

REVIEW

Maize proteomics: An insight into the biology of an important cereal crop

Olga Pechanova¹, Tomáš Takáč², Jozef Šamaj² and Tibor Pechan³

¹Mississippi State Chemical Laboratory, Mississippi State University, Mississippi State, MS, USA

²Centre of the Region Haná for Biotechnological and Agricultural Research, Department of Cell Biology, Faculty of Science, Palacký University, Olomouc, Czech Republic

³Institute for Genomics, Biocomputing and Biotechnology, Mississippi Agricultural and Forestry Experiment Station, Mississippi State University, Mississippi State, MS, USA

Maize (*Zea mays* L.) is the most grown cereal crop in the world (839 million tons in 2012). According to its agro-economical importance, maize has received tremendous attention from research communities of academic, state, and industry origin. In this manuscript, we aspire to provide readers with the first comprehensive review of proteomics studies performed on maize within a 1987–2012 time period. The following topics are presented here: maize proteome profiling, developmental proteomics, response to abiotic and biotic stress, maize phosphoproteomics, tissue-specific wild-type versus mutant analyses, heterosis, seed viability, maize allergens, and safety assessment of genetically modified maize. Tissues, organelles, subcellular compartments, secretomes, methods, phenomena, and pertinent proteins were summarized and referenced in tables and figures to provide readers with expediently accessible information in the context of up-to-date achievements. This review illustrates maize proteomics as a firmly established research area with laboratories around the world diligently advancing our knowledge of diverse aspects of maize biology.

Keywords:

Development / Maize / Plant proteomics / Stress / Tissue / *Zea mays*



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1 Introduction

Originating in the highlands of Mexico [1], between 5000 and 10 000 years ago [2, 3], maize (*Zea mays* L.) has become the

most extensively cultivated cereal crop, followed by wheat and rice (world production for year 2012 is estimated to be 839, 653 and 465 million tons, respectively, according to United States Department of Agriculture World Agricultural Supply and Demand Estimates report from October 11, 2012). Maize represents a staple food for a substantial part of the world's population, as well as a major nutrient source for animal feed. Besides being a world-feeding crop, maize is also a very versatile commodity that has numerous industrial uses ranging from production of adhesives to textiles to biofuels. Furthermore, since the beginning of the 20th century, maize has served as a model organism for studying the biology and genetics of monocotyledonous plants, and currently is one of the most systematically studied genetic systems [4]. As a cross-pollinating species, maize possesses an extraordinary level of genotypic diversity, which helps researchers to elucidate biological events such as evolution [5], recombination [6], transposition [7], heterosis [8], genomic imprinting [9], and epigenetic phenomena such as paramutation [10]. Maize is

Correspondence: Dr. Tibor Pechan, Institute for Genomics, Biocomputing and Biotechnology, Mississippi State University, Mississippi State, MS 39762, USA

E-mail: pechan@ra.msstate.edu

Fax: +1-662-325-9264

Abbreviations: ABA, abscisic acid; APX, ascorbate peroxidase; BS, bundle sheath; CG, cold germination; DT, desiccation tolerance; ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GBSS, granule-bound starch synthase; GM, genetically modified; LEA, late embryogenesis abundant; LHCI, light harvesting complex of photosystem II; LTP, lipid transfer proteins; M, mesophyll; PAL, phenylalanine ammonia lyase; PEP, phosphoenolpyruvate; PR, pathogenesis-related; SOD, superoxide dismutase; SS, starch synthase

also a representative of the C4 plant species, for which it serves as a model system for C4 differentiation [11]. Maize genetic diversity continues to be successfully exploited in breeding programs as well.

Advances in maize research are now accelerated by the availability of the maize genomic sequence of the B73 line [12] (<http://www.maizesequence.org>) as well as the wealth of genomic and genetic information accessible in public repositories such as MaizeGDB, (<http://www.maizegdb.org/>), PlantGDB (<http://www.plantgdb.org/ZmGDB>), Plant Proteome Database (<http://ppdb.tc.cornell.edu>), TIGR Maize Database (<http://maize.jcvi.org>), and Maize Assembled Genomic Island (<http://magi.plantgenomics.iastate.edu/>), just to name a few. The body of knowledge gained by researching maize biology ultimately leads toward the improvement of its nutritional value, resistance to pests and diseases, improved crop yields, and adaptability to the environment. Furthermore, due to an extensive colinearity among the cereal genomes of maize, sorghum, rice, wheat, and barley [13, 14], this knowledge can be applied to these related crops, and new superior varieties can be developed to meet the world's growing demands for food and feed.

During the last two decades, considerable progress has been made in the analysis of the maize proteome. This review summarizes the efforts of numerous research groups, employing more and more advanced tools of proteomics in maize studies ranging from descriptive to functional—all in regard to a wide variety of biological phenomena, as detailed in the following paragraphs.

2 Maize proteome profiles

The main purpose of proteome profiling (or mapping) is to identify, catalog, and functionally classify gene products from various organs, tissues, and cellular components. Proteome profiling often represents the initial step toward the more specific proteome research in which hypotheses are tested, and a new systematic understanding of protein functions is gained. Earlier, maize-mapping projects used peptide mass fingerprinting for protein identification and had to rely on ESTs databases derived from maize and other plant species, such as *Arabidopsis* and rice. Maize proteome analyses, both profiling and comparative, have become more feasible and informative after genome sequencing of the B73 genotype was completed [12].

Ten tissues, seven subcellular compartments, and two secretomes were profiled in maize, and thirteen 2D proteome reference maps were constructed. Depending on the tissue character, the authors' interests, and methods efficiencies, proteome datasets ranged from a few to thousands of proteins. Details pertinent to maize mapping data are summarized in Fig. 1 and Table 1.

2.1 Maize tissue profiles

Maize tissues proteome profiles/datasets were composed for leaf [15, 16] endosperm [17], egg cell [18], root hair [19], primary root, [20, 21], primary root pericycle [22], root tip [23], rachis [24], proteome associated with starch granules [25], and seed flour [26]. For the purpose of this review, descriptive profiling studies are presented in the form of the table (Table 1.)

2.2 Maize subcellular proteomes

Because whole-cell protein extracts contain a mixture of all cellular proteins, they tell little about less abundant subcellular proteins that often remain below the detectable limit due to masking by highly abundant soluble cytosolic proteins. Subcellular proteomics, however, plays an essential role in unraveling specific functions of cellular compartments as well as mechanisms underlying protein targeting and trafficking (reviewed in [27]). Protein datasets were generated for mitochondria [28], cell wall of primary root elongation zone [29], chloroplast [30, 31], and its several subcompartments such as stroma [32], membranes [33], and nucleoids [34].

A 2DE survey of mitochondrial proteomes from normal and T-cytoplasm maize revealed 58 proteins. One of the proteins, ATPase alpha F1, was encoded by the mitochondrial genome [28]. The remaining 57 proteins were encoded by the nuclear genome, and 27 of them were not previously known to be imported to the mitochondria. The mitochondrial proteome dataset consisted of HSPs, voltage-dependent anion channel proteins, prohibitins, and subunits of several ATPases. Additionally, superoxide dismutase (SOD) and inorganic pyrophosphatase are a few examples of differentially accumulated mitochondrial proteins between normal and T-cytoplasm mitochondrial proteomes indicating that the genotype of the mitochondrion can regulate the accumulation of the nuclear-encoded fraction of its proteome [28].

To profile root cell walls, authors analyzed water-soluble and ionically bound proteins localized in the cell walls of the maize primary root elongation zone, and compared them with total soluble proteins extracted from homogenized tissue [29]. Sixty proteins were identified. As expected, many of them were related to cell wall metabolism. Nine proteins, such as triosephosphate isomerase, alpha-L-arabinofuranosidase, and endo-1,3;1,4-β-D-glucanase, were not previously reported in other cell wall studies, as these proteins seem only to associate with type II cell walls, which are typical for maize. (In the type II cell walls, glucuronarabinoxylans are the principal polymers that interlock the microfibrils, unlike xyloglucans in the type I.)

Plant chloroplasts are important organelles that harbor the photosynthetic apparatus. The first attempt to characterize maize chloroplast proteome was made by Lonosky et al. [30] who identified 26 proteins during five stages of leaf greening (0–48 h postillumination). Although a few core proteins from

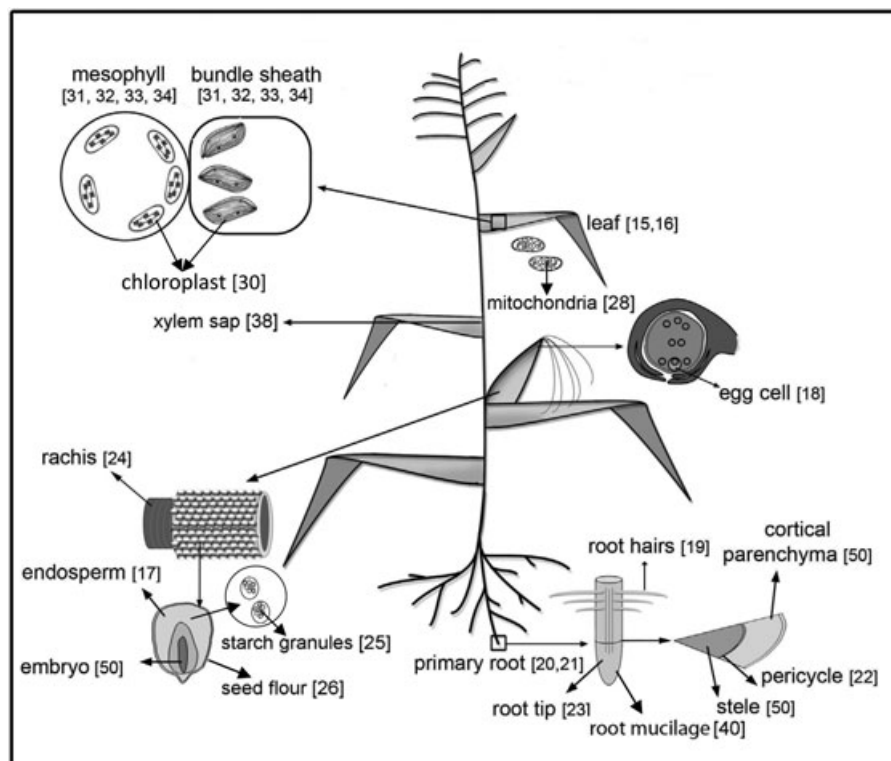


Figure 1. Maize tissue, organelles, and secretomes subjected to proteome mapping. Numbers in brackets refer to the references. (In addition, tissues of Ref. 50 were named to make the scheme more informative.)

the light reaction of photosynthesis (e.g. subunits of ATP synthase) and carbon assimilation cycle (e.g. glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) were detected, the number of identified proteins was not sufficient for global characterization of chloroplast development.

In C₄ plants, chloroplasts are differentiated between distinct and highly specialized bundle sheath (BS) and mesophyll (M) cells. Distribution of the photosynthetic apparatus between these two types of cells was studied extensively by Majeran et al. who established truly comprehensive proteome profiles for stroma [32], membranes [33], and nucleoids [34] of both BS and M chloroplasts. Moreover, distribution of chloroplast metabolic pathways between BS and M were also obtained [31]. In all studies, multiple gel-based and gel-free proteomic approaches coupled to high-resolution and high-sensitivity MS instrumentation were utilized to attain maximum coverage (Table 1). Stromal proteomes were portrayed through 400 identified proteins [32], which covered a broad range of plastid functions such as primary carbon metabolism and photosynthesis (Calvin cycle, oxidative phosphopentose pathway, glycolysis, and starch metabolism), amino acid and lipid metabolism, nitrogen and sulfur assimilation, as well as proteins synthesis, folding, and degradation. In the following study of chloroplast thylakoid and envelope membranes [33], 393 and 483 proteins were identified, respectively, using 1D blue native gel electrophoresis. It is important to mention that while analyzing stromal and membrane proteomes, the maize genome sequence was not yet available

and both proteomes were identified using maize EST assemblies. Repeating the experiment (stroma) and researching existing MS data (membranes) against later available ZmGI assembly yielded much higher proteome coverage with 954 stromal and 882 membrane proteins identified [31]. In the same study [31], analysis of chloroplast from M and BS cells supplied a dataset of 1105 proteins with nearly complete coverage of primary carbon, starch, and tetrapyrrole metabolism. Excellent coverage was also acquired for fatty acid synthesis, redox regulation, detoxification, nitrogen, sulfur, and amino acid metabolism. Analysis of nucleoids from mature and immature chloroplasts [34] resulted in cumulative identification and in-depth characterization of 1092 proteins and helped to elucidate functions of these important components of the plastid gene expression machinery. Obviously, nucleoid-enriched proteome included proteins from DNA replication, organization, and repair, as well as transcription, mRNA processing, splicing, and editing. More importantly, proteins of unknown functions were also found to be nucleoid associated, and they included pentatricopeptide repeat, tetra-tricopeptide repeat, DnaJ, and mitochondrial transcription factor domain proteins. Also in this study, 1717 proplastid proteins were identified [34]. Thus in summary, thousands of proteins were identified and cataloged for various chloroplast tissues that helped to elucidate their functions. Identified proteins were deposited into the Plant Proteome Database (<http://ppdb.tc.cornell.edu>) [35]. For a review on plastid proteomics, see van Wijk et al. [36].

Table 1. Summary of maize tissues, organelles, and secretomes that were mapped/profiled using proteomic approaches

Profiled plant material	Age ^{a)}	Separating technique	Spots/proteins identified ^{b)}	Identification method	Annotated 2D map	Reference
Tissue						
Leaf	14 DAG	2DE	149/73	MALDI-MS	Yes	[15]
Leaf	9-day-old seedling	1DE	n/a/ > 4300	nanolC-MS/MS	n/a	[16]
Endosperm	14 DAG	2DE	496/–	LC-MS/MS	Yes	[17]
Egg cell	n/a	1DE	n/a/5	nanolC-MS/MS	n/a	[18]
Egg cell	n/a	2DE	3/3	nanolC-MS/MS	Yes	[20]
Primary root	5 DAG	2DE	156/74	MALDI-MS	Yes	[21]
Primary root	9 DAG	2DE	–/47	MALDI-MS	No	[19]
Root hair	4 DAG	1DE	n/a/2573	nanolC-MS/MS	n/a	[23]
Root tip	6 DAG	2DE	46/31	MALDI-MS	Yes	[22]
Primary root pericycle	2.5 days old	2DE	–/20	nanolC-MS/MS	Yes	[24]
Rachis	21 DAS	2DE	416/517	1D LC-MS/MS	Yes	[25]
Rachis	21 DAS	2D LC	n/a/701	2D LC-MS/MS	n/a	[26]
Starch granule	n/a	1D LC	–/109	nanolC MS/MS	n/a	[30]
Grain flour	Mature seeds	2DE	40/25	MALDI-MS, nanolC-MS/MS	Yes	[28]
Organelle						
Chloroplast	0–48 HOG	2DE	54/26	MALDI-MS	No	[29]
Mitochondria	Immature ears	2DE	100/58	MALDI-MS	Yes	[32]
Cell walls of primary root elongation zone						
Cell wall protein fraction	28 HAG	2DE	43/60	MALDI-MS	Yes	[32]
Total soluble fraction	28 HAG	2DE	36/34	MALDI-MS	Yes	[32]
Stroma of chloroplasts (total protein)						
Stroma	14-day-old seedling	2DE	400 ^{c,d)}	MALDI-MS; nanolC-MS/MS	Yes ^{e)}	[32]
Stroma	14-day-old seedling	1D LC	–/221 ^{c,d)}	nanolC-MS/MS	n/a	[32]
Stroma	14-day-old seedling	1D LC	n/a/100 ^{c,d)}	nanolC-MS/MS	n/a	[32]
Membranes of BS chloroplasts	12–14-day-old seedling	1D BN	n/a/305 ^{c,d)}	nanolC-MS/MS	No ^{f)}	[33]
Membranes of M chloroplasts	12–14-day-old seedling	1D BN	n/a/393 ^{d)}	nanolC-MS/MS	No ^{f)}	[33]
Chloroplasts (BS and M)	12–14-day-old seedling	1D BN	n/a/483 ^{d)}	nanolC-MS/MS	No	[31]
Nucleoids of chloroplasts (total protein)	12–14-day-old seedling	1DE	n/a/1105 ^{c)}	nanolC-MS/MS	No	[34]
Nucleoid leaf base	9–10-day-old seedling	1DE	1092 ^{c)}	nanolC-MS/MS	No	[34]
Nucleoid leaf tip	9–10-day-old seedling	1DE	n/a/678 ^{c)}	nanolC-MS/MS	No	[34]
Nucleoid young seedlings	7–8-day-old seedling	1DE	n/a/710 ^{c)}	nanolC-MS/MS	No	[34]
Proplastids	9–10-day-old seedling	1DE	n/a/829 ^{c)}	nanolC-MS/MS	No	[34]
Secretome						
Xylem sap	23 DAS	2DE	154/–	nanolC-MS/MS	Yes	[38]
Root mucilage	3-day-old seedling	1DE	n/a/2848	nanolC-MS/MS	No	[40]

a) DAG: days after germination; DAP: days after pollination; DAS: days after silking (rachis); DAS: days after sawing (xylem sap); HOG: hours of greening; HAG: hours after germination; b) –/– authors did not specify the exact number of identified spots or proteins, or this information is not clear from the text.

c) Number reflects the combined proteome from chloroplasts suborganelles of both bundle sheath (BS) and mesophyll (M) cells.

d) The number of identified proteins given as presented in corresponding manuscript identified by searching against Maize EST assemblies. These data were lately upgraded by researching against ZmGI assembly [31].

e) 2DE proteome reference maps were produced separately for each BS stroma and M stroma.

f) 2DE map was established for major complexes and proteins analyzed by 2D Blue Native (BN)-PAGE coupled to MALDI-MS 2DE and/or LC-MS/MS identification.

2.3 Maize secretomes

Plant secretomics is a rapidly emerging research area in the field of plant proteomics. It studies proteins secreted by various tissues, organs, or cells into the extracellular spaces where they perform specific functions. To capture a truly representative secretome is, however, technically very challenging due to the ease of proteinaceous contamination from broken cells or symplast. Plant secretomes were recently reviewed by Agrawal et al. [37].

Two articles characterized constitutively secreted maize proteins. Alvarez et al. described the composition of xylem sap [38], an exudate that plays an important role in root-to-shoot communication via transportation of water and dissolved nutrients from plant roots to its aerial parts. As typical for xylem sap proteins, 42% of 2DE spots were present in various isoelectric isoforms, and many were glycosylated. Hence, 154 identified spots corresponded to 54 distinct protein species. Xylem sap proteins belonged to the three major functional categories: cell wall metabolism, plant defense, and proteolysis. The SignalP algorithm [39] confirmed the presence of signal peptide for 97% of identified proteins, and the remaining 3% were nonclassical secreted proteins. Furthermore, by staining with ProQ-Emerald (Molecular Probes), the authors also established a xylem sap 2D glycoproteome reference map.

Recently, Ma et al. reported a comprehensive proteome of root mucilage [40], an exudate that is continuously secreted by maize primary root tips, and which among other functions also serves as a significant source of carbon for rhizospheric microbes [41]. Mucilage from 3-day-old primary roots was analyzed in a combined 1DE and nanoLC-MS/MS approach, which resulted in 2848 protein identifications. Most of the identified proteins belonged to metabolism, especially cell wall related, but also energy production, amino acid, and lipid metabolism. Additionally, proteins involved in the secondary metabolism of terpenes, flavonoids, and phenolics were also abundant. However, only 16% of proteins possessed a secretory pathway signal peptide, and a subset of 165 cytosolic proteins was also found in the mucilage sample, raising the question about mucilage purity.

For analysis of secretomes or subcellular compartments, sample purity is the most critical parameter. To conduct purity checks for possible contaminants from the symplast, cytosol, or other relevant compartments, tests are performed using immunological reactions with known protein markers. Rubisco and pyruvate phosphate dikinase are, for example, accepted markers for evaluation of contamination from BS and mesophyll, respectively [31,32]. Unfortunately, not all authors subjected studied samples to the rigors of purity verification, consequently leaving their conclusions in question [38,40].

Due to advances in MS technologies and completed sequence of the maize genome, maize proteome profiling is now progressing from identification of “the most abundant gel protein spots” to a more thorough characterization of proteomes, identifying thousands of proteins in one experi-

ment/publication. A wealth of information is being obtained, allowing for pathways reconstruction and models development. Combining the comprehensive proteomic data with genomic, transcriptomic, and metabolomic information creates a foundation for a truly integrated system biology approach. With continuous progress in functional annotations of maize proteins, generated protein datasets represent essential resources for targeted investigations. Numerous public repositories compiling data from large-scale proteome analyses are now available, such as PRIDE (www.ebi.ac.uk/pride) [42] or Plant Proteome Database (PPDB; <http://ppdb.tc.cornell.edu>) [35].

3 Maize developmental proteomics

Physiological changes during maize development are initiated by the regulation of gene expression, however they are a direct consequence of changes in protein abundance and function. Therefore, comprehensive studies have been conducted to assess the proteomes of diverse maize organs, tissues, and cell types during development. For a recent review on plant developmental proteomics, see Takáč et al. [43].

Endosperm is a tissue of important nutritive value for both embryo development and seed germination. Vigorous signaling occurs between the embryo and endosperm in order to coordinate their development [44]. Early stages of endosperm development were characterized by increased abundance of proteins related to cell division, detoxification, respiration, energy production, and accumulation of ubiquitin-proteasome complex components [45]. Proteins related to glycolysis, storage protein synthesis, and protein folding were abundant during more advanced stages of endosperm development.

Because starch is a commodity of substantial economic importance, much attention has been paid to starch synthesis, which occurs in endosperm [45–47]. Generally, the activity of enzymes from the starch biosynthetic pathway followed the rates of starch and amylose accumulation. These enzymes showed low or undetectable activity before 14 days after pollination. However, enzyme activity progressively increased and reached maximum at 30 days after pollination, while it declined subsequently [46].

Several proteomic studies compared wild-type and mutant plants to investigate maize root growth [48–50]. Differential 2DE gel-based study was performed on wild-type plants and *rtcs* mutant defective in the initiation of shoot-borne roots and embryonic seminal roots at various developmental stages [48]. Genes related to ethylene production, such as *S*-adenosylmethionine synthetase and ethylene inducible proteins, were differentially expressed in 10-day-old coleoptile nodes, suggesting a role for ethylene in shoot-borne root growth. The expression of histone *H2B.2* correlates with increased mitotic activity in young developing wild-type root, in contrast to the *rtcs* mutant, which forms crown roots at the coleoptile node. Small G protein showed exclusive accumulation in 5-day-old *rtcs* coleoptile nodes, indicating a possible

signaling function of this protein in shoot-borne roots. Another signaling protein, calmodulin, was preferentially expressed in 10-day-old wild-type coleoptile nodes, suggesting involvement in crown root formation. Additionally, actin and profilin were preferentially accumulated in 10-day-old wild-type coleoptile nodes, indicating a connection between the dynamic actin cytoskeleton and enhanced meristematic activity in the coleoptile node during crown root development [48].

The primary root proteome of wild-type maize and the *rum1* mutant affected in lateral root and seminal root initiation was studied using 2DE coupled to nanoLC-MS/MS [49]. This study showed that synthesis of benzoxazinoids, compounds playing an important role in wounding and pathogen response, takes place before the initiation of lateral roots. Additionally, thiamin- and stress-related protein GST was less abundant in mutant plants. These data indicate that control of compounds involved in lignin biosynthesis might be critical both before and after emergence of lateral roots [49]. The *rum1* mutant was also used to investigate lateral and seminal root formation in tissue-specific proteomic analysis [50]. Embryo, stele, and cortical parenchyma were analyzed using differential proteomics. Proteins related to signal transduction were significantly overrepresented in cortical parenchyma as compared to embryo tissue. Differential accumulation of diverse globulin isoforms was found in wild-type and in the *rum1* mutant. Interestingly, a trypsin inhibitor (Hageman factor inhibitor protein), which might be involved in the regulation of the specific degradation of globulins, was exclusively expressed in wild-type embryos [50]. Furthermore, proteomes of primary maize root pericycle cells prior to their first division and during lateral root initiation were investigated using laser dissection/capture microscopy of root cryosections [22]. Interestingly, actin, pathogenesis related (PR) 10, and two isozymes of GST were highly abundant in the pericycle.

Considerable efforts were dedicated to dissect differences in chloroplast proteomes between BS and M cells, two highly specialized and functionally distinct types of cells that share the photosynthetic apparatus of C4 plants. Studies focused on stroma [32], thylakoid, and envelope membranes [33], nucleoids [34], chloroplasts [31], and leaf development [16]. These elegant and comprehensive quantitative investigations exploited multiple proteomic approaches and revealed that lipid biosynthesis, nitrogen import, and enzymes of the Calvin cycle were predominantly located in the M chloroplasts, while enzymes of starch synthesis and sulfur import were mostly found in BS chloroplasts [32]. Differences were also observed for distribution of antioxidants and other proteins. For example, peroxiredoxins were more abundant in M chloroplasts due to their high linear electron flow and higher production of ROS. On the contrary, defense-related β -D-glucosidase was more abundant in BS chloroplasts, where it can be development associated. Chloroplast membrane systems are composed of photosynthetic thylakoid-containing constituents of the electron transport chain and envelope membrane harboring various transporters and the protein import apparatus. Some differences between BS and M

chloroplast membranes [33] included increased protein flux of M chloroplasts, higher rates of thylakoid proteins translation in M chloroplasts, and strongly reduced linear electron transport rates in BS thylakoids. These variations are in agreement with the unique biochemistry of M and BS cells. Protein expression patterns along the leaf and BS developmental gradient identified five major developmental transition phases of plastids during their biogenesis, which were characterized by specific proteome profiles and helped to elucidate structural and metabolic changes during maize leaf development and C4 differentiation [16]. An extensive overview of the distribution of metabolic pathways and homeostasis machinery across BS and M chloroplasts was reported recently [31] and it showed strong differences in cell-type specific chloroplasts proteome composition. Recently, proteomes of highly enriched nucleoids (plastid chromosomes consisting of DNA folded together with proteins and RNA) of proplastids and mature chloroplasts isolated from the base and tip of maize leaf, respectively, were also studied by proteomic methods [34]. This study revealed that mRNA processing, splicing, and editing, as well as ribosome assembly are processes associated with the nucleoid. Moreover, proteins involved in RNA metabolism were predominant in proplastids, while transport- and photosynthesis-related proteins were overabundant in fully photosynthetic mature chloroplasts. Altogether, these studies considerably improved our knowledge about spatial and temporal organization of C4 photosynthesis during maize leaf development.

The process of greening after etiolation was studied in maize seedlings by a label-free quantitative proteomics approach [51]. The abundance of photosystem II subunits was substantially increased upon light exposure, indicating that its biogenesis is triggered by the transition from dark to light. Additionally, phosphoenolpyruvate (PEP) carboxykinase, a key enzyme of the PEP carboxykinase C4 pathway, was more tightly regulated by light compared to key enzymes of the NADP-malic enzyme C4 pathway. Further, NADPH-protochlorophyllide oxidoreductase, an enzyme involved in chlorophyll biosynthesis, as well as key enzymes of carbon fixation were strongly dependent on light [51].

Finally, a differential gel-based proteomic approach was employed to study differences between mature and germinating maize pollen [52]. In particular, pectin methylesterase, inorganic pyrophosphatase, glucose-1-phosphate uridylyltransferase, and Rab GDP dissociation inhibitor were highly upregulated, suggesting their roles in pollen tube growth. On the other hand, defense-related proteins, pollen allergens, and some metabolic enzymes were downregulated during pollen germination and tube growth [52].

All of the above proteomic studies contributed to a better understanding of maize development at the cellular, tissue, and organ levels because they identified new developmentally regulated proteins. The major advantage of proteomic studies is that they temporally integrate information on multiple biochemical and physiological processes. Some of these studies

revealed that maize root initiation and growth are accompanied by increased mitotic activity, lignin biosynthesis, as well as small G protein and ethylene signaling [48, 49]. Other studies showed that particular stress response pathways, like those involved in pathogen resistance, are also employed in plant development [22, 49, 52].

4 Maize stress proteomics

4.1 Maize response to abiotic stress

Diverse abiotic stress factors considerably limit crop yields in maize. Therefore, systematic studies of maize adaptation to diverse stress conditions at the proteome level are of immense importance, as they may lead to improvement of maize tolerance toward environmental stresses. Recently, highly dynamic plant proteome responses to abiotic stress were comprehensively reviewed [53]. An overview of studies on abiotic stress is given in Table 2. The most common proteins responsive to various abiotic stresses are listed in Table 3.

4.1.1 Anoxia

Adaptation of plants to low oxygen (anoxia) was studied in maize roots by gel-based proteomic approach [54]. Such adaptation can be facilitated by plant acclimation using hypoxia. Metabolic enzymes, such as alcohol dehydrogenase, GAPDH, and enolase were preferentially synthesized during hypoxic acclimation. In addition, GLU1 (beta-glucosidase) protein involved in hormone metabolism [55] and protection against pathogens was quite abundant during hypoxia acclimation. These data indicate the cross-talk between hormonal and metabolic pathways during the plant adaptation to anoxia.

4.1.2 Water stress

Water deficit has serious consequences on maize growth and yield. Proteomes of maize leaves of two different genotypes exposed to drought stress varied significantly. Quantitative variation of ABA45 and OSR40 proteins, but not that of cystatin or beta glucosidases, was linked to differences in abscisic acid (ABA) accumulation between these genotypes [56]. More recently, changes in hormones and phenylpropanoid metabolites, as well as proteins in maize xylem sap under water deficiency were investigated by combined metabolomic and proteomic approaches [57]. Xylem sap transports compounds involved in drought adaptation from roots to shoots. Proteins involved in cell wall metabolism (e.g. peroxidase, xyloglucan endotransglycosylase, and pectin methylesterase) and defense (e.g. thaumatin-like protein, zeamatin-like protein, class IV chitinase, and β -1,3-glucanase) were differentially regulated in xylem sap in response to drought. Together

with changes in phenylpropanoids (e.g. coumaric, caffeic, and ferulic acids), it may lead to the reduction of lignin biosynthesis in the xylem vessels and to stiffening of the cell walls [57]. This, however, requires a complex stress-responsive regulatory mechanism, which controls protein abundances in the xylem sap.

Desiccation tolerance (DT) was also investigated in maize embryos during their development and germination [58]. Eleven proteins involved in drought tolerance were identified, such as late embryogenesis abundant (LEA) protein EMB564, globulin 2, putative cystatin, class I HSP, NBS-LRR resistance-like protein RGC456, etc. These proteins accumulated during embryo maturation and during desiccation of desiccation-tolerant embryos, but decreased during embryo germination. On the other hand, small HSP precursor and Rhd6-like 2 were less abundant. These stress-related proteins might be involved in DT during embryo maturation mimicked by desiccation treatment. In another study, water soluble and loosely ionically bound cell wall proteins were analyzed in different regions of the maize root elongation zone under water deficit [59]. Induced changes in the root cell wall proteome were related to ROS metabolism and involved SOD, oxalate oxidase, probable germin protein, and three peroxidases. Additionally, water stress caused changes in the abundance of proteins involved in cell wall remodeling. Examples include oxalate oxidases, alpha-L-arabinofuranosidase, alpha-1,4-glucan-protein synthase, beta-galactosidases, chitinases, endo-1,3;1,4- β -D-glucanases, xylosidases, beta-D-glucosidases, peroxidases, etc. Altogether, these studies revealed that drought is tightly connected with hormones such as ABA but also with ROS metabolism and cell wall remodeling.

4.1.3 Nutrient deficiency

Nutrient deficiency, especially lack of phosphorus (P), negatively affects plant growth and yield. Maize roots exposed to a P deficit showed changes in a wide spectrum of proteins with diverse functions [60]. Some of the proteins, such as nuclear Ran B1 (small GTPase-regulating nuclear transport and cell cycle), cytoskeletal proteins (e.g. actin and tubulins), and purine rich element-binding protein (regulating gene expression) might be involved in linking changes in external P concentrations to such processes as signaling, adjustment of cellular/root morphology, and gene expression.

4.1.4 Temperature stress

Maize plants grown in different temperatures (13°C versus 24°C) and light (high versus low) conditions were used for proteomic analysis of chloroplast thylakoid membranes. These showed differences in the accumulation of proteins constituting the major light harvesting complex of photosystem II (LHCII), as well as in minor antenna chlorophyll

Table 2. An overview of maize proteomic studies focused on abiotic stress response

Plant material	Stage/age	Type of stress	Stress duration ^{a)}	Separation and identification methods	Number of differentially expressed proteins	Affected cellular processes/pathways	Reference
Excised root tips	Seedlings with root length of 110 mm	Hypoxia, anoxia, normoxia	4 h	2DE, MALDI-MS	48	Carbohydrate metabolism, postanoxic oxidative stress resistance, signal transduction, translation, hormone metabolism	[54]
Etiolated part of the leaf blade	Plants in fifth leaf stage	Drought	4–14 d	2DE, Edman microsequencing	60	Cell wall metabolism and lignification, defense, senescence	[56]
Xylem sap	15-d-old plants	Drought	7–12 d	2DE, LC-MS/MS	39	Cell wall metabolism, defense, phenylpropanoid metabolism, programmed cell death	[57]
Embryos	28, 52, and 72 d after pollination	Dessication	0.07 ^{b)}	2DE, MALDI-MS/MS	111	Protein metabolism, stress response, carbohydrate and energy metabolism, signal transduction	[58]
Root tip sections (cell wall fraction)	Seedlings with 10-mm-long primary roots	Drought	24, 48 h	2DE, MALDI-MS/MS	152	ROS metabolism, defense and detoxification, carbohydrate metabolism, cell elongation	[59]
Root tip fragments	24-d-old seedlings	Phosphorus deficiency	17 d	2DE, MALDI-MS/MS	106	Phytohormone biosynthesis, carbon and energy metabolisms, protein synthesis and fate, signal transduction, cell cycle, cellular organization, defense, secondary metabolism	[60]
Seedlings	14-d-old seedlings	Salt, salt + nitric oxide	48 h	2DE, MALDI-MS/MS	82	Defense and detoxification, carbohydrate and energy metabolism, cell structure and division, redox metabolism, signal transduction	[67]
Chloroplast (thylakoids)	Seedlings with full expanded third leaf	Different temperature and light regimes	28 d	2DE, immunoblotting	21	Major LHCl complexes, the minor antenna chlorophyll proteome	[61]
Whole plants	14-d-old seedlings	Solar and UV light	4 and 8 h	2D DIGE, LC-MS/MS	178	Photosynthesis, metabolism, translation, PTM, transport, oxidative-stress defense	[63]
The growing zone of the leaf	23-d-old seedlings	Salt	8 d	2DE, MALDI-MS	Not specified	Cell wall loosening, growth	[64]
Chloroplasts	21-d-old seedlings	Salt	1, 2, and 4 h	2DE, MALDI-MS	20	Photosynthetic machinery	[65]
Root	21-d-old seedlings	Salt	1 h	2DE, MALDI-MS	14	Sugar signaling, cell signaling, cell wall modification, cell elongation	[66]
Shoots	Not specified	Arsenic	24 h	2DE, MALDI-MS	74	Protein biosynthesis, signaling, primary metabolism (Krebs cycle)	[68]
Anthers	Microspores ^{c)}	Cold	12 d	2DE, MALDI-MS/MS	39	Energy-yielding metabolism, protein biosynthesis, detoxification, transport, cell structure	[62]

a) h: hours; d: days.

b) Until water content in embryos decreased to 0.07 g.g⁻¹

c) Late uninucleate to early binucleate stage.

Table 3. List of the most common proteins that are differentially accumulated in maize tissues after various abiotic stresses

Protein	Type of abiotic stress	References
Alcohol dehydrogenase	Annoxia, phosphorus deficiency, nitric oxide, salt stress	[54, 60, 67]
Enolase	Annoxia, desiccation, drought, phosphorus deficiency	[54, 56, 58–60]
Malate dehydrogenase	Annoxia, drought, desiccation, phosphorus deficiency, nitric oxide, salt stress, arsen	[54, 56, 58, 60, 67, 68]
Beta glucosidase	Annoxia, drought, phosphorus deficiency, cold	[54, 59, 60, 62]
Ascorbate peroxidase	Desiccation, drought, phosphorus deficiency, nitric oxide, salt stress, cold	[58–60, 63, 67]
Glutathione transferase	Desiccation, phosphorus deficiency, nitric oxide, salt stress	[58, 60, 67]

proteins CP29, CP26, and CP24 [61]. Decreasing temperature increased the LHCII/minor antenna ratio, which was associated with changes in pigment composition and in the spectroscopic properties of LHCII complexes. Additionally, low temperature combined with high light increased non-photochemical quenching. The data suggest that temperature along with light affect proteins of photosystem II and photoprotection.

However, stress, in some specific situations such as androgenic development of plants from *in vitro* cultured microspores, might also have some positive effects. A recent proteomic and biochemical study on maize anthers revealed that cold pretreatment was associated with differential abundance and activity of antioxidative enzymes such as SOD: superoxide dismutase (APX) and SOD [62]. These enzymes probably play a role in the induction of maize androgenesis from cold-pretreated anthers. In addition, new proteins such as Ran GTPase and actin identified by this study were likely associated with early cell divisions of induced microspores.

4.1.5 Radiation

UV-B irradiation might cause genotoxic stress with detrimental consequences for the plant. Maize leaves from the W23 inbred line (similar to commercial corn) and two genotypes from high altitudes (naturally more exposed to UV-B) responded to damaging UV-B irradiation by changed proteome profiles [63]. Some proteins showed multiple isoforms, while pyruvate phosphate dikinase was posttranslationally modified by phosphorylation. This study suggests that more abundant proteins accumulating in high-altitude plants might be involved in higher UV-B tolerance.

4.1.6 Salinity

Maize is salt sensitive, and salinity considerably inhibits shoot growth. It was shown that expansins, proteins involved in cell wall loosening and growth, are more abundant under salt stress conditions in leaves of salt-resistant hybrid SR03 than in the salt-susceptible maize variety Pioneer 3906 [64]. Additionally, expansins require acidic apoplastic pH for their cell wall loosening function, which is maintained in the SR03

hybrid line, but not in Pioneer 3906 (both under salt stress conditions). Next, proteomic experiments on short-term effects of salt exposures (up to 4 h) performed on maize chloroplasts revealed 12 more abundant and eight less abundant salt-responsive proteins in these photosynthetic organelles [65]. It was proposed that salt-enhanced accumulation of the 23 kDa polypeptide of the photosystem II, ferredoxin NADPH reductase, and the FtsH-like protein might help to attenuate damaging effects of NaCl on the photosynthetic apparatus. On the other hand, the calcium-sensing receptor protein was less abundant after short-term salt stress and might be implicated in sensing of Na⁺ ions, which accumulated heavily in chloroplasts incubated with NaCl. A more recent study on maize root proteome and phosphoproteome affected by short term (1 h) application of NaCl reported several new salt-inducible proteins and phosphoproteins [66]. For example, fructokinase, UDP-glucosyl transferase BX9, and 2-Cys-peroxyredoxine were phosphorylated, whereas isocitrate-dehydrogenase, calmodulin, maturase, and a 40-S-ribosomal protein were dephosphorylated after adjustment to saline conditions, suggesting putative involvement of calmodulin and sugar-signaling pathways in salt acclimation of maize roots. In addition, proteins involved in cell elongation, such as 14–3-3 protein and xyloglucan endotransglycosylase, were also changed after short-term salt treatment, probably reflecting root growth adjustment to salt stress.

A recent study revealed a protective role of nitric oxide against salt stress in maize [67]. Salt treatment resulted in increased nitric oxide production and an accumulation of signaling G-proteins. It also increased activation of antioxidant enzymes, such as SOD, APX, and glutathione reductase, which control hydrogen peroxide levels and prevent its cytotoxicity.

4.1.7 Metal toxicity

Arsenic toxicity was assessed by gel-based proteomics on maize shoots treated with sodium arsenite [68]. This treatment induced changes in protein abundance of protein kinase C inhibitor, translation initiation factor eIF-5A, cysteine synthase, malate dehydrogenase, ATP synthase, transposase and guanine-binding protein. Among them, eIF-5A was completely repressed linking arsenic toxicity with severe growth

retardation. The complete downregulation of ATP synthase, malate dehydrogenase, and cysteine synthase suggests an interference of arsenic toxicity with key aspects of primary metabolism.

In summary, numerous proteomic studies have provided clear evidence that maize responds to a changing environment involve multiple levels of molecular events including cell wall modification, dynamics of the photosynthetic apparatus, and changes in xylem sap protein abundance, to name a few. Some of the differentially regulated proteins, which are listed in Table 3, appear to be involved in universal stress responses. It can be concluded that maize strongly responds to environmental stresses by modification of its diverse metabolic machineries (e.g. glycoside and oligosaccharide metabolism) as well as triggering the oxidative stress responses (e.g. ascorbate peroxidase (APX) and GST).

4.2 Maize response to biotic stress

Despite its enormous genetic diversity, maize suffers from a variety of diseases that are caused by an array of phytopathogens, many of which produce toxins. Whether they affect the ear or foliar parts of the plant, damage by the pest translates to decreased crop yield and subsequent economic losses. Good understanding of the maize interactions with its pathogens on the molecular level is therefore extremely important, as it can identify resistance gene candidates and leads to the development of resistant varieties. The plant disease proteomics was recently reviewed by Rampitsch et al. [69]. An overview of proteomic studies on biotic stress in maize is given in Table 4.

4.2.1 *Aspergillus flavus* and aflatoxins

Aspergillus flavus is a fungal pathogen that causes *Aspergillus* ear rot and contamination of grains with aflatoxins, carcinogenic secondary metabolites. Aflatoxin contamination radically reduces grain value and has a detrimental impact on the economy worldwide. A notable number of proteomic investigations have been conducted to elucidate *A. flavus*-maize interactions with a major goal to identify resistance-associated proteins. Such proteins could be used as markers in marker-assisted breeding to develop resistant commercial varieties of maize.

Most of the proteomic investigations were understandably conducted in the kernel tissues. For instance, comparing 2DE maps of embryo proteins from two aflatoxin-resistant and five susceptible maize genotypes revealed several unique and up-regulated proteins (at least fivefold) in resistant genotypes [70]. Resistance-associated groups of proteins included globulins (1 and 2), LEA proteins (3 and 14), osmotic stress-related proteins WSI18 and aldose reductase, heat stress-related protein HSP16.9, as well as glyoxalase I [71]. Endosperm proteins exhibited a similar pattern. Higher levels in resistant

inbreds were observed for globulin 2, LEA3, LEA14, peroxiredoxin antioxidant, HSP17.2, cold-regulated protein, antifungal trypsin-inhibitor [72], and PR-10 [73]. These data indicate that constitutive defenses play an imperative role in kernel resistance to *A. flavus* infection and/or aflatoxin accumulation (Fig. 2). Inducible defenses are, nevertheless, also necessary, as demonstrated for PR-10 transcripts [73]. Thus three main categories of proteins appear to be involved in kernel resistance: (i) storage proteins, (ii) stress-related proteins, and (iii) antifungal proteins. While the role of antifungal proteins in antifungal combat is apparent, it was suggested that high constitutive levels of storage and stress-related proteins enhance kernel stress tolerance, which simultaneously helps the tissue to enhance the resistance to aflatoxin accumulation [74]. LEA proteins protect higher plants from damage caused by diverse environmental factors, especially drought [75]. Glyoxalase, on the other hand reduces the levels of methylglyoxal, which was shown to induce aflatoxin production in vitro [71].

Rachis (cob) is another maize tissue involved in resistance [76]. Rachis is the middle part of the maize ear that provides mechanical support to the grains and delivers essential nutrients to the developing kernels. *Aspergillus flavus* exploits the rachis as a conduit to spread throughout the ear and to enter and infect the kernels. Proteome changes due to rachis maturation and responses to *A. flavus* infection 10 and 35 days postinoculation were studied by a series of comparative 2D DIGE analyses of aflatoxin-resistant and susceptible inbred lines. Numerous proteins were found as resistance related where disease-/stress-related proteins, antioxidant enzymes, and proteins from the phenylpropanoid pathway seem to play the most principal role. The findings further indicated that resistant rachis relies primarily on constitutive defenses (Fig. 2), while susceptible rachis is more dependent on inducible defenses when given enough time. Indeed, the strongest response of susceptible rachis occurred 35 days postinoculation with robust induction of a variety of PR proteins. Similar to kernel tissues, high constitutive levels of abiotic stress-related proteins in young resistant rachis seemed to enhance its capability to resist infection in synergy with higher tolerance to abiotic stress. Figure 2 summarizes some of the components of constitutive defenses present in the maize ear tissues that were discovered by proteomic investigations.

Finally, maize silks have also been shown to assist in resistance [77]. Silks are believed to serve as a main entry for *A. flavus* [78] and their antifungal properties have been reported [79]. Comparative 2DE analysis of several resistant and susceptible inbred lines led to the identification of a few antifungal proteins. Three PR proteins (PRm3 chitinase, chitinase I, and chitinase A) were present at higher amounts in silks of resistant inbreds. Moreover, silk extracts from resistant lines showed higher antifungal activity than those from susceptible inbreds. These findings suggest that silk chitinases contribute to *A. flavus* resistance.

From the studies reviewed above, it is clear that maize resistance to *A. flavus* and aflatoxins is a complicated

Table 4. An overview of inducible defenses in various maize tissues in response to common maize pathogens.

Plant material	Stage/age	Pathogen	Stress duration ^{a)}	Separation and identification methods	Number of differentially expressed proteins	Upregulated/induced proteins	Reference
Rachis	Young (21 DAS ^{b)})	<i>A. flavus</i>	10 d	2D DIGE, MALDI-MS/MS	27 (R genotype)	R: PR-1, GAPDH, calmodulin	[76]
Rachis	Mature (35 DAS ^{b)})	<i>A. flavus</i>	35 d	2D DIGE, MALDI-MS/MS	19 (S genotype) 30 (R genotype) 48 (S genotype)	S: fumarate hydratase, GST R: GAPDH, GST, endo-1,3- β -glucanase flavin-containing monooxygenase S: chitinases, PR-5, permatin, xylanase I inhibitor, endo-1,3- β -glucanase, P21 protein, profilin-4, ATP surfonylase, PR-10 (transcript)	[76]
Endosperm	7 d after pollination	<i>A. flavus</i>	0, 3, 8, and 15 d	2D, nanoLC-MS/MS	Not a large-scale study		[73]
Kernel	Developing (the blister stage)	<i>F. graminearum</i>	48 h	LC-MS/MS	878 (R&S genotypes)	R&S: PR-10, PR-5, chitinases, proteinase inhibitors, xylanase inhibitors, thaumatin-like protein, germin-like protein, LOX PAL	[83]
Silk	Not specified	<i>F. graminearum</i>	48 h	2DE, LC-MS/MS	10 (S genotype)	S: chitinase A, PR-10, PR-5, profilin-4, PRm3 chitinase, GAPDH, carbonyl reductase, win 1 precursor	[83]
Embryo	After 20-h germination	<i>F. verticillioidea</i>	24 h	2DE, MALDI-MS; nanoLC-MS/MS	9 (S genotype)	S: catalase 2, Cu/Zn SOD, GST, HSP17.2, peptidyl-prolyl cis-trans isomerase, fructose-bisphosphate aldolase	[84]
Extracellular matrix	Cell suspension cultures 3 d after transfer to fresh media	<i>F. verticillioidea</i>	6 h	1DE; 2DE, MALDI-MS; nanoLC-MS/MS	Not specified	Secretion of xylanase inhibitor, GAPDH, and HSP phosphorylation of apoplastic peroxidases	[86]
Leaf	2-leaf stage	Black-streaked dwarf virus	5 d	2DE, MALDI-MS/MS	123 (S genotype)	S: peroxidase 39, APx2-cytosolic ascorbate peroxidase, catalase 3, oxygenase, caffeic acid 3-O-methyltransferase, cinnamyl alcohol dehydrogenase, UDP-glucose pyrophosphorylase, GAPDH	[90]
Leaf	6- or 7-leaf stage plants	<i>Curvularia lunata</i>	24 h	2DE, MALDI-MS/MS	R: 9 (identified) S: 5 (identified)	R: germin-like protein, oxygen-evolving enhancer protein 1 and 2, rubisco, 23-kDa oxygen-evolving complex S: 23-kDa oxygen-evolving complex, oxygen-evolving enhancer protein 2 rubisco	[91]
Leaf	6- or 7-leaf stage plants	<i>Curvularia lunata</i>	24, 36, 48, 60, and 72 h	2DE, MALDI-MS/MS	R: 23–46	R: 22-kDa drought-inducible protein, glutathione peroxidase, translation initiation factor (eIF-5A), ascorbate peroxidase, S: ascorbate peroxidase, ras-related proteins	[92]

a) h: hours; d: days.

b) DAS: days after silking.

Analyses were performed in resistant (R) and/or susceptible (S) genotypes. Only most upregulated proteins are listed. For complete lists of upregulated (and downregulated) proteins, see corresponding references

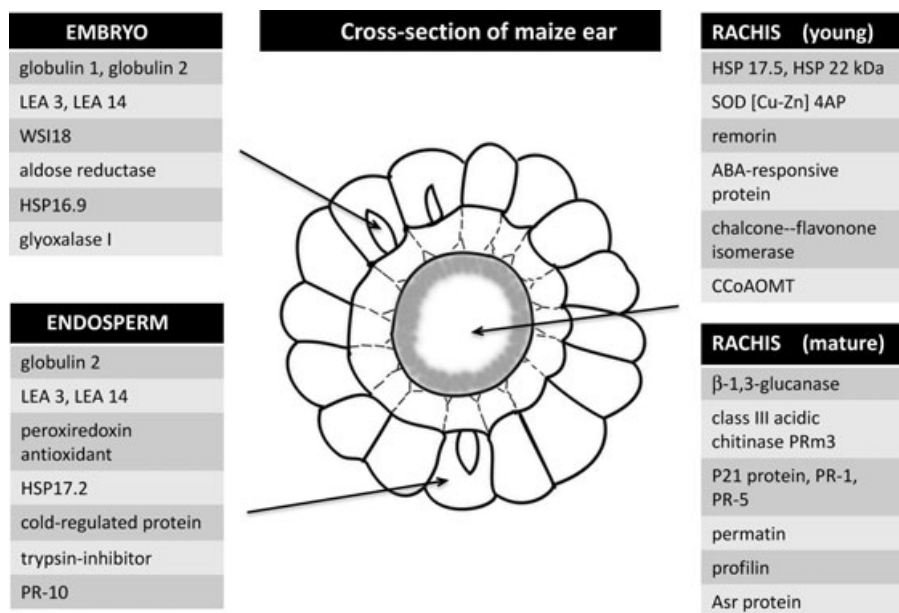


Figure 2. Constitutively expressed defense-related proteins essential for the resistance to aflatoxin accumulation. Depicted here are the possible gene candidates involved in the resistance in maize embryo, endosperm, and rachis—the most critical tissues in regard to contamination itself and spreading of infection. WSI: water stress inducible; TI: trypsin inhibitor; ASR: ABA, stress, and ripening induced; CCoAOMT: caffeoyl CoA 3-O-methyltransferase. For a complete list of rachis proteins, see Pechanova et al. [76].

network of multiple defenses coming from different tissues of the maize ear. While defense mechanisms of embryo, endosperm, rachis, and silk have partly been elucidated, to complete the resistance picture it would be interesting to see the contribution of other kernel tissues, pericarp, and aleurone. Rachis might be able to obstruct *A. flavus* from entering the kernels from the cob interior. However, *A. flavus* entering the ear via external silks quickly colonizes kernel surfaces, enters the wounded kernels, and contaminates them with aflatoxins [80]. Thus pericarp, which provides a protective coat and immediate physical barrier between the seed endosperm and exterior, might play a vital part during the *A. flavus*–maize encounter. Chemical analysis of waxes from pericarp of resistant and susceptible maize genotypes strongly supports its involvement in resistance [81].

4.2.2 *Fusarium* pathogens

Fusarium species, predominantly mycotoxin-producing *F. proliferatum*, *F. verticillioides*, and *F. graminearum*, are other fungal pathogens associated with maize diseases. *Fusarium* spp. can infect maize in all stages of development and cause extensive damage to different parts of the plant. *Fusarium graminearum*, for instance, causes *Gibberella* ear rot and stalk rot [82]. Proteome changes caused by this fungus were evaluated in developing kernels of two inbred lines 48 h postinoculation [83]. A total of 878 proteins were differentially expressed as shown by iTRAQ MS/MS analyses. The most upregulated defense proteins PR-10, chitinases, proteinase inhibitors, xylanase inhibitors, thaumatin-like and germin-like proteins appear to be the major contributors to the resistance, as they were present at significantly higher lev-

els in mock- and/or *Fusarium*-treated tolerant inbred CO441 when compared to susceptible B73 genotype. Also higher in abundance in CO441 were cinnamyl alcohol dehydrogenase, 4-coumarate-CoA ligase, and phenolic O-methyltransferase, which might contribute to the resistance by reinforcing kernel cell walls via enhanced lignification. In addition, *Fusarium*-infected B73 silks responded with upregulation of antifungal PRm3 chitinase, PR-10, PR-5, and several other proteins, as revealed by 2DE MSMS [83].

F. verticillioides causes *Fusarium* ear rot during both the pre and postharvest periods. When 20-h-old germinating embryos of susceptible inbred W64A were treated with *F. verticillioides* for 24 h [84], two main groups of defense proteins increased in abundance: (i) antioxidant enzymes catalase 2, SOD, and GST that protect cells from oxidative damage [85], and (ii) proteins involved in protein synthesis, folding, and stabilization such as eukaryotic translation initiation factor eIF 5A, small HSP 17.2, peptidylprolyl cis-trans isomerase, and cyclophilin. Carbohydrate metabolism also seemed to be affected by *F. verticillioides*. As shown from the expression of several housekeeping enzymes, gluconeogenesis appeared to be activated while glycolysis was repressed after the fungal challenge. Finally, several PR proteins such as β -1,3-glucanases, chitinases, and P23 protein seemed to be both inducible and/or constitutively expressed, as confirmed by immunological reactions.

Extracellular matrix (ECM) is without a doubt one of the most crucial components of plant defense machinery. Upon the elicitor encounter, ECM is the first compartment to take action due to its unique location within the plant cell. Elicitor-induced changes in ECM mediate the first line of defense; therefore, it is essential to understand events occurring in this compartment during the plant's earliest

interaction with an invading pathogen. ECM of maize suspension culture was found to be participating in *F. verticillioides*-induced defenses via (i) accumulation of a secreted putative xylanase inhibitor, (ii) rapid dephosphorylation of several apolastic peroxidases, and (iii) accumulation of cytosolic GAPDH and HSP in the cell wall [86]. Dephosphorylation of identified extracellular peroxidases strongly suggests that phosphorylation regulatory apparatus is governing their activity. Peroxidases are vital components of the plant apoplast where they are involved in reinforcement of plant cell walls via catalyzing the H_2O_2 -mediated cross-linking of cell wall components [87]. Increased production of H_2O_2 was indeed detected, and it represents an undeniable sign of oxidative burst caused by *F. verticillioides*. Secretion of novel defense protein, xylanase inhibitor, is a response to the fungal xylanases that are often exuded by the pathogen in order to hydrolyze the host cell wall and gain access to the plant nutrition source [88].

Maize response to the fungal pathogens *A. flavus* and *Fusarium* spp. that cause maize ear rot and contamination with mycotoxins shared several similar features [76, 83]. In both cases, maize tissues exhibited similar proteome-level defensive patterns as they responded with numerous PR proteins, detoxifying enzymes, and enzymes from the phenylpropanoid pathway. These findings support a previous study [89], which showed that both fungi share a common genetic resistance mechanism for the ear rot diseases.

4.2.3 Other maize pathogens

The interaction of maize with several other pathogens was also assessed through the proteome analyses. For example, Li et al. [90] reported that 123 proteins in leaves of susceptible line Ye478 responded to a 5-day-infection with a black-streaked dwarf virus. This pathogen causes maize rough dwarf disease with extensive damage to the maize leaves. Identities of 91 responsive proteins revealed defense mechanisms such as enhanced synthesis of oxidative stress-related proteins (e.g. peroxidase 39, APx2-cytosolic APX, and catalase isozyme 3), release of defensive secondary metabolites (e.g. benzoxazinoids and phytoalexins), modification of cell walls via cinnamyl alcohol dehydrogenase and caffeic acid 3-O-methyltransferase, and changes in signaling via RanA1protein, lipoxygenases, etc. Also affected by virus infection were metabolic pathways of carbohydrates and amino acids.

Curvularia lunata is a mold that causes a maize leaf spot. To gain an insight into the *C. lunata*–maize interaction, Huang et al. examined changes in the maize leaf proteome after infection with this pathogen [91, 92]. Using 2DE MS/MS, authors demonstrated that fungus-resistant inbred lines responded more vigorously to 24 h pathogen infection than susceptible genotype. Although 100 spots were differentially expressed, only ten were analyzed and eight were identified.

Among them, germin-like protein GLP and translation initiation factor eIF-5A appeared to be important players in fungal resistance [91]. When long-time exposure to *C. lunata* was applied (up to 72 h), more proteins responded in a time-dependent progressive increase in number of upregulated spots [92]. Twenty-seven of the most up/downregulated spots were identified, and they were associated with photosynthesis, respiration, and signal transduction. Moreover, stress proteins 22 kDa drought-inducible protein, glutathione peroxidase, and APX imply that drought-inducible and oxidation stress-related proteins also contribute to the resistance [92]. Similar to the maize–*A. flavus* interactions, these proteins are possible mediators between maize stress tolerance and resistance to *C. lunata*.

Proteomic approaches to study biotic stress in maize clearly showed that maize response to the pathogen is an intricate collaborative network of a broad range of proteins, metabolites, signaling molecules, cellular processes, and signaling pathways, engaging multiple organs and cellular compartments. This is not surprising since maize resistance to a majority of diseases is a quantitative trait that involves multiple genes and is usually affected by the environment [93]. For example, it was estimated that QTLs for maize disease resistance might cover as much as 89% of the maize genome [93]. Moreover, constitutive defenses are an inseparable part of the maize defense machinery as they prime the maize plant for forthcoming encounters with pathogens. Their importance was shown for maize resistance to *A. flavus* and aflatoxins [70–73, 76]. Although resistant maize germplasms are continuously being developed, the transfer of resistance into elite lines is difficult due to the lack of selective markers. Elucidation of resistance mechanisms through proteome-based analysis in conjunction with genomic and functional analyses will help geneticists and breeders to develop resistant maize varieties.

4.2.4 Symbiotic interactions

Fortunately, not all encounters of plants with microbial organisms are deleterious. Plants often live in favorable long-term relationships with symbiotic organisms, during which they benefit each other. *Trichoderma* spp., for example, are symbiotic fungi that help the plant host to enhance growth, alleviate stresses, and control pathogens [94]. By infecting plant roots, these fungi facilitate the increase of plant biomass and trigger localized defense as well as systemic resistance to other phytopathogens throughout the whole plant. To gain an insight into this unique relationship, proteome-level alterations during maize interaction with *T. harzianum* strain T22 symbiont were examined in maize seedlings. Colonization of maize roots with T22 induced changes in the proteome of both roots [95] and shoots [96]. In roots, 20 proteins were upregulated and 11 were repressed. Much stronger reaction, however, was observed in maize shoots where 117 and 50 proteins were induced and repressed, respectively [96].

In the T22-treated plant, there is a higher need for nutrients due to the boosted metabolism, therefore it was not surprising that more proteins from energy-yielding glycolysis and the TCA cycle were needed to power a symbiont-enhanced growth. Better growth is associated with cell wall expansion, and logically, enzymes participating in that event were also affected. In shoots, both UDP-Glc dehydrogenase and UDP-GlcUA decarboxylase were upregulated as well as phenylalanine ammonia lyase (PAL), the key enzyme of the phenylpropanoid pathway. A few photosynthesis-associated proteins and several stress-/defense-related proteins were also in higher abundance to provide the growing plant with a carbon source and to participate in a systemic resistance induced by colonization of roots, respectively [96]. Examples of enhanced stress/defense proteins include oxalate oxidase, peroxidase, GST, NBS/LRR resistance protein-like proteins, and β -glucosidase. Finally, other upregulated processes included amino acid metabolism and processing of genetic information. Proteomic findings presented in this study helped to elucidate some of the molecular mechanisms of T22-induced plant growth and T22-mediated induction of systemic resistance in plant. These findings support the concept of *Trichoderma* being a bio-control agent for maize [96].

5 Maize phosphoproteomics

Protein phosphorylation is the key regulatory mechanism that controls a broad range of cellular processes. The phosphorylation signaling network is a very dynamic assembly of numerous kinases and phosphatases that modify specific targets and amend their activities. It has been estimated that approximately 30% of all plant proteins are phosphorylated [97], thus systemic analysis of phosphorylation-mediated cell events via phosphoproteomics and MS has become an increasingly important and exciting research area. Establishment of plant phosphoproteomes has been making remarkable progress (reviewed in [98]) and is advancing from qualitative to quantitative (reviewed in [99]). The maize phosphorylation network is slowly being unraveled as well. Gel-resolvable phosphoproteins can be detected via ProQ-Diamond (Pro-Q DPS; Molecular Probes) and PhosTag™ (Perkin Elmer), unique fluorescent dyes that selectively bind to the proteins' phosphate moiety. They were used to examine phosphoproteomes of maize leaves [100], roots [66], and starch granules [47].

Numerous exogenous and endogenous stimuli trigger specific plant mechanisms that are activated by signal transduction pathways. For instance, maize leaf phosphoproteome was found to be responsive to mechanical wounding [100]. One hundred and twenty-five phosphorylated spots were detected via Pro-Q DPS after a short period (10–180 min) of mechanical wounding. Maize wound-inducible leaf phosphoproteome was unveiled via 21 identified proteins such as PEP carboxylase, PEP carboxykinase, pyruvate

Pi dikinase, HSPs, NBS-LRR protein, and 14–3-3 protein, and involved processes such as central metabolism, protein folding, and cellular signaling. Cellular pathways engaged in wound signaling are crucial for plant survival because they lead to systemic defenses throughout the whole plant and to the healing process at the damaged site, which could otherwise serve as an entry for pathogen invasion.

Salt-inducible phosphoproteome of maize roots was also defined [66]. Within a subset of 2DE-resolved and PhosTag™-stained phosphoproteins, 16 were responsive to 1-h salt treatment, of which ten proteins were phosphorylated and six were dephosphorylated. More details about this study can be found in Section 4.1.

As indicated by the following study, phosphorylation also seems to be actively involved in the regulation of maize starch biosynthesis [47]. Three proteins (granule-bound starch synthase (GBSS), branching enzymes BEI1b, and starch phosphorylase) were found phosphorylated when Pro-Q DPS was applied to inspect the phosphoproteome of starch granule-associated proteins in maize endosperm.

Some of the advantages of gel-based dye-specific techniques are that they eliminate the need for radiolabeling and antibodies and allow for establishment of 2D phosphoprotein reference maps. However, they can only be applied to a portion of phosphoproteomes. On the other hand, more comprehensive characterization of phosphoproteomes can be achieved via shotgun approaches, especially if preceded by phosphopeptides enrichment such as strong-cation exchange, as it was in the case of germinating embryos [101]. In this study, 776 proteins were identified, among them 39 kinases, 16 phosphatases, and 33 unique phosphoproteins containing 36 precise *in vivo* phosphorylation sites. According to the functional annotations, these proteins are involved in DNA repair, transcription, RNA processing, protein translation, and protein folding, implying that phosphorylation plays an integral role in seed germination.

The data above confirm the importance of phosphorylation for several aspects of maize biology. Maize phosphoproteomics is, however, still in its infancy, and the few studies published to date concentrated mainly on an untargeted proteome-wide survey of the constituents of phosphorylation signaling machinery. Systemic discovery of phosphoproteins, quantification of phosphorylation sites, and their assignment to the corresponding kinases will be challenging. According to ProFITS, a database of Protein Families Involved in the Transduction of Signalling in the maize genome (<http://bioinfo.cau.edu.cn/ProFITS/kinase.php>), 1052 genes in the maize genome were characterized as putative kinases. In addition to the large-scale mapping, in-depth analysis of phosphorylation-activated downstream events will be also necessary to fully unravel the regulatory networks and to construct signaling pathways. As more phosphorylation-related data are gained, establishment of a maize phosphoproteome database will hopefully occur in the near future.

Table 5. An overview of differential expression of proteins between wild-type (wt) maize and mutants

Plant material	Mutation	Phenotypic manifestation	Stage/age ^{a)}	Separation and identification methods	Number of differentially expressed proteins	Affected cellular processes/pathways	Reference
Endosperm	<i>op2</i>	Opaque starchy endosperm	15, 25, and 35 d after pollination	2DE, immunodetection; micro-sequencing	36 total Higher in wt: 17 Higher in <i>op2</i> : 19	Glycolysis, catabolism, amino acid biosynthesis	[103, 104]
Roots	<i>lrt1</i>	Does not initiate lateral roots	9 d after germination	2DE, MALDI-MS	18 total Higher in <i>lrt1</i> : 15 Higher in wt: 3	Lignin metabolism, transcription, defense,	[21]
Primary root	<i>rum1</i>	Does not initiate lateral and seminal roots	2.5 d old	2DE, nanoLC-MS/MS	14 total Only in <i>rum1</i> : 5 Only in wt: 4 Higher in <i>rum1</i> : 3 Higher in wt: 2	Stress response and defense, detoxification, citrate cycle, lignin metabolism, thiamine biosynthesis	[49]
Embryo	<i>rtcs</i>	Does not initiate embryonic seminal roots and postembryonic shoot-borne roots	25 d old	2DE, nanoLC-MS/MS	23 total Higher in <i>rtcs</i> : 11 Higher in wt: 12	Energy-yielding primary metabolism, protein fate and biosynthesis, cell fate, disease and defense, transport, transcription, development	[108]
Coleoptilar node	<i>rtcs</i>	Does not initiate embryonic seminal roots and postembryonic shoot-borne roots	5 d after germination	2DE, nanoLC-MS/MS	5 total Only in <i>rtcs</i> : 2 Higher in <i>rtcs</i> : 1 Higher in wt: 2	Signal transduction, defense, protein synthesis, metabolism	[48]
Coleoptilar node	<i>rtcs</i>	Does not initiate embryonic seminal roots and postembryonic shoot-borne roots	10 d after germination	2DE, nanoLC-MS/MS	14 total Only in <i>rtcs</i> : 4 Higher in <i>rtcs</i> : 5 Higher in wt: 5	Signal transduction, defense, metabolism, protein fate, cell fate, hormonal regulation	[48]
Embryo	<i>rum1</i>	Does not initiate lateral and seminal roots	30 d after pollination	2DE, nanoLC-MS/MS	38 total Only in <i>rum1</i> : 9 Only in wt: 14 Higher in <i>rum1</i> : 5 Higher in wt: 10	Primary metabolism (glycolysis), protein fate and biosynthesis, disease and defense	[50]
Cortical parenchyma	<i>rum1</i>	Does not initiate lateral and seminal roots	30 d after pollination	2DE, nanoLC-MS/MS	14 total Only in <i>rum1</i> : 5 Only in wt: 6 Higher in <i>rum1</i> : 1 Higher in wt: 2	Metabolism, signal transduction, protein fate and biosynthesis, disease and defense, energy	[50]
Stele	<i>rum1</i>	Does not initiate lateral and seminal roots	30 d after pollination	2DE, nanoLC-MS/MS	34 total Only in <i>rum1</i> : 11 Only in wt: 19 Higher in <i>rum1</i> : 2 Higher in wt: 2	Metabolism, signal transduction, protein fate and biosynthesis, disease and defense, energy, cell cycle, cell fate	[50]

a) d: days.

6 Wild-type versus mutant proteomic analyses/pleiotropic effects of mutants

By comparing proteomes of maize mutants and their corresponding wild-type genotypes, number of studies attempted to elucidate the impact of a disrupted gene on the biology of the maize plant. Such investigations were conducted to assess a pleiotropy, which is described as the genetic effect of a single gene on multiple phenotypic traits. An overview of maize wild-type versus mutant proteomics studies is given in Table 5.

6.1 Endosperm

A pleiotropic effect of the *opaque-2* (*o2*) mutation alters endosperm development via changes in the expression of multiple proteins that are under direct or indirect control of the transcriptional activator O2 [102, 103]. The most profound effect of *o2* is increased lysine and tryptophan levels and decreased zein amounts resulting in improved nutritional value of kernels [102]. The effect of the *o2* mutation on mature endosperm was assessed via analysis of wild-type, isogenic (W64A) and near-isogenic (F2) maize mutant lines, that differ at the *o2* locus, and their reciprocal hybrids with wild-type genotypes [103]. Thirty and 36 protein spots differed between wild-type and F2 and W64A mutants, respectively, with 14 proteins affected in both backgrounds. Subsequent immunodetection revealed the identity of two O2-affected proteins, b-32 protein, and globulin. Additionally, proteomic analysis confirmed that relative to the wild-type genotype both mutants had lower levels of 22 kDa zeins, major storage proteins of maize endosperm.

When developing kernels were explored, 36 endosperm proteins were altered in expression between *o2* and wild-type plants during multiple developmental stages and throughout the seven genetic backgrounds [104]. An additional 19 proteins were identified from assorted metabolic pathways that were not previously known to be affected by *o2* mutation, such as glycolysis, catabolism, and amino acid synthesis. Seventeen proteins, among them pyruvate orthophosphate dikinase and acetohydroxy acid synthase, were more abundant in wild-type endosperm, and most were affected early in development. Remaining proteins, such as GAPDH, aspartic proteinase precursor, and sorbitol dehydrogenase, were more abundant in *o2* mutants, and these were usually affected later in endosperm development, implying that these proteins might be under indirect control of transcriptional factor O2 [104]. When mature endosperm proteins from W64A/*o2* mutant and 45 genetically diverse inbred lines were compared [105], 59 different isoforms of zeins were detected, reflecting their immense diversity. Due to allelic variations, each inbred line contained a unique pattern of zein isoforms that differed both qualitatively and quantitatively, and only six isoforms were present in all inbreds. As in the previous study, the most affected were 22 kDa zeins.

6.2 Roots

Roots are essential organs for plant growth and development, as they continuously supply the plant with water and dissolved nutrients. The maize root system consists of several root types that are formed during different stages of development [106]. Primary and seminal roots are preformed during embryogenesis, while shoot-borne roots are formed postembryonically later in development [106]. Several mutants that affect the maize root system have been used to investigate their pleiotropic effect in maize.

Lateral roots comprise a majority of absorbing power due to their enormous surface area and are of special interest to researchers. Hochholdinger et al. compared the protein patterns of primary roots of the *lrt1* mutant (does not initiate lateral roots) and wild-type seedlings (both 9-day-old) [21]. Authors discovered that 10% of the proteins detected by 2DE were at least threefold more abundant in the *lrt1* mutant. Enzymes involved in lignin metabolism, such as caffeoyl-CoA-3-O-methyltransferase, beta-glucosidase, *L*-APX, and 1,4-benzoquinone reductase, were among the most upregulated proteins (4.0- to 16.4-fold compared to wild-type). These results suggest that the accumulation of primary root proteins might be a consequence of nonexistent signals from absent lateral roots, which could otherwise repress their expression. Lack of such control provides indirect evidence of communication between the lateral roots and the primary root system [21].

In a similar study, gel-based comparisons were made between primary root proteomes of wild-type and *rum1* mutant [49], which is impaired in both lateral and seminal root initiation. Proteomes were compared at the developmental stage prior to lateral root formation (2.5-day-old). Fourteen proteins, primarily those related to lignin metabolism, stress response, and citrate cycle, were differentially expressed. Results of this study are described in more detail in Section 3. Interestingly, 1,4-benzoquinone reductase was also predominantly expressed in primary roots of *lrt1* mutant (16.4-fold) [21] (see above). Findings of both studies thus indicate that control of lignin biosynthesis in primary roots occurs both before lateral roots initiation and after their emergence [49].

Formation of shoot-borne roots is dissimilar from embryogenetically preformed primary and lateral roots, as shoot-borne roots are initiated from coleoptile nodes by dedifferentiation after seed germination [107]. Shoot-borne root initiation was studied by examining the protein patterns of coleoptile nodes of *rtcs* mutant (does not initiate embryonic seminal roots and postembryonic shoot-borne roots) and its wild-type counterpart for differences in their protein patterns [48]. Details from this study are discussed in Section 3.

6.3 Embryo

Since primary and seminal roots are preformed during embryogenesis [107], the molecular mechanisms of root

development can be studied in embryos. To identify proteins associated with embryonic seminal root formation, immature embryos from the *rtcs* mutant (does not initiate embryonic seminal roots and postembryonic shoot-borne roots) were compared with wild-type embryos (form 2.9 seminal roots per seedling on average) [108]. At this early stage of seminal root development (immature; 25 days old), wild-type embryos differ from the *rtcs* by the presence of seminal root primordia. At the protein level, 23 proteins exhibited at least twofold higher expression in wild-type embryos. Very likely due to the enhanced meristematic activity of the seminal root primordia, the accumulated proteins were pertinent to the carbohydrate metabolic pathways and were represented by two isoforms of phosphoglycerate kinase, enolase, and malate dehydrogenase. Moreover, elongation factor 1- γ 3, protein disulfide isomerase, and co-chaperone protein SBA1 are proteins involved in protein synthesis, a very active process during growth and development. Thus, expression of these proteins seems to be under control of the RTCS gene [108]. Coleptilar nodes of *rtcs* mutant seedlings and their wild-type counterpart were also studied to elucidate shoot-borne root initiation [48], and they are discussed in more detail in Section 3.

When 30-day-old *rum1* and wild-type embryos were compared [50] (also discussed in Section 3), 38 proteins showed differential expression, with 14 proteins in *rum1* and 24 proteins in wild-type upregulated. Differential accumulation was mainly evident for 11 isoforms of globulin in both types of embryo extracts. Globulins' function in embryonic root formation is not yet clear. A trypsin inhibitor, which might be involved in the regulation of globulin degradation, was expressed only in wild-type embryos. Preferential accumulation of ribosomal proteins in wild-type embryos can also be explained by their higher meristematic activity translated to the increased protein synthesis rates. Finally, differential expression of glycolytic enzymes suggests that these proteins are required to power embryonic root development. A little overlap was found between the two differentially expressed datasets (wild-type versus *rtcs* and wild-type versus *rum1*), in which both mutants do not initiate seminal root formation and differ only by postembryonic lateral root initiation capability. As suggested by Muthreich et al. [108], this is possibly due to slightly different embryo ages (25-day-old *rtcs* versus 30-day-old *rum1*) and/or dissimilar impacts of RTCS and RUM1 genes on the downstream pathways during embryogenesis.

6.4 Other tissues

In the study referenced above [50], the authors also analyzed cortical parenchyma and stele tissues, in which 14 and 34 proteins, respectively, showed dissimilar accumulation between *rum1* and wild-type plants. When compared to the embryos, signal transduction-related proteins were more abundant in the differentially expressed dataset of cortical parenchyma,

while proteins related to protein fate were significantly under-represented in the differentially expressed dataset of stele.

For many plant cells, starch granules comprise the major carbohydrate storage; therefore, the biosynthesis of starch polymers amylose and amylopectin plays a central role in their metabolism [47]. Genetic, immunological, and proteomic approaches have been used to investigate proteomes and phosphoproteomes of maize endosperm starch granules [47]. Mutant versus wild-type proteomic survey confirmed that starch synthases (SSI, SSIIa, SSIII), branching enzymes (BEI, BEIIa, BEIIb), GBSS, and starch phosphorylase were indeed associated with internal granules. Moreover, GBSS, BEIIb, and starch phosphorylase were found phosphorylated in granules. This study provided a more complex view of granule proteomes, as several proteins not previously known to be located within maize starch granules were identified.

7 Heterosis-associated maize proteomes

By definition, heterosis, or hybrid vigor, is the genetic superiority of heterozygous F1-hybrid offsprings over those of their homozygous-inbred parents [8]. Heterosis has immense agronomic value and for decades has been utilized in maize breeding to produce superior high-yielding hybrids. However, the molecular mechanisms underlying this event are still not completely understood. One of the approaches to study heterosis at the molecular level is to compare the gene expression of hybrids and their parental inbred lines in order to identify heterosis-associated genes and gain insight into the processes governing this phenomenon.

7.1 Manifestation of heterosis in embryos

Phenotypic manifestation of heterosis is typical for adult plants, however, hybrid superiority can already be observed during maize early development, as demonstrated by morphological [109] and gene expression analyses [110, 111]. At the protein level, early manifestation of heterosis was confirmed in germinating embryos from five elite hybrids and corresponding parents by comparing their protein patterns [112]. Cumulatively, over 1400 hybrid proteins exhibited nonadditive expression patterns including dominance, partial dominance, and overdominance, suggesting that these events play an important role in regulating maize seed germination. Identified proteins fell into nine functional categories, which indicates that heterosis in germinating embryos is a complex trait. For example, detoxification via GST, catalase, and hydroxyacid oxidase might be needed to deal with an oxidative stress initiated by imbibition. HSPs are produced upon various stresses but also under nonstress conditions, such as germination, to act as molecular chaperons. In summary, the majority of heterosis-related proteins fell to metabolism, detoxification, chaperoning, and signal transduction via gibberellic and ABA signaling pathways, crucial developmental

processes during which the future fate of the plant is established [112].

Although heterosis in the germinating embryos is clearly evident, hybrid vigor can already be observed during embryo development. For instance, reciprocal maize hybrids manifested heterosis during embryogenesis for several important traits such as length, weight, and the time point of seminal root primordia formation [113]. The 2DE/MS/MS analyses of 25-day-old embryos (seminal root primordia absent) and 35-day-old embryos (seminal root primordia present) showed that 141 protein spots (24%) exhibited nonadditive accumulation in at least one hybrid. Functional classification revealed that development and protein metabolism were the most abundant categories among the nonadditively accumulated proteins. They were followed by redox regulation, glycolysis, and amino acid metabolism. At the later stage of embryogenesis (35 days postpollination), upregulation of glucose metabolism was most profound, which may be a result of more vigorous embryo development in the hybrids [113]. Better performances of hybrid embryos were also verified by morphological and histological dissection. Enhanced involvement of the identified processes supported the notion that more resources and energy are used in the heterosis-driven growth.

7.2 Manifestation of heterosis in roots

The primary root is the first organ emerging from the germinating seed. Three and a half days after germination, primary roots of hybrids exhibited distinct protein expression from their parental inbreds [114]. The 2D gels revealed that 150 detected hybrid proteins (49%) accumulated in nonadditive fashion. For 17 proteins, expression in the hybrid significantly exceeded both parental inbred lines. Among them were mainly proteins from metabolism and defense such as PAL, aconitate hydratase, cytoplasmic, alpha-1,4-glucan-protein synthase, SOD (Mn) 3.4, mitochondrial, and a few HSPs. An additional seven protein spots of PAL, together with caffeoyl-CoA 3-O-methyltransferase 1, chalcone-flavonone isomerase, and numerous other proteins (59 total) were expressed in the hybrid at significantly lower levels than in both parents. Interestingly, PAL was abundantly present in proteome of 2.5-day-old maize pericycle that resides on primary roots and from which lateral roots are formed [22]. Given that lateral root density is significantly enhanced in hybrid plants [109], PAL seems to play a pivotal role in lateral root density [114]. Moreover, defense-related proteins GST, UDP-glucosyltransferase BX8, and several HSP also appear to be essential for the manifestation of heterosis during early development of root.

Maize has become a model organism for studying heterosis, but a complete understanding of this phenomenon continues to be a daunting task. This is because of the immensely complex nature of heterosis and the involvement of multiple traits and organs, which ultimately

combine into a superior phenotypic manifestation—a large agronomic yield. A complete understanding of heterosis on the molecular level will be critical for further increase of crop performance.

7.3 Parental imprinting

In maize, phenotypic differences between reciprocal F1 hybrids are commonly observed due to parental imprinting. Comparative proteomic analyses of reciprocal F1 hybrids and their parental lines proved to be an excellent technique for identification of genes associated with uniparental dominance, which subsequently allows for proper selection of parents for breeding events. In the study of Kollipara et al. [115], reciprocal crosses were made between recombinant inbreds tolerant and nontolerant to cold germination (CG) and desiccation. Resulting F1 hybrids, exhibiting contrasting phenotypes (high and low tolerance) for CG or DT traits, were selected for differential profiling of transcripts and proteins. Using 2DE, the authors compared the protein patterns of embryo axes from reciprocal F1 hybrids in order to identify CG- and DT-associated proteins. They discovered that 117 and 205 proteins showed at least a 1.5-fold difference in expression for CG and DT, respectively, with 56 and 57 of the proteins identified. Due to the complexity of both traits, multiple biochemical pathways seemed to be affected. With regard to the DT trait, 30 different globulins comprised the largest group of differentially expressed proteins. For the GC trait, differentially expressed proteins were more diverse and included mainly proteins from proteolysis, glycolysis/gluconeogenesis, cytoskeleton organization, and stress response.

8 Other maize phenomena analyzed by proteomic approaches

8.1 Seed viability assessment

Seed viability is an important agronomic trait, during which dormant seed maintains the capability to germinate and establish a new plant. During storage, however, seeds tend to lose viability due to the aging process, which subsequently leads to their deterioration and incapability to germinate [116]. Loss of seed viability is accompanied by changes in their proteomes [117]. To identify proteins associated with seed deterioration in maize, a few studies were performed. Utilizing gel-based approaches, maize seed viability was assessed via artificial aging [118, 119].

Wu et al. [119] compared protein profiles of embryos dissected from seeds with degrees of viability ranging from highly viable to zero-viable (dead), which were prepared by exposure to 41°C and 100% relative humidity for 1–6 days (controlled deterioration treatment) to mimic natural aging. Twenty-five proteins showed significant differences in protein expression between the two types of embryos (highly

viable and dead). A majority of these proteins (19) was more abundant in the viable embryos and was related to stress response, protein folding and stabilization, nutrient reservoir, and metabolism. Higher amounts of small HSPs, LEA proteins, and antioxidant enzymes glyoxalase, thioredoxin peroxidase, SOD, and GST indicated that these enzymes play an important role in seed viability maintenance via their protective properties to the cells. For example, LEA proteins are well-known enhancers of plant tolerance against diverse abiotic stresses, while the antioxidants detoxify cells from harmful chemicals. The authors did not address the possible role of fructose-bisphosphate aldolase. This protein was more than 100-fold downregulated, which suggests that sugar metabolism is also affected during the aging. On the contrary, two isoforms of CAAX prenyl protease were present at higher amounts in dead seeds where they very likely contributed to the deterioration process by digesting embryonal proteins [119].

When seeds were artificially aged by exposure to 50°C for 5 or 13 days [118], their embryos exhibited impaired metabolism, elevated proteases, degraded storage proteins, altered signal transduction and transcription pathways, disrupted defense systems, and abnormal cell division and growth, which resulted in a remarkable decrease in seed vigor and eventually led to seed deterioration [118]. Forty proteins were altered in abundance and a portion of them was from glycolysis, TCA cycle, electron transport chain, and oxidative phosphorylation. Downregulation of these proteins in aged seeds obviously resulted in disrupted mobilization of energy supplies required for a successful germination. Similar to the previous study, a few proteins responsible for protein degradation were upregulated in artificially aged seeds, which could lead to the breakdown of storage proteins, such as globulin 2 and vicilin-like protein. These two storage proteins were indeed found in diminished amounts in the deteriorated seeds, resulting in reduced supplies of amino acids that are essential for the synthesis of new proteins required for germination. Abnormal cell division due to altered expression of cyclin-dependent kinase A and mitogen-activated protein kinase is another factor affecting seed viability. Finally, oxidative damage to seeds due to ineffective protection from antioxidant system further contributed to vigor decline.

The causes of seed deterioration during aging are not completely understood. They can certainly be attributed to genetic predisposition, but improper storage also may play a role. To understand the mechanism of aging and signals that trigger it is critical for lengthening the seed life span. As shown from the studies reviewed above, the aging process is a very complicated trait that involves alterations in many cellular mechanisms ranging from basic metabolism that provides energy and support for vigorously dividing embryonic cells, to processes leading to death, such as high activities of cellular proteases and abnormal cell division [118, 119]. The quantitative trait of seed longevity was also confirmed by genetic analyses [120].

8.2 Safety assessment of genetically modified (GM) maize

With the introduction of recombinant DNA technology, new varieties of maize with improved agronomic traits have been developed. However, as for any GM crop, insertion of foreign DNA into the maize genome has raised public concerns about the potential risks to human health and the environment. To evaluate the possible unwanted effects, safety assessments of GM maize are necessary. They are achieved by comparison of GM varieties with non-GM counterparts of the same genetic background. Safety assessments were conducted for MON810 (Bt maize, Monsanto), varieties of GM maize that were developed to resist European corn borer (*Ostrinia nubilalis*). Differences between MON810 and non-GM maize were investigated via untargeted MS-based proteomic analyses of grains from plants grown in fields [26, 121–123] and growth chambers [124, 125] to account for environmental influences.

For the field-grown plants, interesting results were obtained in flour samples prepared from the kernels [26]. For example, glucose and ribitol dehydrogenase homolog was detected exclusively in insect-resistant Bt maize, while endochitinase A was unique to a near-isogenic non-GM variety. In addition, transgenic maize overexpressed triosephosphate isomerase 1 and globulin 1, while downregulating cytosolic 3-phosphoglycerate kinase and aldose reductase. Since the two compared lines ideally should differ only by the presence of an inserted *Cry1Ab* gene in MON810 maize, the authors speculate that glucose and ribitol dehydrogenase homolog in Bt flour might have already been present in the wild-type maize before transgene insertion. Similarly, an absence of endochitinase A in Bt flour can be attributed to the insertion event. Interestingly, *Cry1Ab* protein was not found among the Bt flour unique proteins (unmatched 2DE spots) likely due to detection limits, but it was confirmed by immuno-assay.

When milky-starchy stage seeds (used in forages) were analyzed [121], very few 2DE spots were differentially accumulated (less than 1.2%) with expression change levels below 1.8-fold. Examples of upregulated proteins in MON810 cultivars included HSPs, triose-phosphate isomerase, sorbitol dehydrogenase, and adenosine kinase. The authors concluded that variability between the GM and non-GM is variety specific and cannot be solely attributed to the Bt transgene. However, similar to the previous study, they did not take into consideration that nontransgenic control lines were not entirely isogenic, which could contribute to variability. Near-isogenic lines were also used to assess maize plant-to-plant variability by comparing them to samples from individual GM plants and pools of GM plants [122]. Variability was found in both cases, as well as the masking of significant differences in pooled samples.

A more thorough study that involved analysis of gene, protein, and metabolite expression in control and two transgenic lines, Bt maize and RR maize (herbicide-tolerant Roundup Ready) was performed by Barros et al. [123]. By considering

samples from different locations and multiple growing seasons, the authors concluded that the environment indeed has a strong effect on gene and protein expressions as well as on metabolite production. Five proteins, 65 genes and 15 metabolites were differentially expressed among two GM plants and one control during three growing seasons at one location. Dissimilar protein patterns were also revealed for the three different locations. More of the detected variations were caused rather by the environment than by genetic manipulation. Interestingly, gene expression values for a known maize allergen LPT protein were significantly lower in the transgenic variety than in the non-GM variety. Regrettably, none of the five differentially expressed proteins could be identified.

To specify effects of the environment and the transgene, two subsequent generations of seeds from both GM and isogenic non-GM lines were scrutinized in a series of comparative analyses [124]. Unlike in previous studies, a significant number of proteins were found differentially regulated in MON810 seeds by both the environment and by insertion of the *Cry1Ab* gene. Moreover, environmental influence on GM proteome was significantly stronger than that resulting from gene manipulation. One hundred differentially expressed proteins were modulated by the environment, as opposed to 43 proteins affected by the transgene. A known maize allergen, 50 kDa gamma zein, was found as a newly expressed protein in MON801 [124].

A controlled environment was also applied to inspect changes in leaf proteomes among the four Brazilian MON8110 lines and four non-GM isogenic counterparts [125]. Although alterations in the MON8110 leaf proteomes are not directly implicated in human health, their identification may help to assess the risks to the environment. During a two-year study focused on measuring qualitative differences, 12 newly expressed leaf-specific proteins were observed in two GM varieties. Homology-based identification was obtained for two proteins, putative NAD-dependent epimerase/dehydratase and phosphatase 2C. With regard to the physiological parameters (aerial part weight, main leaf length, chlorophyll, and total protein contents), some differences were observed, but they were not consistent between GM and non-GM genotypes, and cannot be considered as reliable indicators of insertion-caused changes.

Finally, in the attempt to determine maize allergenic potential possibly induced by transgene [126], no statistically significant differences between MON810 and nontransgenic seeds were detected for known maize allergens, lipid transfer protein (LTP), trypsin inhibitor, thioredoxin, 50 kDa zein, and glutelin. On the other hand, 14 novel allergens were identified in both GM and non-GM varieties via immuno-assay of plasma from maize-allergic patients against maize seed protein extracts. As reported in previous studies, many of the newly identified maize allergens were stress-related defense proteins such as LEA, dehydrin, chaperonin CPN60–1, cysteine proteinase 1 precursor, UDP-glucose pyrophosphorylase, and triacylglycerol lipase.

Despite their immense agronomic potential, safety of GM crops is persistently being questioned and scrutinized. A number of “omics” technologies have been implemented to assess the safety of GM maize and to determine possible unintended alterations to its genome during transformation that could negatively affect the maize physiology (for a review on methodologies see [127]). These concerns are legitimate since such unwanted alterations have previously been reported [128]. They included deletions and rearrangements of host DNA at the site of insertion [128]. Contemporary safety assessment studies are predominantly focused on untargeted profiling. However, they face two big challenges—the immense natural variability of maize and the strong effect of the environment [129]. Careful experimental designs must therefore be implemented to account for interfering variables and to produce accurate data, as misrepresentation of data can have significant consequences on the marketing of GM cultivars.

8.3 Search for maize allergens

Food-induced allergies can have serious health implications. Since maize is one of the most globally grown crops, the focus of scientists is turning toward the identification of its allergens. The first maize allergen was discovered by Pastorello et al. only a decade ago [130]. A 9 kDa LTP was found via immuno-assays between protein extracts from commercial maize flour and sera from 22 anaphylactic patients. Apparently, this defense protein (belonging to the PR proteins) is very stable and accounts for 4% of all soluble proteins in maize seeds and flour [131]. LTP protein was later found in five other maize hybrids and proved to be heat stable, hence likely present in cooked maize-containing foods [132]. Owing to the advances in MS technologies, much stronger allergenic potential of maize seeds was revealed recently [133]. By exploring genotype B73, six additional maize allergens were identified. Vicilin, globulin-2, 50 kDa gamma-zein, endochitinase, thioredoxin, and trypsin inhibitor were detected via 2DE/nanoLC MS/MS, thus significantly expanding the repertoire of maize allergens. Interestingly, a set of important defense proteins was among the maize sensitizers.

Allergenic properties of transgenic MON810 maize have also been evaluated. Fourteen novel allergenic proteins were detected. However, they were found in both the GM and non-GM varieties. More details about this study can be found in Section 8.2.

Pollen allergies are also common due to pollen being easily widespread. Via gel-based and immunological comparison of maize pollen with pollen of native timothy grass (*Phleum pratense*), Petersen et al. [134] demonstrated that European maize pollen has a less complex allergen pattern, as it contains only four grass pollen allergen groups (1, 3, 12, and 13). Its allergenic potential is thus much lower than that of common grasses. Immuno-detection followed by N-terminal sequencing validated the presence of expansin, profilin, and

polygalacturonase, with expansin being the most prevalent pollen sensitizer. No LTP was found in investigated maize pollen samples.

Maize allergies, especially the food borne, can cause severe health problems particularly in the highly allergic patients. Before the era of high-throughput proteomics, very little was known about the potential allergenic properties of maize. Proteins were routinely resolved by 2DE, however, their identification had to rely solely on immuno-detection and N-terminal sequencing, which were not always successful. Recent advances in MS and maize sequence data allow for unambiguous identification of novel allergens, significantly broadening our knowledge about the harmful potential of the maize to the allergic population.

9 “Preproteomics” era

Maize proteomics had been pioneered prior to the term itself even being coined. Today, “proteomics” evokes use of high-throughput analysis aided by sophisticated mass spectrometers and software algorithms. However, 2DE, immunodetection, N-terminal sequencing, and bioassays have their rightful place in the history of protein research, because they provided valuable knowledge, and set the stage for the next “high-tech” act. For instance, the above approaches contributed to elucidation of following phenomena: genetic variability due to allelic polymorphism [135], nonadditive inheritance in hybrids [136,137], organ-specific genetic variability [137], coleoptile genetic diversity [138], relation of protein amounts to phenotypic variability [139,140], and detection of polymorphic loci and genetic markers that can be subsequently employed by breeders [141,142]. A key enzyme (cysteine proteinase) in maize resistance to Lepidoptera insects was also identified using “classical” methods [143].

10 Concluding remarks

With the progress of MS-based proteomics and maize genome sequence availability, researchers were given tools to explore previously uncharted areas of maize biology. As documented by this review, proteomics approaches were instrumental in gaining new knowledge for a wide range of topics. Proteomes of leaf, endosperm, egg cells, root hairs, primary root, primary root pericycle, root tip, rachis, starch granules, and seed flour were mapped, as were subcellular proteomes of mitochondria, cell wall of primary root elongation zone, and chloroplasts with their subcompartments such as stroma, thylakoids, envelope membranes, and nucleoids. Proteins of maize secretomes of xylem sap and root mucilage were also defined. Developmental studies were focused on endosperm, root growth, and leaf. In developing leaves, considerable effort was devoted to the biogenesis of plastids in mesophyll and BS cells. Insight into mechanisms of maize response to anoxia, water stress, nutrient deficiency,

temperature stress, UV radiation, salinity, and metal toxicity was gained, equally so for biotic stress caused by *Aspergillus*, *Fusarium*, black-streaked dwarf virus, and *Curvularia*. Knowledge of signaling and regulatory networks was broadened by methods of phosphoproteomics. Proteome differences between maize mutants and wild-type genotypes were identified for roots, embryos, cortical parenchyma, and starch granules.

New maize allergens were identified, and other phenomena were assessed, including heterosis, parental imprinting, seed viability, and safety of GM maize.

In concert with MS, reviewed studies utilized separation methods such as LC and 2DE (see Table S1 in Supporting Information). Most of the proteomic investigations employed gel-based techniques (83 2DE versus 16 1DE versus 6 LC), some using phosphoprotein (2) and glycoprotein (1) specific stains and 2D DIGE (4). Despite being labor intensive, time consuming, and limited to the detection of proteins soluble under native condition (creating a general bias against hydrophobic proteins), 2DE clearly remains the method of choice for resolving maize proteins and their qualitative and quantitative analyses. One can only speculate why this is contrary to the LC methods typically preferred in proteomics studies, generally. Obviously, 2DE must be attractive to maize researchers, perhaps due to pattern visualization, the chance to repeat identification attempts on particular spots (when MS fails), computational ease of spot-based quantification and differential studies, improved detection limits due to the use of sensitive dyes, the availability of phosphoprotein- and glycoprotein-specific dyes, visualization of protein isoelectric isoforms, and access to existing 2DE-related robotics equipment for spot excision and in-gel digestion.

Regardless of particularities, because it is the proteins that are the functional translations of genes, and because posttranscriptional regulations lead to miscommunication between mRNA and protein amounts, the assessment of cellular proteomes is of immense interest to researchers. Future development will likely bring a progress in automated robotic sample preparation, better resolution in protein/peptide separation, even faster scanning, more accurate and sensitive mass spectrometers with PTM-friendly modes of fragmentation and quantification methods based on spectral counting, ion intensities, and SRM. Advances in the technology will result in better reproducibility, higher confidence, more complete proteome coverage, wider dynamic range of detection, and identified protein sets rich in modified proteins. No doubt, investigators will continue to exploit ever more powerful proteomics techniques for deeper understanding and consequent improvement of one of the most important crops in the world—maize.

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