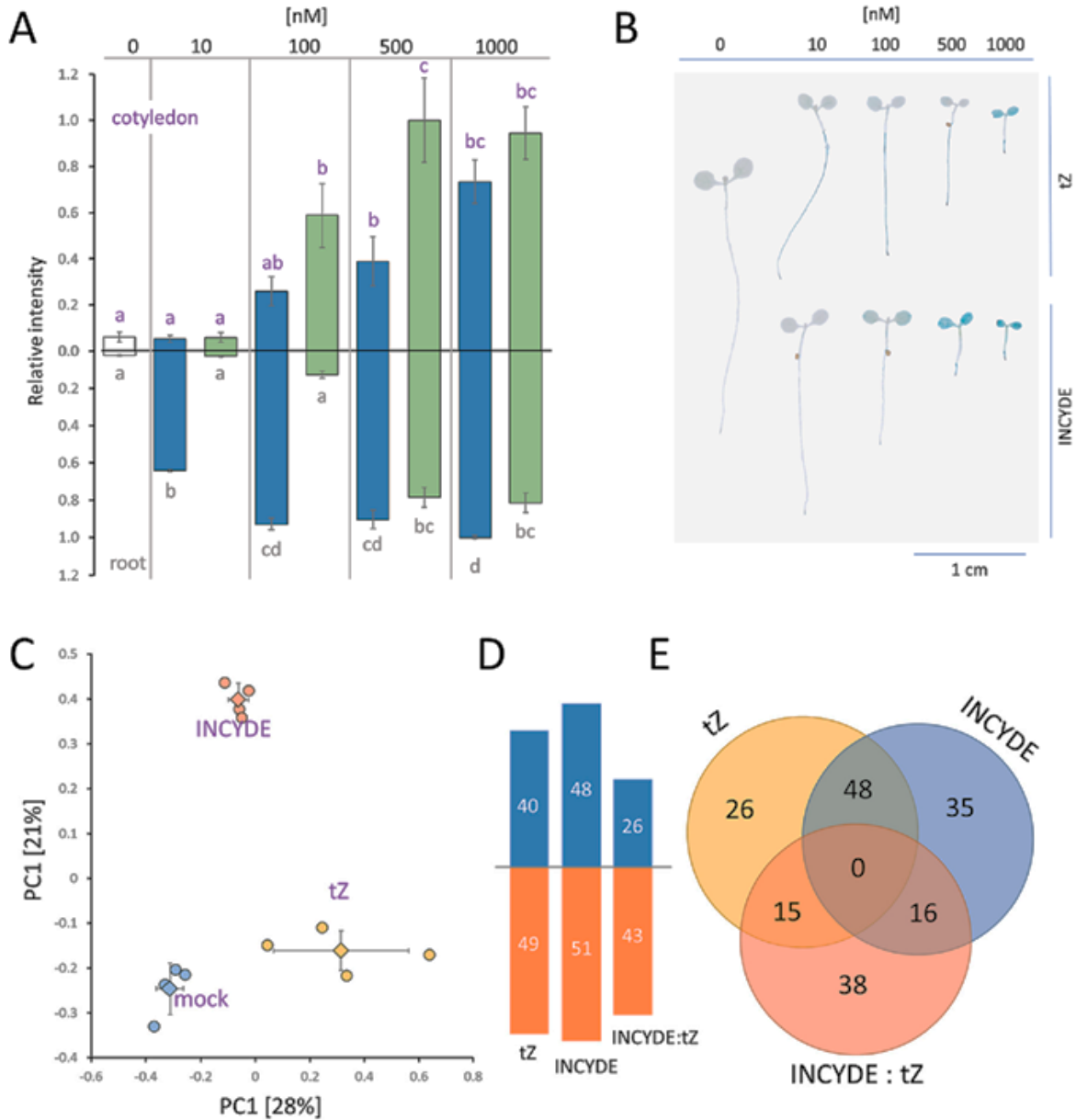
Figure 23. Differences between *trans*-zeatin (tZ) response and effects of cytokinin dehydrogenase inhibitor INCYDE. (A) Comparison of normalized ARR5 promoter activity visualized by histochemical staining and (B) representative images of seven-day-old ARR5::GUS reporter line cultivated on the medium supplemented with *trans*-zeatin. Results represent means and standard error, different letters indicate significant differences (Kruskal-Wallis, n>15, p < 0.05); (C) Proteome profile separation after 24 h incubation with 500 nM INCYDE or tZ. Principal component analysis based on quantitative data of 178 differentially abundant proteins; (D) Differentially abundant proteins accumulated (blue) and decreased (orange) compared to mock (tZ—tZ vs. mock; INCYDE—INCYDE vs. mock) or tZ-treated samples (INCYDE:tZ); (E) Overlap between tZ and INCYDE-responsive proteins (Adapted from Berková et al., 2020; Included Publication 8).

The ribosome composition reflects external stimuli and may have a significant impact on plant responses. Our previous analysis of plants with modulated active cytokinin levels revealed a putative cytokinin effect on ribosome composition (Černý et al., 2013a), and a similar effect was found in response to INCYDE. In total, the abundances of 34 ribosomal proteins were altered in the shoot proteome, representing 29 different ribosomal subunits (Figure 24). However, the most prominent INCYDE-responsive paralogs formed only 50% of the detected ribosomal protein isoforms. This indicates that the total ribosome population was not completely altered, or that the alteration was cell-specific, and that the localized distribution was lost in the total shoot protein extracts.
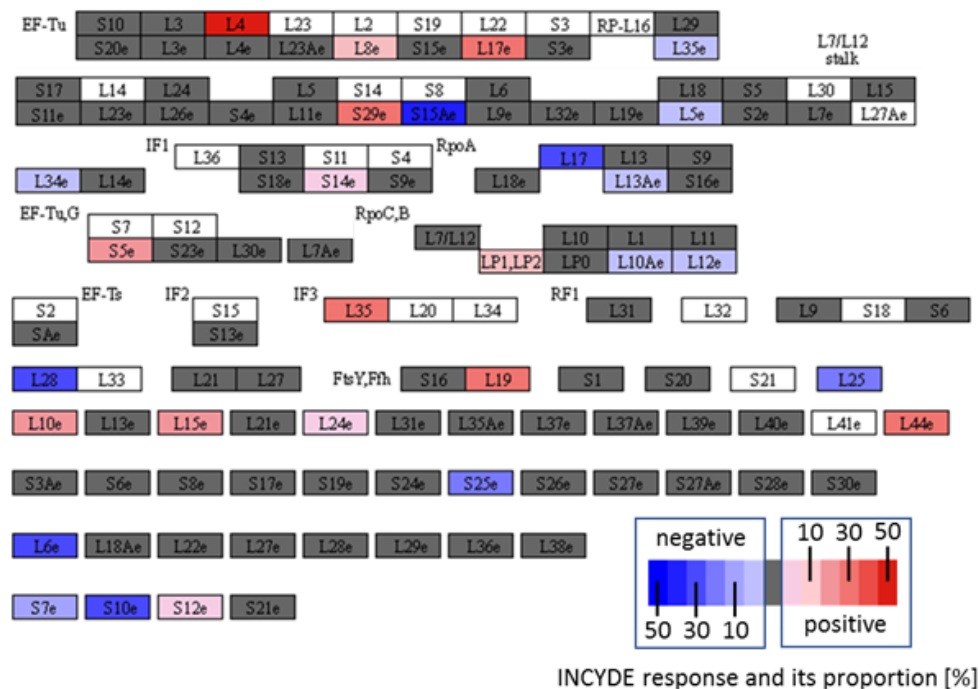
Figure 24. A graphical representation of all identified ribosomal subunits in shoots of plants exposed to INCYDE for 168 h. Blue and red gradients indicate subunits with one or more INCYDE-responsive paralogs. The contribution of the paralog to the subunit composition [%] is indicated; white—not detected; grey—no significant difference compared to the mock-treated plants (Berková et al., 2020; Included Publication 8).

Finally, we searched our proteomics data for changes that could shed light on the reported INCYDE-promoted growth under suboptimal conditions. We found that INCYDE attenuated abscisic acid signaling. That was reflected in a depletion of transcription factor NFYC4, accumulation of ABI1, and depletion of at least 21 additional stress-responsive proteins. The comparison of INCYDE response proteins with our database of previously

identified phytohormone-responsive proteins (Černý et al., 2016) found that the INCYDE response was predominantly opposite to that of jasmonic acid or abscisic acid treatment. Thus, we believe that the INCYDE-repressed degradation of cytokinin inhibits stress perception in plants.

5. Proteomics of biotic interactions

5.1 Biotic interactions

The endosymbiotic theory postulates that mitochondria and plastids were once separate prokaryotic microbes. Consequently, biotic interactions formed the plant cell and have been shaping plants ever since. In their natural environment, plants have to interact and cope with many different organisms simultaneously at any time. The complex nature of biotic interactions is far beyond the scope of this work, and only the key points relevant to the described experiments will be summarized. Some of these interactions are beneficial and provide an advantage. For instance, approximately 80% of biological nitrogen fixation is produced in symbiotic associations with bacteria. Similarly, most terrestrial plants are supported by arbuscular mycorrhiza, which is formed by an interaction with obligate symbiotic fungi (Schüßler et al., 2007). Mycorrhizae play an essential role in plant growth and disease protection. Fungi supply plants with inorganic nutrients and water in exchange of carbohydrates. Conversely, this mutualism may occur even at an immeasurably low profit for fungi. That is characteristic for orchidaceous mycorrhizae. Orchids start their lives as myco-heterotrophs and depend on their fungal benefactors. Some orchids are nonphotosynthetic and are full myco-heterotrophs throughout their life. Yet even those that become photosynthetic at maturity retain their mycorrhizal fungi for protection and nutrient recovery (Bidartondo, 2005).

Plants interact indirectly with their neighbors by releasing phytochemical compounds into the environment. This long-distance communication called allelopathy is usually (but not exclusively) facilitated by volatile compounds and has beneficial or detrimental effects on target organisms (Cheng and Cheng, 2015). Allelopathy might be a part of resource competition between organisms, but it also contributes to the coexistence of closely related species (Zhang et al., 2021). Besides the direct effect on competitors in a plant's proximity, plants may produce volatiles conveying information about their identity to attract pollinators or enemies of attacking herbivores (Cusumano et al., 2015).

Adverse biotic interactions have shaped plant evolution and facilitated the development of a unique plant immune system. The attack by deleterious organisms activates the plant defense response, and the outcome can be broadly characterized as an incompatible and compatible interaction, representing successful and failed defense, respectively (Glazebrook, 2005). However, this does not fully reflect plants' resistance. A resistant host will not show infection symptoms but may still mediate pathogen replication if the host-pathogen interaction is compatible and the plant does not respond to the pathogen's presence. There are several distinct barriers protecting plants against pathogens and pests. Some mechanisms are constitutive and form the innate immunity. For instance, many plants produce a range of secondary metabolites with antimicrobial properties and toxic proteins, including alkaloids, glucosinolates, lectins, and protein inhibitors (Heldt and Piechulla, 2021). The next levels of induced defense are formed by pathogen-associated molecular patterns-triggered immunity, effector-triggered immunity, and RNA interference. Examples of pathogen molecules that are recognized by plant receptors are fungal chitin fragments, lipopolysaccharides, peptidoglycans, and bacterial flagellin (Newman et al., 2013). Binding of these molecules to the plasma membrane receptors triggers transcriptional reprogramming via the activation of calcium-dependent protein kinases and mitogen-activated protein kinase cascades (Buchanan et al., 2015). The resulting effect includes reactive oxygen species burst at the cell surface, strengthening of the cell wall, production of phytoalexins, jasmonates, or secretion of chitinases. Pathogens have evolved mechanisms to counter this process by delivering effectors into plant cells, which, in turn, may activate effector-triggered immunity, the second level of induced defense (Han, 2019). This recognition is facilitated by disease resistance proteins and may culminate in a hypersensitive response and programmed cell death containing the pathogen at the site of infection (Mukhtar et al., 2016). It also elicits systemic signaling and induces the accumulation of salicylic acid in uninfected tissues, leading to the production of so-called pathogenesis-related proteins with antimicrobial activity.

Proteins form an indispensable part of the plant defense system, those present in plant cells, as well as those that are secreted into the apoplast or even the environment. The conventional plant protein secretion pathway requires N-terminally located transit peptides that target proteins via the endoplasmic reticulum and the Golgi apparatus to the plasma membrane

(Wang et al., 2017). Interestingly, despite the presence of cell walls, plants may secret proteins via alternative unconventional protein secretory pathways. These Leaderless Secreted Proteins that are devoid of the consensus N-terminal peptide sequence may be secreted by at least four distinct mechanisms, namely, a pore-mediated translocation across the plasma membrane, an ABC transporter mediated secretion, an autophagosome/endosome-based secretion, and a Golgi bypass (Padmanabhan and Manjithaya, 2020). The exact extent of these alternative pathways in plants has not yet been fully elucidated. However, it has been confirmed that the extracellular vesicles in the plant secretome contribute to the plant defense system (Regente et al., 2017).

5.2 Peptide-based identification of microorganisms in plants

The detection of microbes in general is important for our understanding of biomes and it is also an essential step in disease control. Traditional approaches for microbial detection and identification employ microbial cultivation. However, that approach was not reliable and has been replaced by molecular methods based on antibody detection and PCR. The sensitivity of PCR is still beyond the reach of proteomics analyses, but unlike enzyme-linked immunosorbent assays, the present-day MS proteomics techniques have shown high sensitivity as well as specificity that can be utilized for the identification of microorganisms (e.g., Strejcek et al., 2018; Hayoun et al., 2020; Included Publication 9). Nonetheless, the detection of microbes within the proteome of its host is more complicated. A major obstacle for high-throughput shotgun LC-MS proteomic approach is the presence of evolutionary conserved sequences. Some of these could be a result of plant-microbe interaction and horizontal gene transfer, but the most abundant peptides originate from constituents of primary metabolism, including ribosomal proteins, histones, and enzymes of energetic metabolism. As illustrated in Figure 25, the theoretical overlap in tryptic peptides is not that significant. However, the shared peptides have a higher abundance and the correct assignment of the species of origin may be a challenge and should not be underestimated in non-model species with limited genome annotations.
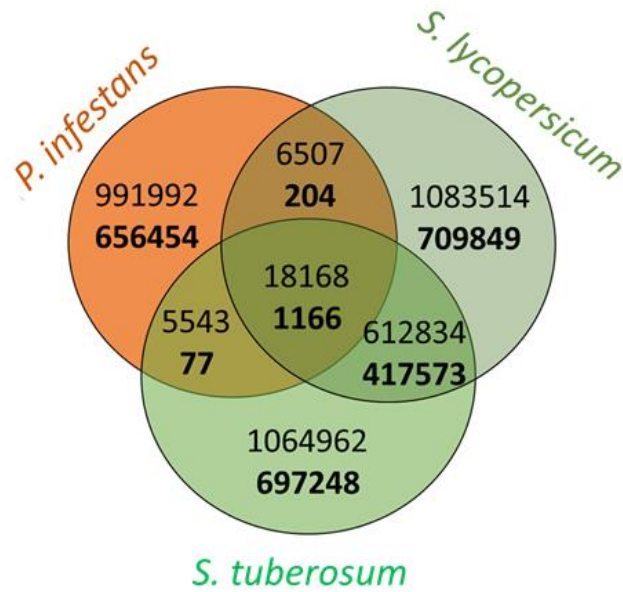
Figure 25. Evolutionarily conserved peptide sequences in three model genomes digested by trypsin *in silico*. Numbers corresponding to low-stringency (maximum three miscleavages, 6–30 amino acids per peptide) and high-stringency criteria peptides (maximum one miscleavage, 8–25 amino acids per peptide) are represented in plain and bold font, respectively (Adapted from Berka et al., 2020a; Included Publication 9).

We have encountered this issue in our own experiments with phytopathogenic oomycetes, namely, *Phytophthora infestans*. To elucidate the source of putative *P. infestans* proteins, we analyzed the time-course response of proteomes of infected leaves. We inoculated detached leaves of *Solanum tuberosum* (cv. Kerkovske rohlicky) with a mixture of *P. infestans* isolates, sampled 72 and 96 h after the inoculation, and analyzed proteome profiles (Figure 26A). The set of 146 *P. infestans* proteins (containing at least two detected unique peptides and not identified in the mock-treated samples) were analyzed in detail. Functional enrichment by STRING (Figure 26B) showed that these were enzymes belonging to amino acid metabolism, ribosomal proteins, ROS metabolism enzymes, CAZymes, and components of the proteosynthetic machinery and proteasome. Surprisingly, only six detected proteins were annotated as secreted effectors.
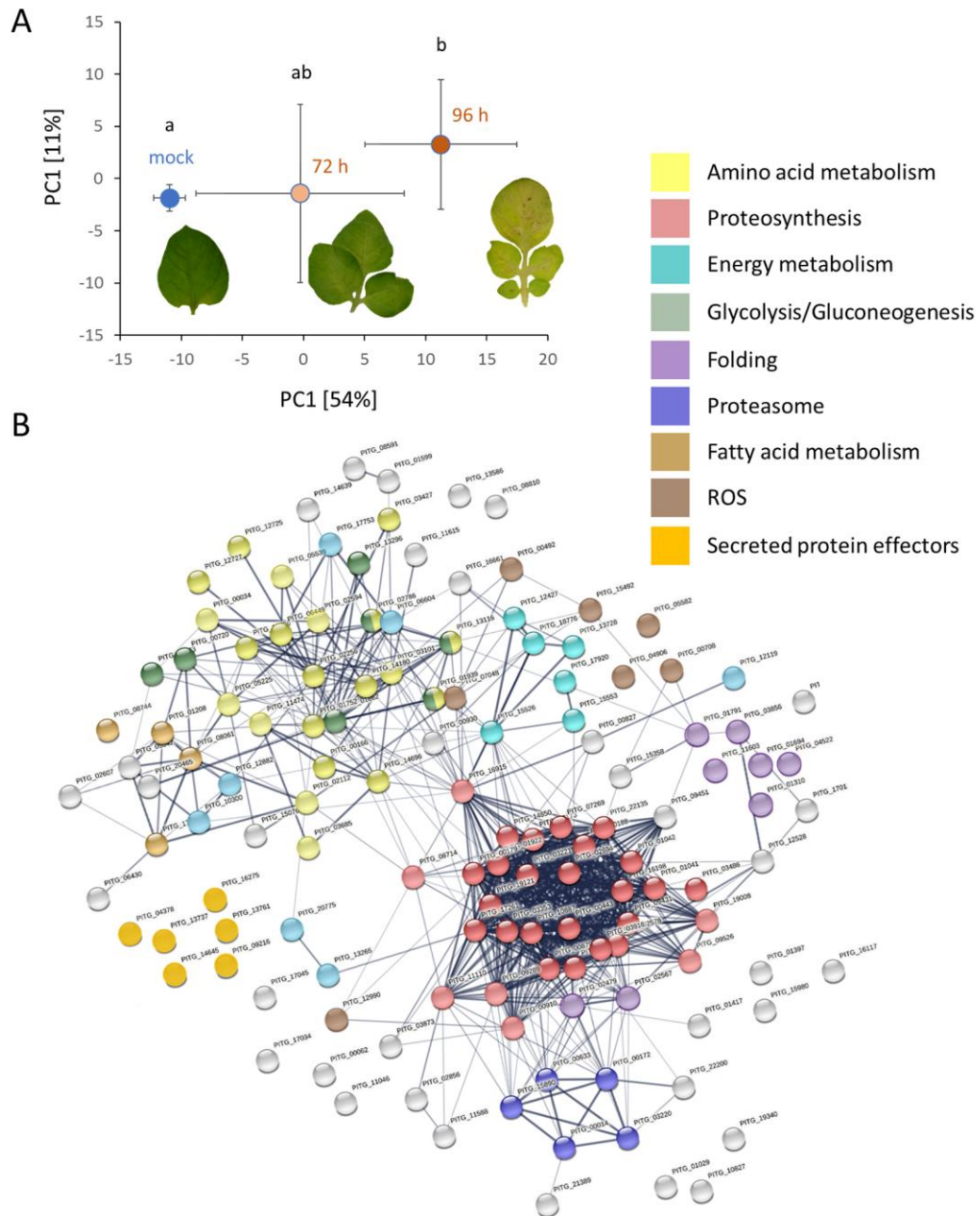
Figure 26. Detection of *P. infestans* in inoculated detached leaves of *Solanum tuberosum*. (A) Representative images of plant material and separation of proteome profiles of *S. tuberosum* leaf 72 and 96 h after inoculating with *P. infestans*. Different letters indicate significant differences (Kruskal–Wallis test, p < 0.05, n=5); (B) Interactions and functional clusters of high-confidence *P. infestans* proteins highlighted by STRING. Color-coding of proteins is denoted by the functional designation given by KEGG pathway enrichment, only the nine most significant categories are highlighted (Adapted from Berka et al., 2020a; Included Publication 9).

Finally, we performed qPCR and compared the estimated protein abundances with the *P. infestans* DNA content. As illustrated in Figure 27, more than 300 out of 802 quantified proteins showed a strong correlation with the *P. infestans* DNA amount. *P. infestans* employs a biphasic infection strategy and minimal symptoms are exhibited by the plant at the initial biotrophic phase (e.g., Zuluaga et al., 2016). Nevertheless, we were able to identify more than 90 of these putative *P. infestans* marker peptides in samples collected in our field experiments, providing direct evidence that this technique is a suitable method for *P. infestans* monitoring. It is not likely that this relatively expensive method would become a method of choice for *Phytophthora* control, but it provides a more exact and PCR-independent estimation of the disease progress.
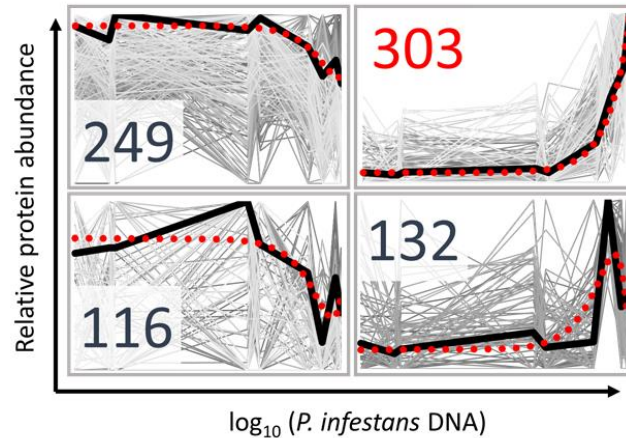
Figure 27. Identification of putative *P. infestans* marker peptides. Correlations between P. infestans DNA and protein relative abundances. Numbers indicate the size of the protein profile cluster. The mean profile and polynomial regression are represented by black and red curves, respectively (Adapted from Berka et al., 2020a; Included Publication 9).

5.3 Putative role of HSP70 in *Plasmodiophora brassicae* infection

Plasmodiophora brassicae Wor. is a root-infecting protist pathogen in the eukaryotic kingdom that causes an economically important clubroot disease of plants in the Brassicaceae family. The disease is characterized by enlargement and uncontrolled cell division leading to the development of galls on infected roots. Club-root disease has become an increasingly serious economic threat for agriculture with reported yield losses up to 91% (Hwang et al., 2012). Furthermore, galls contain long-lived resting spores that are released

into the soil and can persist for many years and serve as an inoculum for the infection of subsequent crops (Howard et al., 2010).

We analyzed *Plasmodiophora* proteome in the root gall of *Arabidopsis* and found that its most abundant protein was an orthologue of *Phytophthora infestans* HSP70 (Malych and Berka, 2019). The role of HSPs has been documented in malaria disease caused by *Plasmodium* spp. (Przyborski et al., 2015), and several studies have highlighted the role of HSPs in plant-biotic interactions. For instance, it has been demonstrated that HSP90 and HSP70 are important for plant defense signal transduction pathways (Kanzaki et al., 2003) and that a mutation in HSP genes may increase susceptibility to pathogens (Jacob et al., 2017). Furthermore, pathogens like *Pseudomonas syringae* may directly hijack the plant's HSPs and recruit these to facilitate the pathogen's virulence (Jelenska et al., 2010).

To confirm the role of HSP in *Plasmodiophora* infection, we analyzed different stages of clubroot development. In total, 5,287 proteins were identified, including 3,704 *Arabidopsis* and 1,583 *P. brassicae* proteins, respectively (Figure 28; Kopecká, 2020). Results showed a clear proteome profile separation of different infection stages (Figure 28A), and a detailed analysis revealed quantitative data for 21 *Plasmodiophora* HSPs (Figure 28B). The previously identified CEO96729 was again one of the most abundant *Plasmodiophora* proteins and represented on average 24% of all *Plasmodiophora* HSPs. This protein also showed the highest correlation with the infection progress (Pearson's correlation coefficient r>0.9) and its amount was proportional to the estimated *Plasmodiophora* protein content in *Arabidopsis* tissue. Interestingly, mRNA analysis showed that the mRNA of its *Arabidopsis* orthologue *HSP70-14* was also significantly upregulated (Kopecká, 2020). However, the upregulation was not reflected in the HSP70-14 protein content. A similar decrease was found for all 18 detected *Arabidopsis* HSP70s, and the abundance of HSP70s showed a strong correlation with the estimated *Arabidopsis* protein content, which represented only 56±2% of the root gall proteome in the fifth stage of its development.

HSPs are proteins with a role in the unconventional protein secretion as a part of the mechanism via chaperone-mediated autophagy (Padmanabhan and Manjithaya, 2020) as

well as the proteins that are being secreted. It has been shown that secreted human HSPs participate in the immune response and facilitate stress signal propagation and priming. It is tempting to speculate that the most abundant *Plasmodiophora* HSP70 has a direct role in preventing the host's immune response and that it interferes with the host's HSPs. However, the existence of this mechanism in plants has not yet been reported.

Protein ID	Family	1	1	2	3	4	5
CEO94635	HSP90						
CEP03176	HSP90						
CEO95605	HSP70						
CEO96729	HSP70						
CEO98236	HSP70						
CEO98303	HSP70						
CEP02017	HSP70						
CEO94485	HSP40						
CEO97199	HSP40						
CEP02046	HSP40						
CEO95979	HSP20						
CEO96076	HSP20						
CEO96287	HSP20						
CEO96288	HSP20						
CEO98046	HSP20						
CEO98050	HSP20						
CEP00014	HSP20						
CEP00299	HSP20						
CEP00597	HSP20						
CEP00666	HSP20						
CEP03223	HSP20						
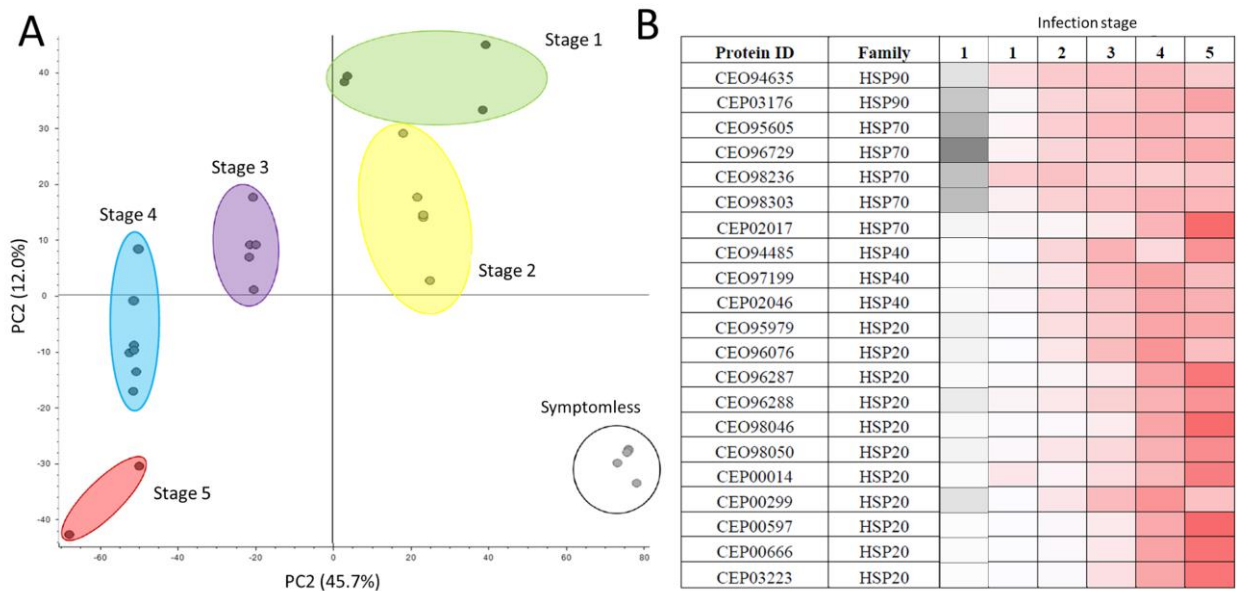
Figure 28. Relative protein abundances of *Plasmodiophora* HSP family proteins during the infection. (A) PCA separation of root gall proteomes and (B) a heatmap representation of an average HSP profile in up to five biological replicates. Relative protein abundances in the first stage of infection and median-normalized abundances of individual proteins are represented by gray and red heatmaps, respectively (Kopecká, 2020).

5.3 Proteomics offers insight to the mechanism behind *Pisum sativum* L. response to *Pea seed-borne mosaic virus* (PSbMV)

Pea seed-borne mosaic virus (PSbMV; family *Potyviridae*) is a non-persistent seed-borne and aphid-borne pathogen that has been dispersed globally by seed stock exchange. Its host range is naturally limited to leguminous plants and includes at least 47 plant species belonging to 12 families. The symptoms are often difficult to see, and the effect on crop yield may vary. The effect is most serious when seeds with infection are sown, and aphid population growth is encouraged by favorable weather (Konečná et al., 2014; Makkouk et al., 2014).

The eukaryotic translation initiation factor confers resistance to PSbMV (Smýkal et al., 2010), implying that translation and proteome dynamics play a role in resistance. In our study, we inoculated *Pisum sativum* plants of two contrasting cultivars, namely, the resistant cultivar B99 (viral replication is not detectable) and the susceptible cultivar Raman (severe symptoms of viral infection). We analyzed proteome profiles at two different time points (10 and 20 days post-inoculation) that best reflect a plant's struggle before the onset of severe symptoms in the sensitive cultivar (Figure 29).
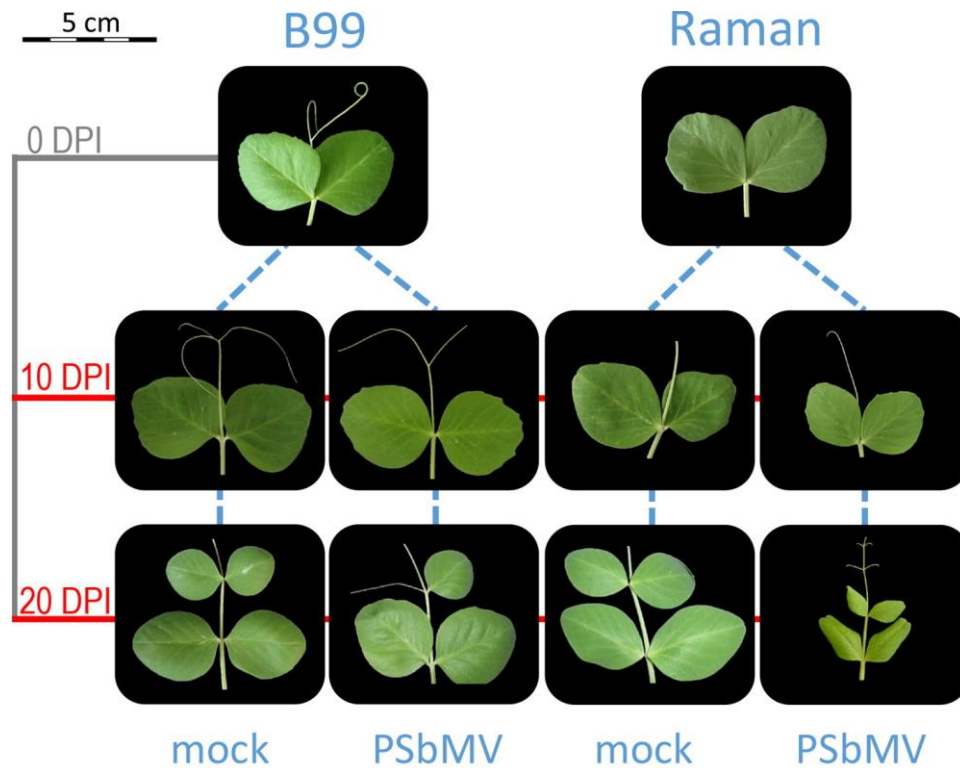
Figure 29. Leaves of PSbMV-sensitive and resistant pea cultivars B99 and Raman collected 10 and 20 days post inoculation (Adapted from Cerna et al., 2017; Included Publication 2).

First, we determined virus presence in all PSbMV-inoculated plants. Only four viral proteins produced by proteolytic processing of two polyproteins encoded by potyviral RNA were detectable in our samples, namely, Helper component proteinase, cytoplasmic inclusion protein, viral genome-linked protein, and capsid protein. These four proteins were detected only in the sensitive PSbMV-inoculated Raman cultivar, and similar results were confirmed by RT-qPCR.

The analysis of *Pisum sativum* leaf proteome revealed over 2,300 proteins, and 116 proteins demonstrated statistically significant and reproducible changes over all experimental replicates. The identified proteins represented a broad range of primary and secondary metabolism, including enzymes of amino acid biosynthesis, protein metabolism, photosynthetic proteins, CAZymes, lipid metabolism, proteins of signaling, redox homeostasis, and stress response (Figure 30). Surprisingly, a majority of these PSbMV-responsive proteins were found to be differentially abundant in both cultivars, when compared to the mock inoculation samples. That implies that a seemingly unaffected resistant plant, with no detectable levels of PSbMV, was actively suppressing viral replication.

Only 13 and 23 differentially abundant proteins were specific for B99 and Raman, respectively. Among these, there was an evident increase in proteins participating in lipid and amino acid metabolism in the resistant and susceptible cultivars, respectively. One of the B99-specific responsive proteins is Phospholipase D, an important member of signaling cascades that participates in many physiological processes, including membrane trafficking, cytoskeletal reorganization, and wounding. Our dataset also contained a non-specific lipid transfer protein with lipase activity, patatin, and five homologs of enzymes known to participate in lipid metabolism. The levels of all of these proteins increased in the resistant cultivar following PSbMV inoculation, but the same response was delayed or even absent in the susceptible cultivar. This decrease in lipid metabolism was also apparent in the fatty acid pool, which was significantly lower in infected Raman plants.

Similar to our analysis of *Plasmodiophora* disease, an interesting candidate and putative resistance/susceptibility determinant could be the protein HSP70. Two HSP70s were differentially abundant at 20 days post-inoculation in our dataset, but only one was B99 specific. Its *Arabidopsis* ortholog, HSP70-3 (>91.5% identity), interacts with the RNA-dependent RNA polymerase of the *Turnip mosaic virus* within virus-induced membrane vesicles and is believed to have an important function in viral replication (Dufresne et al., 2008).
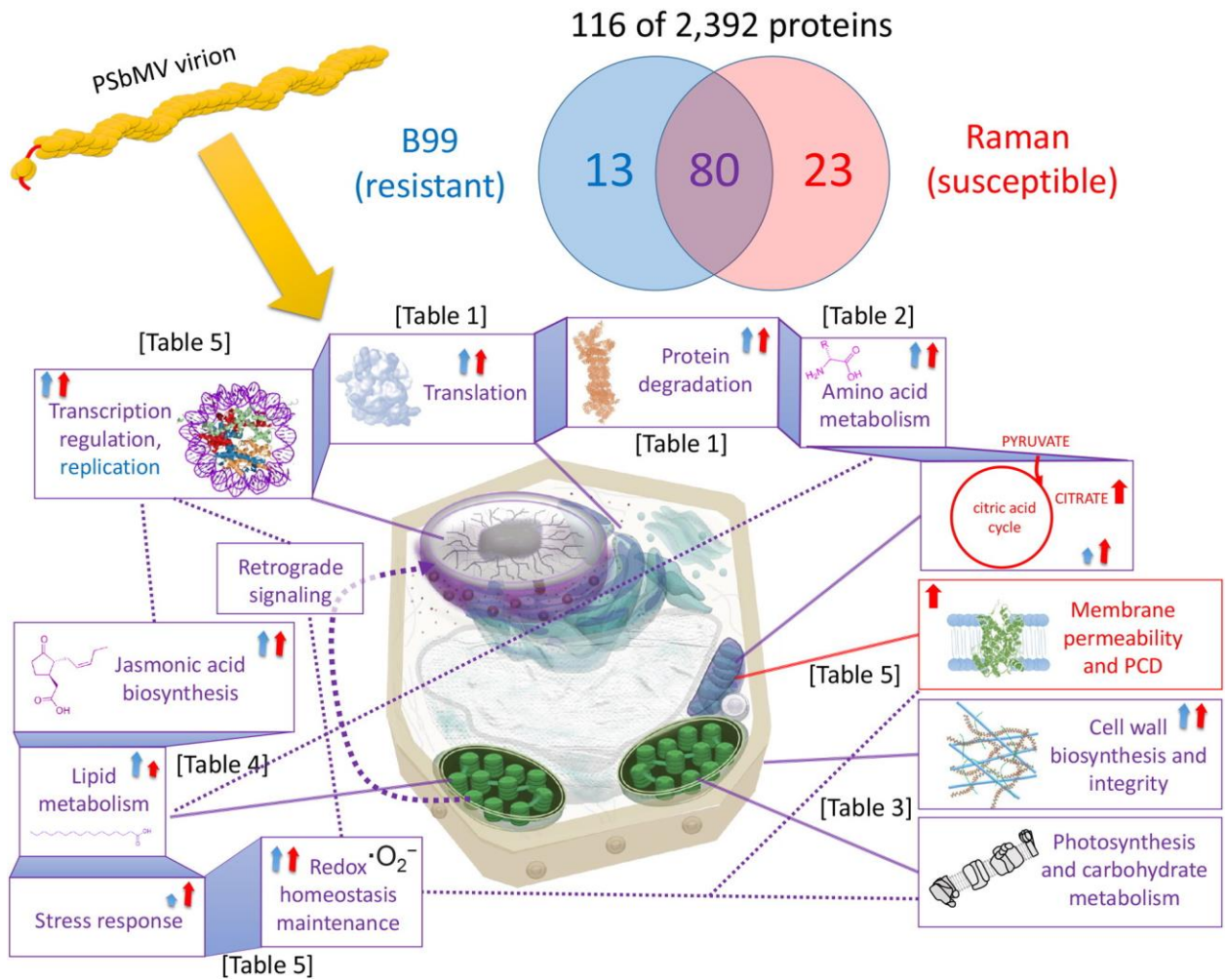
Figure 30. Processes revealed by proteomics analysis of PSbMV-sensitive and resistant pea cultivars B99 and Raman in response to PSbMV inoculation. The referenced tables are part of the Included Publication 2 (Cerna et al., 2017; Included Publication 2).

5.4 Balancing positives and negatives - *Acremonium alternatum* story

The outcome of the interaction between a host plant and fungus is based on a fine-tuned balance and shares many common attributes with plant interactions with pathogens (Kogel et al., 2006). For instance, *Acremonium alternatum* may suppress clubroot disease in *Arabidopsis* (Jäschke et al., 2010) and promote *Arabidopsis* growth (Figure 31; Malých, 2020). However, we found that its positive effect is lost on a medium supplemented with 1% sucrose (Figure 31A-B).
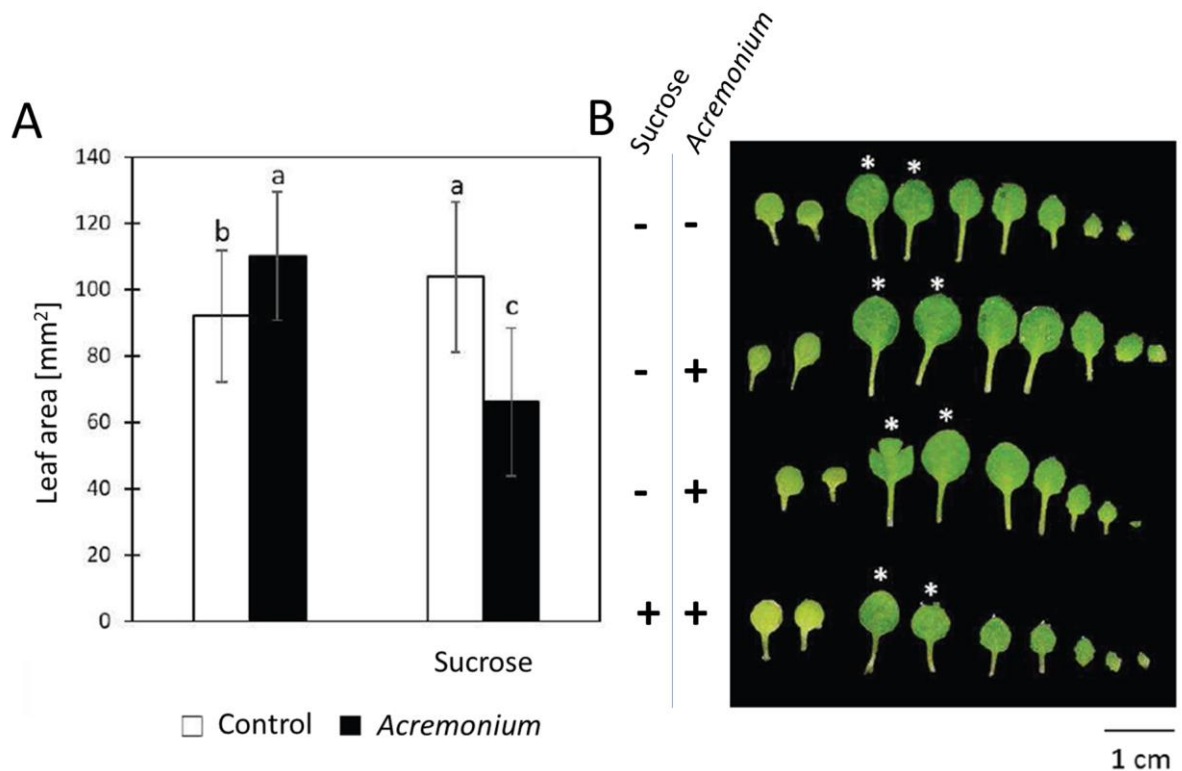
Figure 31. The growth-promoting effect of *Acremonium* is lost in the medium supplemented with sucrose. (A) Total leaf area (n=60) and (B) representative leaf series of 14-day-old *Arabidopsis* plantlets seven days post-inoculation. Data represent means and standard deviation; Different letters indicate significant differences (ANOVA, $p<0.05$); Asterisks indicate true leaves present at the inoculation stage (Adapted from Malých, 2020).
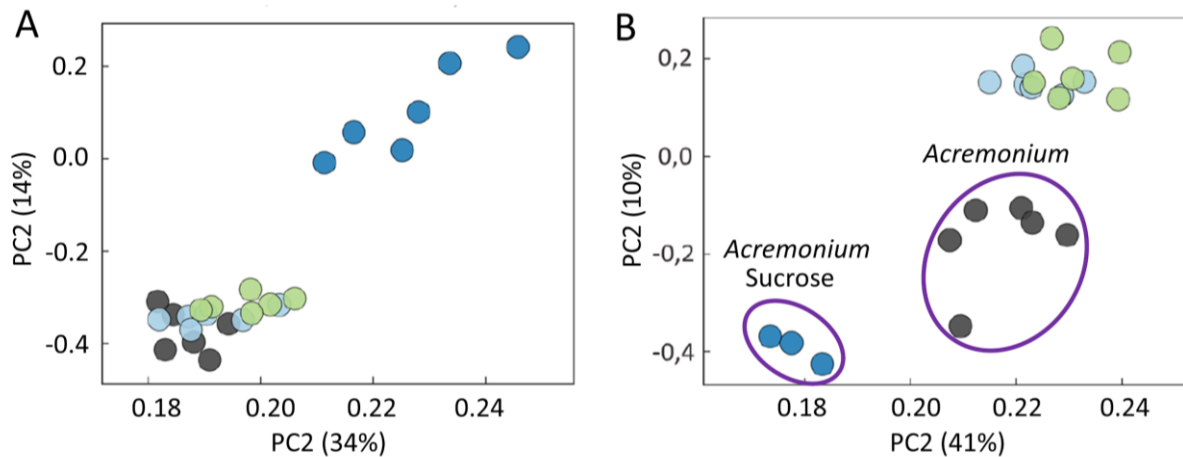
Figure 32. *Acremonium* response is tissue-specific. PCA separation of (A) shoot and (B) root proteomes of 14-day-old *Arabidopsis* plantlets inoculated with *Acremonium*. Light blue - control; light green - 1% sucrose; gray - *Acremonium;* blue - *Acremonium* and 1% sucrose (Adapted from Malých, 2020).

We analyzed the root and shoot tissue proteomes of these plants and identified over 4,600 proteins. As illustrated in Figure 32A-B, the proteomes of inoculated plants grown on the medium supplemented with sucrose were clearly separated (PC1), which was in line with the observed phenotype and metabolome (Malých, 2020). The detailed pairwise comparison of inoculated plants grown in the presence and absence of sucrose revealed more than 40 putative markers of the endophyte-pathogen shift. These included the accumulation of stress-response proteins, namely, flavonoid biosynthetic enzymes, enzymes involved in jasmonic acid biosynthesis, mitogen-activated protein kinase, and caleosin family protein involved in biotic stress. The protein of interest is also TOR1 which was significantly less abundant in inoculated plants on sucrose medium. This protein regulates the direction of organ growth and its decrease could correspond to the observed growth inhibition.

6. Conclusions and future perspectives

Plant proteome analyses are essential for the understanding of biochemical pathways involved in plant signaling and adaptation processes, including those affected by biotic and abiotic stressors. In the last decade, proteome fractionation, enrichment and mass spectrometry-based analysis have undergone rapid evolution. It has been demonstrated that an extensive fractionation can identify most of the expected *Arabidopsis* proteins. Furthermore, proteomics extraction methods have been optimized to facilitate a multi-omics analysis from a single sample (Valledor et al., 2014; Salem et al., 2020). New methods have also provided deeper insights into the complexity of protein complexes and protein-protein interactions (Mair et al., 2019; Van Leene et al., 2019). However, proteomics, like all omics analyses, is prone to errors and misinterpretation. By extending the sensitivity and increasing the amount of data that these analyses are rapidly generating, manual validation of all identified molecules has become obsolete and for large-scale experiments mostly impossible. Besides the data processing, the experimental design is in most cases restrained by budget and does not allow a sufficient number of biological replicates. It has been demonstrated that an RNAseq experiment may require more than 20 biological replicates to reach at least 85% of differentially expressed genes (Schurch et al., 2016), and it is unlikely that a proteomics analysis would cope any better. That undersampling is usually compensated by increasing the ratio threshold for observed significant changes, yet this could be a mistake for large or highly abundant proteins.

False positives may originate not only from the experimental design and analysis but also from overinterpretation. For example, plant responses to phytohormone cytokinin have been extensively studied in *Arabidopsis* at the transcriptional level, and the number of putative cytokinin-regulated genes identified in the last 15 years has reached more than 10,000. That is clearly a considerable overestimate and a problem in distinguishing between signaling-related events and any response that originates solely as a consequence of altered growth or perhaps even due to experimental error. Similarly, our comparison of phytohormone-responsive proteomes revealed that the proteins found in the largest number of

phytohormone-response proteome analyses are an ATP synthase subunit and ribulose bisphosphate carboxylase (Černý et al., 2016; Included Publication 1). These proteins are highly abundant in plant total protein extracts, and the change may reflect an altered turnover. Nevertheless, it is also possible that their presence indicates a potential bias in the proteomics data. It is thus crucial to validate the results of any proteomics analysis. A functional validation with a corresponding mutant phenotype is the best, yet usually also the least convenient. Especially in the case of non-model organisms, the mutants are not readily available, and validation is often impossible. In most of our experiments, the proteomics analyses have been supported and complemented by enzymatic assays, metabolomics, hormonomics, or transcriptomics data. That helped us in confirming at least a portion of the observed changes on the protein level, but the majority of our interpretations have been deduced from indirect evidence based on previously observed effects and available annotations of orthologous genes/proteins in databases.

The dependency on genome sequencing and annotation is the most pressing dire problem for present-day proteomics. Ambitious projects like 10KP (https://db.cngb.org/10kp/) aim to fully sequence and assemble several thousands of plant genomes by 2022. That will be a significant improvement, yet still a long reach with an estimated 500,000 species of land plants (Corlett, 2016). Furthermore, functional annotations are missing even for the best-characterized plant model *Arabidopsis*. The database UniProt lists only 16,036 reviewed *Arabidopsis* proteins, representing less than 50% of the predicted protein-coding transcripts. The second obstacle is spatial and temporal resolution. That is a well-recognized issue in all omics analyses, and Nature Methods crowned the spatially resolved transcriptomics method of the year 2020 (Marx, 2021). Finally, accumulated evidence has shown that many proteins form multiprotein complexes and assemblies critical for their function. That presents new challenges but could be critical for our understanding of the molecular mechanisms of the plant cell (McWhite et al., 2020).

History teaches us that our well-being depends critically on plants. Crop failure and famine have shaped the history of mankind around the world. Early civilizations in Mesopotamia and Peru failed due to human-induced salinization and crop production losses (Shahid et al.,

2018). In our past, as well as in our present, famines have ignited countless wars and migrations (e.g., Feng et al., 2010; Lee, 2018). It is the duty of science to elucidate the mechanisms of plant stress response and adaptation to improve agriculture, and proteomics analysis has the potential to become the best approach for this challenge.

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7.1 Online resources

10KP: 10,000 Plant Genomes Project. Available at: https://db.cngb.org/10kp/ [Accessed April 4, 2021].

The official website of the Nobel Prize - NobelPrize.org Available at: https://www.nobelprize.org/ [Accessed April 4, 2021].

PeptideAtlas, a multi-organism, publicly accessible compendium of peptides identified in a large set of tandem mass spectrometry proteomics experiments. Available at: http://www.peptideatlas.org/ [Accessed April 4, 2021].

PXD020324   Environment and its impact on barley grain composition. Available at: https://www.ebi.ac.uk/pride/archive/projects/PXD020324 [Accessed April 4, 2021].

PXD020480 *Arabidopsis* proteome in the tripartite interaction between cytokinin, light and temperature. Available at: https://www.ebi.ac.uk/pride/archive/projects/PXD020480 [Accessed April 4, 2021].

PXD020627 Barley root proteome and metabolome in response to cytokinin. Available at: https://www.ebi.ac.uk/pride/archive/projects/PXD020627 [Accessed April 4, 2021].

PXD025075 Barley embryo proteome analysis. Available at: https://www.ebi.ac.uk/pride/archive/projects/PXD025075 [Accessed April 4, 2021].

PXD025095 Epulorhiza-responsive proteins found in tubers of Dactylorhiza. Available at: https://www.ebi.ac.uk/pride/archive/projects/PXD025095 [Accessed April 4, 2021].

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8. Abbreviations

2-DE	Two-dimensional gel electrophoresis
ACN	Acetonitrile
ANOVA	Analysis of variance
ARR	*Arabidopsis* response regulator
C18	Octadecylsilane reversed phase chromatography
CAZymes	Carbohydrate-active enzymes
CKX	Cytokinin dehydrogenase
DAB	3,3'-diaminobenzidine
GMO	Genetically modified organism
HSP	heat-shock proteins
ICA	Independent component analysis
IEF	Isoelectric focusing
INCYDE	2-chloro-6-(3-methoxyphenyl)aminopurine
KMD	Kiss me deadly family
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LEA	Late embryogenesis abundant
MS	Mass spectrometry
NBT	Nitroblue tetrazolium
Off-gel	Agilent 3100 OFFGEL Fractionator
PAGE	Polyacrylamide gel electrophoresis
PCA	Principal Component Analysis
PCD	Programmed cell death
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PhyB	Phytochrome B
PSbMV	Pea seed-borne mosaic virus
PTM	Post-translational modification,

qPCR   Quantitative polymerase chain reaction

qTOF   Quadrupole time-of-flight

RT-qPCR   Reverse transcription polymerase chain reaction

RuBisCO   Ribulose-1,5-bisphosphate carboxylase-oxygenase

SCX   Strong-cation-exchange chromatography

SDS-PAGE   Sodium dodecyl sulfate-polyacrylamide gel

TUBE   Tandem Ubiquitin Binding Entities

VIP   Variable Importance in Projection

9. List of Included Publications

Included Publication 1

Černý, M., Novák, J., Habánová, H., Cerna, H., and Brzobohatý, B. (2016). Role of the proteome in phytohormonal signaling. *Biochim. Biophys. Acta - Proteins Proteomics* 1864, 1003–1015. doi:10.1016/j.bbapap.2015.12.008.

Included Publication 2

Cerna, H., Černý, M., Habánová, H., Šafářová, D., Abushamsiya, K., Navrátil, M., et al. (2017). Proteomics offers insight to the mechanism behind Pisum sativum L. response to pea seed-borne mosaic virus (PSbMV). *J. Proteomics* 153, 78–88. doi:10.1016/j.jprot.2016.05.018.

Included Publication 3

Pavlů, J., Novák, J., Koukalová, V., Luklová, M., Brzobohatý, B., and Černý, M. (2018). Cytokinin at the Crossroads of Abiotic Stress Signalling Pathways. *Int. J. Mol. Sci.* 19, 2450. doi:10.3390/ijms19082450.

Included Publication 4

Černý, M., Habánová, H., Berka, M., Luklová, M., and Brzobohatý, B. (2018). Hydrogen peroxide: Its role in plant biology and crosstalk with signalling networks. *Int. J. Mol. Sci.* 19. doi:10.3390/ijms19092812.

Included Publication 5

Černý, M., Berka, M., and Habánová, H. (2019). "Fractionation techniques to increase plant proteome coverage: Combining separation in parallel at the protein and the peptide level," in *Functional Proteomics. Methods in Molecular Biology, vol 1871* doi:10.1007/978-1-4939-8814-3_6.

Included Publication 6

Dufková, H., Berka, M., Luklová, M., Rashotte, A. M., Brzobohatý, B., and Černý, M. (2019). Eggplant Germination is Promoted by Hydrogen Peroxide and Temperature in an Independent but Overlapping Manner. *Molecules* 24, 4270. doi:10.3390/molecules24234270.

Included Publication 7

Berka, M., Luklová, M., Dufková, H., Malých, V., Novák, J., Saiz-Fernández, I., et al. (2020b). Barley root proteome and metabolome in response to cytokinin and abiotic stimuli. *Front. Plant Sci.* 11, 1647. doi:10.3389/FPLS.2020.590337.

Included Publication 8

Berková, V., Kameniarová, M., Ondrisková, V., Berka, M., Menšíková, S., Kopecká, R., et al. (2020). *Arabidopsis* Response to Inhibitor of Cytokinin Degradation INCYDE: Modulations of Cytokinin Signaling and Plant Proteome. *Plants* 9, 1563. doi:10.3390/plants9111563.

Included Publication 9

Berka, M., Greplová, M., Saiz-Fernández, I., Novák, J., Luklová, M., Zelená, P., et al. (2020a). Peptide-based identification of Phytophthora isolates and phytophthora detection in planta. *Int. J. Mol. Sci.* doi:10.3390/ijms21249463.

10. Supplementary materials