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Review

Deciphering the soybean molecular stress response *via* high-throughput approaches

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As a result of thousands of years of agriculture, humans had created many crop varieties that became the basis of our daily diet, animal feed and also carry industrial application. Soybean is one of the most important crops worldwide and because of its high economic value the demand for soybean products is constantly growing. In Europe, due to unfavorable climate conditions, soybean cultivation is restricted and we are forced to rely on imported plant material. The development of agriculture requires continuous improvements in quality and yield of crop varieties under changing or adverse conditions, namely stresses. To achieve this goal we need to recognize and understand the molecular dependencies underlying plant stress responses. With the advent of new technologies in studies of plant transcriptomes and proteomes, now we have the tools necessary for fast and precise elucidation of desirable crop traits. Here, we present an overview of high-throughput techniques used to analyze soybean responses to different abiotic (drought, flooding, cold stress, salinity, phosphate deficiency) and biotic (infections by *F. oxysporum*, cyst nematode, SMV) stress conditions at the level of the transcriptome (mRNAs and miRNAs) and the proteome.

Key words: soybean, transcriptome, miRNA, proteome, stress conditions

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INTRODUCTION

Soybean is a plant of the *Fabaceae* family, which originates from East Asia. It is considered as one of the most important crops in the world. Because of its high protein (38–45%) and oil (approx. 20%) content, as well as its ability to perform symbiotic nitrogen fixation, soybean is called the Miracle Bean and presents a variety of benefits to farmers, industry, food processors and consumers (http://wwf.panda.org/what_we_do/footprint/agriculture/soy/facts/). At first, the soybean meal was obtained as a byproduct of oil production which is now the second most highly consumed oil in the world. Soybean is used mainly for the production of oil and animal feed (source of protein), but also widely used in food production (oil, soy milk, soy flour, tofu and food additives), as well as for industrial goods (e.g. cosmetics, plastics, paints). Moreover, the oil extracted from this plant is one of the primary raw materials for biodiesel production (Tyczewska *et al.*, 2014).

In the past 30 years, the world production of soybeans has tripled and in the 2013/2014 season it exceeded 283 million tons. In 2013, soybeans ranked as the 8th among the top food and agricultural commodities production worldwide (http://faostat3.fao.org/browse/rankings/commodities_by_regions/E). Currently, soybean is grown in many regions of the world, primarily in the North and South Americas, and in Asia. Greater use of soybean in various industries has resulted in an intense increase in its consumption; since the 70s in the 20th century this consumption has increased by over 200 million tons (Garrett *et al.*, 2014). About 400 000 hectares of soybean grown in the European Union represent only 3% of the demand at our continent for the animal feed industry. Hence, the European Union annually imports 20 million tons of soybean from the North and South Americas. One of the major difficulties in the cultivation of soybean in Europe is the climate which is characterized by cool springs and early summer droughts. To overcome this problem, soybean varieties insensitive to the European temperate climate should be cultivated.

The process of innovation in agriculture is based on achieving the so called biological progress which is the dominant factor determining the growth of agricultural productivity. It is associated with the introduction of changes that affect technological and practical values of plants and animals related to productivity, health quality, suitability for processing and consumer expectations (Mańkowski *et al.*, 2012). Also, adverse changes in the environment caused by human activities pose new challenges for farmers, who must provide an increase in the yield of crops grown under abiotic stresses, like cold, drought or high salinity of the soil.

Nowadays, improvements in yield, quality, abiotic and biotic stress tolerance are major targets in soybean breeding programs. Abiotic stress is defined as a negative impact of non-living factors on the living organisms in a specific environment; as a natural part of every ecosys-

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Abbreviations: 2-DE, two dimensional electrophoresis; ABA, abscisic acid; CD, Chundou (soybean variety); DEG, differentially expressed gene; DGE, digital gene expression tag; EST, expressed sequence tag sequencing; GABA, gamma-aminobutyricacid; GO, gene ontology; HB, Harbin xiaoheidou (soybean variety); HDEG, highly differentially expressed gene; hpi, hours post inoculation; L10, Liaodou 10 (soybean variety); LC, liquid chromatography; LC-MS, liquid chromatography–mass spectrometry; LEA, late-embryogenesis abundant; miRNA, micro RNA; MS, mass spectrometry; N, nitrogen; NGS, next generation sequencing; Pi, phosphate; PS I, photosystem I; PSII, photosystem II; qRT-PCR, quantitative reverse transcription polymerase chain reaction; QTL, quantitative trait loci; RAM, root apical meristem; ROS, reactive oxygen species; RNA-seq, RNA sequencing; RT-PCR, reverse transcription polymerase chain reaction; RuBisCO, ribulose bisphosphate carboxylase/oxygenase; SCN, soybean cyst nematode; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SMC, soil moisture content; SMV, soybean mosaic virus; sRNA, small RNA; SSP, seed storage protein; TF, transcriptional factor; YH, Yunhefengwodou (soybean variety)

tem, this affects organisms in a variety of ways. To cope with stress conditions, biological organisms exposed to the environmental stimuli had developed different strategies (Trindade *et al.*, 2011). Because of their stationary life style, plants require efficient short-term strategies based on manipulation of the existing genetic information (restricted to the tolerance, resistance, and avoidance mechanisms only) (Boyko & Kovalchuk, 2008). Therefore, plants acquire resistance to the stressful environment by reprogramming their metabolism and gene expression, gaining a new equilibrium between growth, development and survival (Mazzucotelli *et al.*, 2008).

To ensure proper (temporal and spatial) changes in the gene expression in response to stress conditions – post-transcriptional and post-translational mechanisms, as well as their interactions, must be strictly controlled. The network of such mechanisms is expected to effectively target transcription factors and other regulatory components of the stress signaling, resulting in either activation or repression of their activities (Boyko & Kovalchuk, 2008). Among other things, different families of proteins involved in e.g. signaling (second messengers, plant hormones, signal transducers and transcriptional regulators function), translation, host-defense mechanisms, carbohydrate and amino acid metabolism products known to be associated with the plants' response to stresses are being newly synthesized, accumulated or depleted (Timperio *et al.*, 2008; Hirayama & Shinozaki, 2010).

Several research groups are making efforts to evaluate the mechanisms underlying the soybean response to various stress conditions. Therefore, herein, we wish to focus on the available large scale high-throughput analyses of transcriptomes (protein coding RNAs, as well as non-protein coding RNAs – particularly miRNAs) and proteomes obtained under soybean stress conditions (overview of the described data and references is presented in Table 1).

TRANSCRIPTOME

The term "transcriptome" refers to a pool of RNA molecules which are transcribed in a particular cell or tissue under given conditions. In contrast to the genome, whose sequence is rather fixed during the lifespan of an organism (excluding somatic mutations), the transcriptome is characterized by a high level of plasticity and reflects changes taking place in developmental stages, as well as in response to external stimuli. Transcriptomic studies are also referred to as expression profiling – they answer questions on both: which genes are active under particular conditions and what is the level of expression of the transcribed genes. In 2010, the soybean genome was sequenced (Schmutz *et al.*, 2010) and the latest assembly of *Glycine max* transcriptome deposited in the Phytozome database (Goodstein *et al.*, 2012) predicted 56 044 protein-coding loci and 88 647 transcripts, which can be expressed during the soybean's life cycle.

In recent years, we have observed a tremendous increase in the transcriptomic data generated with high-throughput technologies, like microarrays and Next-Generation Sequencing (NGS) technologies, which are becoming a method of choice in the transcriptomic analyses (Urano *et al.*, 2010). At the beginning of the transcriptome research, methods like Northern blot allowed to analyze transcripts from a single gene. Then, reverse transcription semi-quantitative polymerase chain reaction (RT-PCR) and quantitative PCR (qRT-PCR) broadened the spectrum of analysis to transcripts expressed from more than one gene. Another breakthrough method applied in the transcriptomic field was the expressed sequence tag (EST) sequencing, which is based on sequencing of cloned cDNA which results in obtaining short DNA fragments. Since 1995, when a first microarray was used to study a model plant (*Arabidopsis thaliana*) transcriptome (Agarwal *et al.*, 2014), the possibility of simultaneous detection of multiple transcripts has gradually increased from several genes to the entire genome, but the necessity of knowing the nucleotide sequences of transcripts (or genes) was one of the major limitations in the microarray application. The Next-Generation Sequencing (NGS) technologies revolutionized not only the genome sequencing, but the world of transcriptomic research as well. When compared with microarrays, RNA-seq possesses a number of technological advantages, such as a wider dynamic range and the freedom from predesigned probes (giving possibility of analysis of transcriptomes of plants with no reference genome, assessment of low-abundance transcripts, detection of non-coding RNAs, etc.). It also gives a significant enhancement in coverage depth, detection of novel transcripts, splice variants or gene fusions and reduction in research cost. Constant progress in the available technologies ena-

Table 1 . Summary of the described studies of soybean stress response analyzed with high-throughput technologies focused on transcriptomic, miRNA and proteomic analyses

	Transcriptome studies	miRNA studies	Proteome studies
Drought	Le *et al.*, 2012 Chen *et al.*, 2013 Shin *et al.*, 2015 Tripathi *et al.*, 2016	Li *et al.*, 2011	–
Flooding	Nanjo *et al.*, 2011a	–	Komatsu *et al.*, 2010 Nanjo *et al.*, 2010 Nanjo *et al.*, 2011b Yin *et al.*, 2014a Khan *et al.*, 2015 Yin & Komatsu, 2015
Cold stress	–	–	Tian *et al.*, 2015
Salinity	–	Dong *et al.*, 2013 Sun *et al.*, 2016	Ma *et al.*, 2012 Yin *et al.*, 2015
Phosphate deficiency	Zeng *et al.*, 2015 Wang *et al.* 2016	Xu *et al.*, 2013 Zenga *et al.*, 2010	Chen *et al.*, 2011
Biotic stress	Lanubile *et al.*, 2015	Li *et al.*, 2012b Yin *et al.*, 2013	–

bles us to create gene expression atlases, which may present snapshots of the transcriptome profiles even for the whole life-cycles of plants. Moreover, expression profiling obtained by using high-throughput methods contributes to the development of molecular markers, finding genes responsible for the secondary metabolism and studying the evolution of organs in plant families. It is also necessary for identification of genes and pathways active during different developmental stages and in response to abiotic and biotic stresses. Knowledge based on transcriptomic studies sheds a light on reduction of crop yield caused by environmental stresses, which are major sources of decrease in the world food production. The deduced genes, pathways and networks can eventually be exploited for the formation of tolerant varieties resulting in yield enhancement, even under stress conditions (Akpinar *et al*., 2013).

miRNA

MicroRNAs (miRNA) are a group of small noncoding RNAs abundant both, in animals and plants. miRNAs are involved in growth and development control, in plants e.g., leaf development. Moreover, they have been found to participate in both, abiotic and biotic stress tolerance in plants. These small RNAs suppress expression of target genes by guiding silencing complexes to complementary mRNAs, which results in mRNA cleavage or interruption of translation (Bartel, 2004; Brodersen *et al*., 2008).

Originally, miRNA identification was done by genetic screening, an approach similar to that used in traditional gene investigation. Despite its success in describing miRNAs, such as *lin-4* and *lin-7,* it had considerable flaws, namely randomness, long turnaround time and high cost consumption. An improvement in this method is direct cloning of small RNAs. The first step in this approach is isolation of small RNAs by size fractionation. Next, small RNAs are ligated to adapters at both, 5' and 3' ends. cDNA is obtained by using the prepared sRNAs (small RNAs) in reverse transcription reaction, followed by amplification and sequencing. Initial reduction of input material narrows down the field of view, which results in higher efficiency of miRNA identification. By employing this approach, several groups found miRNAs in plants (Sunkar *et al*., 2005) and animals (Fu *et al*., 2005). Advances in the sequencing technology allowed for the establishment of high-throughput methods that immensely sped up the identification of novel miRNAs. Traditional computational prediction of miRNA is an efficient approach that utilizes the known genome sequences and exploits several of miRNA's traits. miRNA requires high specificity of interaction in order to avoid off-target effects, therefore the target sequences are subjects of strong evolutionary conservation. In contrast, less strict sequence conservation applies for primary miRNA fragments that are not included in the mature miRNA. Scientists take advantage of this arrangement to design specialized algorithms for genome-wide screening. MIRscan and MIRalign are examples of programs used for successful prediction of miRNA in *Arabidopsis* (Adai *et al*., 2005), rice (Li *et al*., 2005), human (Bentwich *et al*., 2005) and *C. elegans* (Lim *et al*., 2003).

Microarrays are other very useful tools for studying expression patterns of known miRNAs in a high-throughput manner. This method is cost and time efficient thanks to the enormous number of probes that can be analyzed simultaneously. Generally, they are easy to use and provide a quick feedback. Unfortunately, there are also some drawbacks to this method, like background signal and cross-hybridization (Zhang *et al*., 2006). Luckily, similarly to the whole transcriptome studies, NGS remedies all of these issues. With proper library preparation, including isolation of small RNA fraction (by either size fractionation or commercial kits) and adapter ligation followed by reverse transcription and amplification, sequencing offers high sensitivity and ability to probe both, the known and novel miRNAs. Furthermore, high reproducibility allows for studying the differential expression of miRNAs under various stress conditions. Hundreds of new miRNAs have been annotated using the genome-wide small RNA-Seq, the latest report stating the deposition of 35,828 mature miRNAs derived from 223 species in miRBase. To date, this technology has been used to find a huge amount of plant miRNAs in crucial species, such as *Arabidopsis thaliana*, *Oryza sativa* or *Triticum aestivum* (Hu *et al*., 2013). Validation of data obtained with high-throughput methods is performed by robust and proven northern blotting or ingenious stem-loop RT-qPCR utilizing special primers allowing for amplification of short miRNA molecules.

PROTEOME

A proteome is the set of proteins expressed from a genome in a given type of cellular compartment, cell or organism, at a given time and under defined conditions. Proteomics and genomics are complementary - life can be described as translation of the relatively static genomes into highly dynamic proteomes that differ from cell to cell and change over time. The proteome, after the transcriptome, is the next layer of cell metabolism adjustment to adverse conditions and therefore to some degree, the proteome reflects the underlying transcriptome. However it is not possible to predict the protein expression levels strictly from the transcriptomic data (Anderson & Seilhammer, 1997). There are thousands of distinct proteins and peptides in every eukaryotic organism, many more than the number of protein-coding genes. All this variety comes from distinct processes that add to the complexity of proteins and multiply the number of components and functions, based on the demands of a cell at a given time. These events are: post-transcriptional splicing and alternative splicing, co- and post-translational protein modification or enzymatic activation of proenzymes (Gracz, 2016; Prabakaran 2012; Khan & James, 1998). Moreover, some proteins can be engaged in intra- or intermolecular interactions to form functional oligomers or protein complexes.

The goal of proteomics is to analyze the varying proteomes of an organism at different times and conditions, in order to highlight the differences between them. In modern proteomics, simultaneous analysis of several thousands of different proteins from complex biological samples is often required. For the correct identification of peptides and proteins, and due to the complex nature of the proteome, constant development of new methods and techniques for sample cleanup, fractionation, concentration, separation and detection becomes a crucial prerequisite.

The complexity of a proteome demands numerous and often complicated methods of studying it and there are many equivalent ways to analyze proteins in the cells (Chandramouli & Qian, 2009; Roe & Griffin, 2006). Among others, these are: gel electrophoresis, chromatography or electrofocusing (Kurien & Scofield, 2015, Ol-

iviera *et al.*, 2014). For cellular localization purposes, immunofluorescence, immunohistochemistry, and immunoelectron microscopy are used (McDonough *et al.*, 2015). Structure prediction and simulation can be achieved with the use of crystallography and cryo-electron microscopy, but it can be also studied indirectly by using computational methods, such as homology modeling, molecular docking or molecular mechanics (Varadi & Tompa, 2015; Shen *et al.*, 2013). Protein fragment complementation assays are often used to detect protein–protein interactions (Waadt *et al.*, 2014). The yeast two-hybrid assay is the most popular of them, but there are numerous variations used both, *in vitro* and *in vivo* (Gietz, 2006).

In order to obtain large amounts of data, we need to employ high-throughput protein analyses. For many years, two dimensional gel electrophoresis has been the most popular method in simultaneous analysis of proteins from cellular compartments, cells, organs or tissues at particular stages of growth and development or from different growth conditions or even different species (Oliviera *et al.*, 2014). This method is based on separation of proteins first by isoelectric focusing, which resolves proteins on the basis of their overall charge. In the second dimension, proteins are separated by their molecular weight on SDS-PAGE. To visualize such separated proteins, the gels are dyed with Coomassie Brilliant Blue or silver. For the identification of proteins present in particular spots, the peptide mass fingerprinting is used which is a core technology in proteomics. Mass spectrometry is an analytical tool used for measuring the mass-to-charge ratio of peptides (or other molecules) present in the tested sample. 2D gels can be seen as a protein screening process with high resolution, high reproducibility, quantitative, label-free intermediate read-out, but with limited analysis depth in terms of protein scope, where around several hundreds of proteins can be identified (depending on the sample, preparation technique and the mass spectrometer) (Han *et al.*, 2008; Oliviera *et al.*, 2014).

For higher resolution of the proteome analysis, liquid chromatography–mass spectrometry (LC-MS) may be applied. LC-MS is a highly sensitive analytical technique that combines the physical separation capabilities of liquid chromatography (LC) with the mass analysis potential of mass spectrometry (MS). Its application can be oriented towards the separation, general detection and potential identification of proteins of a particular mass in the tested samples. LC-MS/MS is most commonly used for proteomic analysis of complex samples where the peptide masses may overlap even with the use of a high-resolution mass spectrometer. Complex biological samples, when run in a modern LC-MS/MS system, may result in over 1000 proteins being identified at a time. The application of techniques based on mass spectrometry for the qualitative and quantitative analysis of the global proteome samples derived from complex mixtures has a pivotal role in our understanding of cellular function (Mitulović & Mechtler, 2006; https://www.broadinstitute.org/scientific-community/science/platforms/proteomics/lcms-overview).

The protein microarray technology is another technique which enables a high-throughput analysis and has progressed rapidly the identification, quantification and functional analysis of proteins in the applied proteome research (Gupta *et al.*, 2016; Chandramouli & Qian, 2009). Multiplex assays enable a precise characterization of proteins and the study of complex protein-protein interactions, but also peptides, low molecular weight compounds, oligosaccharides or DNA. The next generation of microarrays with a capability for high-throughput, ultrasensitive, low-cost biomarker analysis will most probably involve a combination of nanotechnology, surface enzyme reactions, microfluidic networks and advanced data analysis tools. This will undoubtedly accelerate the protein biomarker discovery and characterization of disease-specific pathways.

Ribosome profiling is a technique where the use of specialized mRNA sequencing allows to determine which mRNAs are being actively translated (Weiss & Atkins, 2011; Ingolia 2014). It provides information on all of the ribosomes that are active in a cell at a particular moment. Ribosome profiling targets only those mRNA sequences that are protected by the ribosome during the process of translation (all mRNAs bound with the ribosomes in a sample), nevertheless it involves a similar sequencing library preparation and data analysis as RNA-Seq.

Abiotic stresses usually cause the dysfunction of proteins, therefore it is particularly important for cell survival to maintain proteins in their functional conformations and prevent the aggregation of non-native proteins. Thus, quantitative analysis of gene expression at the protein level is essential for determining plant responses to stress conditions.

SOYBEAN RESPONSE TO STRESS CONDITIONS

Abiotic stresses

Soybean, as most crops in the world, is grown under suboptimal conditions which make it impossible to obtain its maximum yield. These unfavorable environmental conditions, creating potentially damaging physiological changes within plants, are known as stresses (Shao *et al.,* 2008). Abiotic stress factors are related to a non-optimal range of non-living chemical and physical parts of the environment, such as temperature, water content, salinity, inorganic nutrient etc. It has been suggested that they reduce the average yields by more than 50% for most of the major crop plants (Wang *et al.*, 2003).

Drought

Scarcity of water is a severe environmental constraint that limits global food production. The effects of drought range from morphological to molecular and are evident at all stages of plant growth. Plants are, however, most vulnerable to drought during the reproductive stage of growth, as limited water availability during flowering can severely shorten this phase. Also, duration of the seed formation phase is decreased due to stimulation of senescence related water stress. It has been estimated that drought may cause even 40% loss in yield and reduction of seed quality in soybeans, especially when they occur at the late vegetative stage of growth (Thao & Tran, 2011).

In response to the soil water deficit, plants can exhibit either a drought escape, drought avoidance, drought resistance, or drought tolerance mechanisms (Levitt 1980; Price *et al.*, 2002). Under drought escape, plants have the ability to complete their life cycle before a severe stress sets in. Drought avoidance is maintaining of high tissue water potential through such mechanisms as improved water uptake and the capacity to hold the acquired water and reduce the water loss. These can be achieved by extensive and prolific root system (Price *et al.*, 2002), through stomatal control of transpiration, and reduced leaf area (evaporative surface) (Turner *et al.*, 2001; Kavar

et al., 2007). Plants with drought tolerance have the ability to withstand water deficit with low tissue water potential (Ingram & Bartels, 1996). Many of drought adaptive mechanisms – like stomatal closure and decreased transpiration, are well described at the cellular and physiological levels, but the identification of key drought-responsive genes is still needed, especially in such an economically important crop like soybean.

In 2012, Tran and his research group had used for the first time the high-throughput microarrays technique to monitor the soybean leaf transcriptome changes during a drought stress (Le *et al.*, 2012). In this precursor analysis, they managed to identify alteration in the expression of genes involved in response to stress conditions during different developmental stages. Biological material was sampled from late growth vegetative stage (V6) to early bloom phase (R1) and full bloom (R2) stage. The period from late V6 toward the end of R2 was previously recognized as one of the critical periods in which drought can influence the soybean yield in a negative manner. Comparison of transcriptomes from drought treated plants at thestage of V6, R1 and R2 to leaf transcriptomes from well-watered plants revealed 1 458 and 1 818 upregulated and 1 582 and 1 688 downregulated genes in drought-stressed V6 and R2 leaves, respectively. Transcriptional changes of various well-known functional and regulatory genes, including those encoding transcription factors (TFs), kinases, heat shock proteins, late embryogenesis-abundant (LEA) proteins, osmoprotectant biosynthesis-related proteins, hormone-related proteins, transporters and detoxification enzymes, were detected. When the identified up- and downregulated transcripts from stage V6 were compared to those from R2 stage, an overlap was observed for only 41.98% (for genes with elevated expression) and 29.27% (for genes with reduced expression levels), so the altered gene expression profile of the drought-treated V6 leaves was significantly different from that of the drought-treated R2 leaves. This result may suggest that the response to drought stress in soybeans is highly stage-specific. The authors have also expanded a comparative analysis to the species level - they compared the soybean transcriptome datasets to that of drought stressed *Arabidopsis* leaves (35 day old) and observed that many of the stress induced genes are affected in a similar manner in those two distinct plant species. This result suggests that to some degree, there is a common response to drought; but on the other hand, some of *Arabidopsis* orthologs display differential expression pattern under water deficit conditions in soybeans, indicating that there is also a species-specific drought response.

Another example of describing soybean's transcriptomic response to drought by using a microarray technology was an analysis performed by Tripathi *et al.* (2016). Contrary to previously mentioned research, which was focused on the plant's different developmental stages (Le *et al.*, 2012), the authors had performed an analysis on different plant tissues – leaves and roots sampled at six time points after dehydration (0 min, 30 min, 1 h, 2 h, 3 h and 5 h). After microarray data analysis, they picked a set of genes with the most distinct differences in expression levels (≥ 8-fold change) and in this way identified a pool of 2 972 genes in the leaves and 1 394 in the roots. Further analysis of the transcriptome dynamics had shown that the roots are characterized by far more extensive and far more rapid changes than the leaf tissues – in roots, at the first time point (after 30 minutes of dehydration), 128 genes displayed at least 8-fold induction; in the leaves, the first changes had occurred after two hours. After functional analysis of the differentiated genes at the considered time points, in both – the leaves and roots, a shift from signaling (elevated expression level of genes encoding transcription factors, protein kinases, protein phosphatase 2Cs, F-box family proteins and ubiquitin protein ligases) to downstream responses aimed at protecting the plant against drought (induced expression of genes encoding: water channel proteins, membrane transporters, glutathione S-transferase and LEA proteins, osmotin, chaperons) has been observed. From the same plant material, parallel to the transcriptomic analysis, the physiologic, metabolomics and proteomic studies were performed. Collected data enabled the authors to create a toolbox of genes, promoters, proteins and metabolites which can help in reaching the ultimate goal – to enhance tolerance of the soybean varieties to the drought stress conditions (Tripathi *et al.*, 2016).

Many of the stress adaptive mechanisms are still not completely understood or even recognized. Identification of novel drought-responsive genes from drought-tolerant soybean cultivars and comparison with the drought-sensitive ones can help in elucidation of their roles in adaptation to stress conditions. Such approach was used by Chen and coworkers (2013), who employed Illumina sequencing-based Digital Gene Expression Tag profile (DGE) technology to the transcriptome analysis, where two genotypes were chosen for sequencing – a drought-tolerant Jindou21 and a drought-sensitive Zhongdou33. In the next step, 20 libraries were prepared from both genotypes (leaves and roots) and from different time points after dehydration (0, 2 and 10 h). Additional 8 libraries were constructed from the plant material collected after rehydration – from 0.5 and 2h time points. Functions of most differentially expressed genes in the drought-tolerant genotype under dehydration were unknown. However, the differentially expressed genes (DEGs) identified, were successfully annotated according to biological processes using a gene ontology (GO) functional enrichment analysis. The primary enriched biological processes in the drought-tolerant genotype belonged to the carbohydrate metabolism, which provides most of the energy required for cells coping with stress conditions. Other biological processes (aspartate family amino acid and acetyl-CoA metabolic processes), that also contribute to the energy release, were enriched as well. Moreover, similarly to previous studies (Tripathi *et al.*, 2016) genes encoding transcription factors were found in the highly enriched category, as well as protein kinases and molecules functioning in the hormone-mediated signaling pathways. The seven most differentially expressed genes (log2 ratio ≥8) (*Glyma15g03920, Glyma05g02470, Glyma15g15010, Glyma05g09070, Glyma06g35630, Glyma08g12590* and *Glyma11g16000*) identified in this DEGs analysis of the drought-tolerant genotype were indicated by authors as candidate genes for further drought tolerance improvement in the soybean breeding programs.

Recently, two other soybean genotypes were subjected to NGS analysis after application of drought stress conditions – Benning (drought sensitive, elite US soybean cultivar) and PI 416937 (drought tolerant, slow-wilting Japanese landrace) (Shin *et al.*, 2015). Plants from both of these varieties were subjected to dehydration at the R2 developmental stage and sampled in 0, 6, 12 and 24 h intervals. After RNA-seq analysis, the transcripts from both genotypes were classified into different groups depending on their expression profiling. The majority of genes confidently characterized as drought responsive, were genes whose expression levels changed

over the time-course, but there were no genotypic differences between the tested genotypes. The most obvious response shared by the two genotypes was down-regulation of expression of the photosynthesis related genes. A similar pattern of genes engaged in photosynthesis was previously observed in *A. thaliana* and rice under severe water deficit conditions (Chaves *et al.*, 2009). Other genes, which responded to drought in both phenotypes, were responsible for processes like protein transport and chromatin remodeling. Also, at least five of the identified genes fell within the known quantitative trait loci (QTL) underlying the slow-canopy wilting trait and therefore are very good candidates for further research.

Analyses of changes in soybean miRNA pool during drought conditions were also performed. Li and coworkers (2011) used a stress sensitive soybean line HJ-1 to study the stress associated miRNAs. Plants were subjected to drought stress for 48 h, after reaching four leaf stage of growth. Roots of the stressed, as well as control, soybeans were harvested and used for isolation of small RNAs and for the library construction. Subsequent libraries were analyzed with the Illumina sequencer. The results indicated a differential expression of 71 miRNAs during water deficit, where 17 of them (miR1508a, miR1509a, miR1510a−3p, miR1520k, miR171b−5p, miR396e, miR4352b, miR4358, miR4360, miR4364a, miR4365, miR4367, miR4375, miR4390, miR4393b, miR4394, miR4400, miR4410) were up-regulated under drought stress exclusively, and the expression of the remaining 54 miRNAs was also affected by alkalinity, salinity or both of these stresses (Li *et al.*, 2011).

Flooding

Flooding has a severe negative effect on the soybean cultivation as the plant growth and grain yields are markedly reduced in the flooded soil (Githiri *et al.*, 2006). Soybean is one of the most flooding susceptible crop species. In contrary to the flooding tolerant species like rice, soybean has no constitutive or inducible mechanisms for morphological and physiological adaptation to aqueous habitats. Moreover, there are no soybean varieties with clear tolerance to such conditions (Komatsu *et al.*, 2013), but at the early stages of growth it is often influenced by the flooding stress. This stress condition is particularly common in the regions of extreme rainfall, but also in areas with poorly drained soil (impermeable clay or cracking gray clays). Flooding is a complex stress that involves hypoxia as it leads to a decreased oxygen concentration in the soil (oxygen diffuses 10 000 times slower in the water than in the air (Armstrong, 1979)), but also water, and light stresses. Under flooding, plants experience an energy deficiency as a result of restricting mitochondrial oxidative phosphorylation (Gibbs & Greenway, 2003) and loss of cellular function, which inhibit their growth.

Previous comparative analyses between flooding-tolerant and flooding-intolerant plants suggested that an alteration in the carbon and energy metabolisms is responsible for creation of tolerant varieties (Jackson & Colmer, 2005). Flooding experiments conducted on two-day old soybean seedlings revealed numerous changes in the transcriptomes of the treated and control plants (Nanjo *et al.*, 2011a). Response to stress conditions at the transcriptome level was measured with microarrays and the qRT-PCR technique, and the correlation coefficient between gene expression determined by both methods was high, suggesting that the data sets obtained from the different methods were consistent. After 12h of stress conditions, the genes involved in photosynthesis were significantly up-regulated in the treated plants. Similar results were observed with the transcriptome analysis of grey poplar, *Arabidopsis* and rice. Thus, flooding or low-oxygen stresses stimulate the expression of photosynthesis related genes in plants – the phenomenon implies underwater photosynthesis. On the other hand, the flooding stress caused down-regulation of expression of several functional groups of genes, like cellulose synthesis and cell wall degradation, indicating that cell wall biosynthesis is suppressed by flooding. This observation may account for the observed inhibition of seedling root growth and of lateral root formation of the flooded soybean seedlings. Genes categorized in the secondary metabolism, including genes related to the biosynthesis of phenylpropanoids, lignin and flavonoids, were down-regulated as well. Down-regulation of sucrose degradation due to post-transcriptional regulation was also suggested. Moreover, the authors identified three genes encoding small proteins (less than 100 amino acids) of unknown functions that were highly induced in the flooded soybean seedlings. These results suggest that responses to flooding, including transcriptional and post-transcriptional regulation, might play a role in acclimation to this severe stress condition.

Large-scale proteomic analyses (2-DE and nano LC-MS/MS) were also exploited to identify proteins with altered expression levels under the flooding stress in 2-day-old soybean seedlings (Yin *et al.*, 2014a). 53 proteins from 35 protein spots that differentiated on 2-DE gels, and 1379 proteins using nano LC-MS/MS technique were identified in the root tips of soybean under flooding conditions. Among the 9 common proteins (lipoxygenase, alcohol dehydrogenases, phosphoglucomutase, NADP-malic enzyme 4, cytosol aminopeptidase, S-adenosyl methionine synthetase 2, GTP-binding elongation factor Tu and malate dehydrogenase), 2 alcohol dehydrogenases were markedly increased. This is probably due to the fact that anaerobic energy production is a way for plants to adapt to the initial phase of the flooding stress causing low oxygen status in the soil. Between the gel-based and gel-free proteomics analyses, a total of 115 significantly increased or decreased proteins were identified. The analysis indicated that the significantly altered proteins were mainly involved in the amino acid metabolism, glycolysis, the TCA cycle, hormone metabolism, stress, and protein synthesis (Yin *et al.*, 2014a).

Post-flooding proteome responses in soybean hypocotyl were analyzed during the recovery period using a gel-free technique (Khan *et al.*, 2015). 20 proteins, in common between the control and flooding-stressed soybeans that changed significantly in abundance during the post-flooding recovery have been identified using mass spectrometry analysis. They were assigned to the protein metabolism, development, secondary metabolism, and glycolysis categories. The analysis revealed that three proteins involved in glycolysis, nucleotide synthesis and amino acid activation, and complex fatty acid biosynthesis, namely pyruvate kinase, nucleotidyl transferase, and beta-ketoacyl reductase, were increased in the hypocotyl both, under the flooding conditions and during the post-flooding recovery (Khan *et al.*, 2015).

To understand the plant's cellular events occurring in response to the flooding stress, subcellular proteomic approaches were applied by Yin & Komatsu (2015). It has been shown that adaptive responses of soybean to the flooding conditions are regulated at least in part by protein phosphorylation. This is a reversible post-translational modification process where a phosphoryl

group (PO_3^{2-}) is enzymatically added to some amino acids. Phosphorylation is an important regulatory mechanism that occurs in both, prokaryotic and eukaryotic organisms (Cozzone, 1988), but also a common signaling event that occurs upon plant exposure to abiotic and biotic stresses (Ranjeva & Boudet, 1987). It has been already shown that during the flooding stress in soybean, the process of phosphorylation leads to changes in the translational or post-translational regulation of proteins involved in the carbohydrate metabolism (Nanjo *et al.*, 2010). Furthermore, energy-related metabolic processes are particularly sensitive to changes in the protein phosphorylation. It is even more significant considering that, as mentioned above, the expression levels of proteins involved in the energy production increased during the flooding stress (Nanjo *et al.*, 2011b). On the other hand, the levels of proteins implicated in protein folding and cell structure maintenance had decreased. Analysis of proteins isolated from soybean root tips stressed with flooding had shown an increase in the levels of 10 proteins and a decrease in the levels of 4 (Yin & Komatsu, 2015), 10 of them being localized in the nucleus (zinc finger/BTB domain-containing protein 47, 2 glycine-rich proteins, ribosomal protein L1p/L10e, rRNA processing protein Rrp5, U3 small nucleolar RNA-associated protein MPP10, eukaryotic translation initiation factor 4G, calmodulin binding transcription activator, ribosomal protein S24/S35, and DEA(D/H)_box RNA helicase).

Cell wall proteins responsive to flooding were identified using a proteomics technique in 2-day-old soybean seedlings subjected to flooding for 2 days (Komatsu, 2010). It has been shown, based on 2-DE gel analysis, that among $CaCl_2$-extracted cell wall proteins, 16 displayed different accumulation levels. Among the 4 upregulated proteins were 3 methionine synthases and 1 copper amine oxidase. The downregulated proteins included 2 lipoxygenases, 4 germin-like protein precursors, 3 stem 31 kDa glycoprotein precursors, 1 Cu–Zn-superoxide dismutase, 1 copper amine oxidase and 1 unknown protein. Moreover, the proteins differentiating under flooding were classified into several functional categories, like metabolism, defense, secondary metabolism, signal transduction, as well as protein destination/storage (Komatsu *et al.*, 2013).

Cold stress

Cold stress, which includes chilling (< 20°C) and/or freezing (< 0°C) temperatures, represents one of the most harmful abiotic stresses affecting plants. It significantly constraints the spatial distribution and agricultural productivity of plants, affecting their growth and development. Cold stress prevents expression of a full genetic potential of plants because of its direct inhibition of metabolic reactions and, indirectly, through cold-induced osmotic (chilling-induced inhibition of water uptake and freezing-induced cellular dehydration), oxidative and other stresses (Chinnusamy *et al.*, 2007). Low temperature effects include damaged cellular membranes, reduced cellular respiration, increased levels of abscisic acid (ABA), cryoprotectants and reactive oxygen species (ROS) (Balestrasse, 2010).

One way to improve the acreage of soybean cultivation area in the temperate climate is through the understanding of molecular basis of cold stress responses. Plants subjected to cold stress display a broad range of phenotypic alterations that are a result of differential tolerance of particular plant lines to the stress conditions.

Among 41 soybean landraces and cultivars of south China, two were chosen: Guliqing (cold-tolerant) and Nannong 513 (cold-sensitive) to analyze and describe the cold stress responses at a proteomic level (Tian *et al.*, 2015). The cultivars were incubated at 5°C for 12 and 24 h. It has been shown that 57 protein spots on 2DE gels, isolated from the first trifoliate leaves, were found to be significantly altered in abundance. They were further analyzed by MALDI TOF/TOF MS and submitted to search using online MASCOT program against the Swiss-prot and NCBI protein databases. All of the identified proteins were found to be involved in 13 metabolic pathways and cellular processes. Most of the proteins (15) were found to be involved in photosynthesis, including proteins involved in: plastid division, heme and chlorophyll biosynthesis, photosystem I (PSI) and II (PSII), ribulose bisphosphate carboxylase/oxygenase (RuBisCO) proteins, and interconversion of CO_2 and HCO_3. The remaining proteins were ascribed to the following groups: protein folding and assembly, cell rescue and defense, cytoskeletal proteins, transcription and translation regulation, amino acid and nitrogen metabolism, protein degradation, storage proteins, signal transduction, carbohydrate metabolism, lipid metabolism, energy metabolism, and unknown (Tian *et al.*, 2015). It has been established that increased cold-stress tolerance of the Guliqing cultivar is a result of an increase in several biochemical processes: higher/faster protein, lipid and polyamine biosynthesis, more effective sulfur-containing metabolite recycling, and a higher photosynthetic rate, as well as lower production of ROS, lower protein proteolysis and energy depletion under cold stress.

Salinity

Salinity is one of the major negative environmental factors limiting plant vigor and the productivity of agricultural crop plants in many semi-arid and arid regions around the world (Munns & Tester, 2008). This is due to the fact that brackish water is mainly being used in these irrigated areas. High salinity affects plants in several ways: it causes water stress, oxidative stress, ion toxicity, nutritional disorders, alteration in metabolic processes, reduction in cell division and expansion, membrane disorganization and genotoxicity (Hasegawa *et al.*, 2000; Zhu, 2007). Although a great part of cultivated crops is sensitive to the salt stress, legumes are among those threatened the most by yield loss due to their delicate and close symbiosis with *Rhizobia*, which takes place in the roots. High salinity interferes with formation of nodules on the roots of legumes, in consequence impairing a process of atmospheric N_2 fixation. So far, in the soybean roots there have been described only a few of genes, miRNAs and proteins associated with salt stress response and salt tolerance, as summarized below.

Studies on *Glycine max* cv. Williams82, conducted by Sun and colleagues using genome-wide technologies, report differential expression of miRNA under the salt stress (Sun *et al.*, 2016). Five-day old root tips were collected for high-throughput sequencing. The data received, analyzed with miRDeep2, indicate up-regulation of 2 known miRNAs (gma-miR172f, gma-miR390e) and 2 newly identified miRNAs (Gly13, Gly20) in the root apical meristem (RAM). On the other hand, 4 known miRNAs (gma-miR399a/b, gma-miR1512b, gma-miR156g, gma-miR156j) and 3 novel miRNAs (Gly02, Gly03, Gly04) were down-regulated. Additionally, measurement of the auxin level in RAMs suggested that miRNAs responsive to the salt stress are controlled

by an auxin. 17 tested miRNA genes possess in their promoter sequences two or more auxin-responsive elements, implying that auxins regulate the expression of those miRNAs. An expression pattern validation using qRT-PCR revealed that most of the sequencing results, apart from Gly04, were consistent with the qRT-PCR experiment. Known salt-responsive miRNAs affect the RAM activity (Sun *et al.*, 2016), thus controlling root development plasticity under the salt stress conditions.

In 2013, a Chinese group examined salt stress response of *Glycine max* cv. Houzimao (Dong *et al.*, 2013). The plants were inoculated with *B. japonicum* and placed in growth chambers at 27°C. 28 days after inoculation, the plants were treated with NaCl solution and the nodules were harvested 6 h after stress treatment. Small RNAs isolated from nodules were sequenced using Illumina 1 Genetic Analyzer. The obtained results indicate an increased expression of 13 known miRNAs (miR171g, miR171j, miR171o, miR171p, miR171u, miR395b, miR395c, miR408a, miR408c, gma-miR15, gma-miR16, gma-miR39, gma-miR48) and 12 novel miRNAs (miR339i, miR339j, miR339k, miR4416c, miR4416d, Gly1, Gly2, Gly3, Gly4, Gly5a, Gly5b, Gly6) in the nodules. Also, a decreased expression of 5 known miRNAs (miR4416b, miR5559, gma-miR15, gma-miR16, gma-miR31) and 5 novel miRNAs (miR5037e, Gly15, Gly16, Gly17, Gly18, Gly19) was reported in the same tissue. Again, qRT-PCR was employed to validate the differential expression patterns of miRNAs under high salinity conditions. This time, the samples were collected at several points of salt stressed plants in a time span of 24 h, which enabled a time course analysis of miRNA expression. No significant discrepancies were observed, comparing sequencing and qRT-PCR data. Interestingly, some miRNAs exhibited different levels of expression during the 24 h of high salinity stress. For instance, abundance of Gly3 was the highest after 6 h of stressing but went back to initial level further on. Additional miRNA targeted transcripts prediction revealed that over half of targets for the 9 most highly expressed miRNAs were transcription factors, including a zinc finger protein and SBP transcription factors (Dong *et al.*, 2013).

To investigate the proteome expression patterns and to identify the differentially expressed proteins, a soybean salt-sensitive Jackson genotype and a salt-tolerant Lee 68 genotype were analyzed under salt stress using 2-DE (Ma *et al.*, 2012). Among approximately 800 protein spots detected on 2-DE gels, 91 were found to be differently expressed. 78 of those have been identified by MALDI-TOF–TOF. The identified proteins were found to be involved in 14 different metabolic pathways and processes, including photosynthesis (30%), carbohydrate metabolism (15%), redox homeostasis (12%), nitrogen metabolism (12%), metabolite biosynthesis (6%), protein biosynthesis (5%), amino acid and secondary protein folding and assembly (4%), nucleotide metabolism (3%), cellular processes (3%), cell rescue/defense (1%), signal transduction (1%), proteolytic proteins (1%), cell wall-modifying proteins (1%), and unclassified (6%). The authors had shown that the increased tolerance to salinity may be caused by a better ability of ROS scavenging, a more abundant energy supply and ethylene production, and a stronger photosynthesis of the salt-tolerant Lee 68 genotype than the salt-sensitive Jackson genotype (Ma *et al.*, 2012).

It has been demonstrated that application of exogenous calcium enhances salt stress tolerance of soybeans. As a secondary messenger, calcium (Ca^{2+}), is involved in activation of various signaling pathways, influences regulation of plant growth, development and reproduction, and the plant responses to environmental stress (Tuteja & Mahajan, 2007; Qiu *et al.*, 2012). It helps to overcome the inhibition of growth and development and maintain the integrity of the plants' cell function and structure (Guimaraes *et al.*, 2011; Li *et al.*, 2012a). Apart from counteracting harmful effects of salinity, it also increases the soybean biomass and GABA content in the germinating soybean plants (Yin *et al.*, 2014b).

A comparative proteomic approach based on 2-DE and MALDI-TOF/TOF-MS was used to investigate protein profiles in the germinating soybeans and in the embryos (cultivar Yunhe) under NaCl-$CaCl_2$ and NaCl-$LaCl_3$ treatments (Yin *et al.*, 2015). 80 proteins were found to be affected by NaCl-$CaCl_2$ and NaCl-$LaCl_3$ in the germinating soybean cotyledons, and 71 in the embryos. Functional class analysis had shown that most of the cotyledon proteins were seed storage proteins (SSPs), and the remaining were divided into 5 functional classes: metabolism, cell growth/division, proteolysis, transportation and disease/defense. The embryo proteins were divided into 10 functional classes, i.e. metabolism, energy, disease/defense, and protein synthesis (Yin *et al.*, 2015).

Phosphate deficiency

Inorganic phosphorus (Pi) is an essential macronutrient needed for the plant's growth and development. Despite its abundance in soil, Pi displays low mobility that accounts for low availability for plants, a factor limiting the crop yield in 30–40% of arable lands worldwide (Vance *et al.*, 2003). Broad use of fertilizers, although supplements the plants' needs, contributes to serious environmental damage and is economically disadvantageous. Low Pi availability is a severe problem for plants since phosphorus plays a key role in the energy metabolism. This is particularly true for legumes which develop symbioses with rhizobia to form nodules (fixation of atmospheric nitrogen (N) to support plant growth) (Tang *et al.*, 2001; Olivera *et al.*, 2004). As shown before, Pi availability can significantly affect the nodule number, nodule mass, nitrogenase activity and N content in several legume plants (Sa & Israel, 1991; Tang *et al.*, 2001; Olivera *et al.*, 2004; Le Roux *et al.*, 2006). This is due to significant inputs of energy required for atmospheric nitrogen fixation (Olivera *et al.*, 2004). In order to establish more ecological and affordable strategies, like breeding novel phosphate efficient cultivars, understanding of the plant phosphorus uptake and metabolism is needed. Over the years, plants submitted to phosphorus deficiency acquired a few strategies, namely altering expressions of genes and metabolic pathways involved in the phosphate transport, but also enabling an internal recycling process and modifying the root system (Yuan & Liu, 2008).

In 2015, the phosphate-responsive (Pi) genes in soybean roots were determined at the whole-genome scale (Zeng *et al.*, 2015). RNA was extracted from the 7-day old roots, cDNA libraries were constructed and sequenced with Illumina technology. As a physiological result of Pi starvation, it was observed that the shoot biomass of the plants significantly decreased, but no difference was noticed in the root biomass. Also, it has been shown that the Pi concentration in both, the roots and the shoots, had decreased dramatically in plants grown under the Pi-deficient conditions. Moreover, two *Arabidopsis* orthologs, previously described as Pi-responsive marker genes (Cruz-Ramírez *et al.*, 2006), *GmIPS1* and *GmPLDZ2*, were induced under this stress conditions.

Expression analysis indicated that a total of 1612 genes were expressed differentially in the soybean roots under the Pi-deficiency treatment, where 45% (727) were up-regulated, and 55% (885) were down-regulated. Interestingly, many genes with the highest expression levels, which were transcribed exclusively under one condition – either Pi-sufficient or Pi-deficient, encoded signaling-related proteins. For about half (755) of the Pi-responsive genes, the authors had assigned gene ontology (GO) terms, which were arranged in 17 categories including photosynthesis, ferrous ion transport, dUTP metabolic process, plant-type cell wall organization, fatty acid metabolic process, and response to oxidative stress. Several genes engaged in the phosphorus transport were induced under the studied conditions (*GmPHT1;3, GmPHT1;9, GmPHT1;14, GmSPX3, GmIPS1 and GmPLDZ2*). However, genes encoding proteins involved in the uptake and transportation of nutrients other than Pi, such as sucrose, sulfate, Fe, Zn and Cu were also induced. Similar systemic changes in the soybean metabolism were described by Wang and coworkers (2016). Their studies were preceded by a tolerant and sensitive soybean line screening (a similar approach to Chen and coworkers (2013) and drought response studies), and were based on microarray application. Through comparative analyses of the selected two soybean accessions, Chundou (CD) and Yunhefengwodou (YH), 42 candidate genes and three common pathways (methane metabolism, phenylalanine metabolism and phenylpropanoid biosynthesis) highly correlated to low-P stress were identified. Both of the described reports not only promote understanding of a molecular mechanism related to Pi deficit, but also anchor research in improving the Pi usage in soybeans and in designing highly phosphate-efficient soybean varieties.

Soybean cultivar *Glycine max* cv. *Williams82* subjected to a phosphate deficiency stress also had shown differential expression levels of several miRNAs both, in the leaves and the roots (Xu *et al.*, 2013). Seeds were germinated and cultivated for 7 days, followed by a transfer to the Pi-depleted and Pi-sufficient solutions corresponding to the treated and control groups. The RNA for Illumina sequencing was isolated from leaves and roots separately. According to the sequencing results, 13 miRNAs (miR166a-3p, miR166u, miR169c, miR169o, miR169q, miR396j, miR399e, miR2109a, miR492f, miR482g, miR308c, miR1512b, miR3508, miR4376a, miR4416a, miRnov_6, miRnov_7, miRnov_10) and 4 miRNAs (miR396k, miR397a, miR1510d, miRnov_2) were found to be up-regulated in the leaves and roots, respectively. The analysis had also revealed an increased expression of 8 miRNAs (miR399a, miR399b, miR399c, miR399d, miR482j, miR482k, miR482b-3p, miR1510-5p) that was common for both of those tissues. Additionally, 5 miRNAs (miR169c, miR2109a, miR4376a, miRnov_6, miRnov_10) and 6 miRNAs (miR159e-3p, miR169r, miR3522a, miRnov_5a, miRnov_5b, miRnov_9) were down-regulated in the leaves and roots, respectively. The expression patterns observed based on the sequencing data were confirmed by using stem-loop qPCR for a few selected miRNAs. Moreover, the target mRNAs for miR399, miR2111 and miR159e-3p were determined to be a *PHO2* (that indirectly controls Pi transport in plants) and *GmPT5* (important for Pi homeostasis in the nodule development), a kelch-domain containing protein, and a Myb transcription factor, respectively (Xu *et al.*, 2013).

In similar studies performed on *Glycine max* cv. NY205, the seeds were germinated and grown in growth chambers under Pi-sufficient and Pi-deficient conditions for 7 days, after which the soybean roots and leaves were collected separately (Zenga *et al.*, 2010). Differential expression levels were assessed with microarrays using known miRNA probes obtained from the miRBase. The authors reported that an increased expression occurs in the case of 4 miRNAs (miR156/157, miR167, miR168, miR319) in leaves, and 3 miRNAs (miR396, miR474, miR482) in roots exclusively, and 4 miRNAs (miR159, miR894, miR1507, miR1509) that are common for the above- and underground plant samples. Similarly, 13 (miR160, miR396, miR834, miR854, miR1118, miR1311, miR1427, miR1436, miR1508, miR1846, miR1858, miR1879, miR1881), 2 (miR168, miR319) and 4 miRNAs (miR165/166, miR398, miR1450, miR1511) displayed a decreased expression in the leaves, roots and both tissues, respectively. A group of seven miRNAs (miR159a, miR166a, miR319a, miR396a, miR398b, miR399a, miR1507a) responsive to abiotic stress were selected for qRT-PCR analysis. The results indicated up-regulation of miR159a and miR399 expression, and down-regulation of miR166a, miR319a, miR396a, miR398b and miR1507a expression. The miRNA expression patterns were in accordance with those received through microarray analysis, except for miR396a and miR1507a. The diversified abundance of miRNAs implies their role in regulation of the plant response to the phosphorus deficiency stress (Zenga *et al.*, 2010).

Two-dimensional electrophoresis allowed to visualize more than 700 protein spots in the soybean nodules under phosphate starvation. 73 protein spots exhibited differential accumulation in response to the low Pi levels when compared with the nodule protein 2-DE profile at high Pi. Maldi TOF/TOF MS analysis allowed to identify 44 proteins, 17 upregulated and 27 downregulated. Among the upregulated proteins, 16 were identified and separated into 4 functional groups: other metabolic processes, carbon metabolism, transcription, and signaling and stress response. Among the downregulated proteins, several groups were formed. In the largest, the proteins were involved in other metabolic processes, which represented 26% of all of the Pi-starvation downregulated proteins. The following groups were: proteins involved in the stress response, carbon metabolism, amino acid metabolism, transporters, and transcription and signaling (Chen *et al.*, 2011).

BIOTIC STRESS CONDITIONS

Apart from abiotic stress conditions, limitations in the maximum production are largely due to disease pressures (part of biotic stress conditions) that reduce yield. In contrast to the abiotic stresses, a biotic stress response is caused by a vast range of pests and pathogens, including fungi, bacteria, viruses, nematodes, and herbivorous insects (Wrather & Koenning, 2009). Below, we describe a few examples of the soybean molecular response to fungal, virus and nematode infections.

Fusarium oxysporum

One of the well-known pests in soybean cultivation is *Fusarium oxysporum* – a fungal soil-borne facultative parasite present worldwide, which causes seed and seedling diseases, root rot, and vascular wilt (Arias *et al.*, 2013). Managing *Fusarium* infections will, in the long-term, depend on improvements in molecular breeding for resistant genotypes. For this general purpose, recognition of molecular basis of the plant responses to disease conditions is needed. Moreover,

availability of the reference genome sequences and gene annotations for both, *G.max* and *F. oxysporum,* has enabled studies of the molecular interactions between the host plant and its pathogen. To elucidate the comprehensive gene expression profiles for both, the soybean and *F. oxysporum* (Lanubile *et al.*, 2015), the root transcriptomes of plants, non-pathogenic and pathogenic fungi at 72 and 96 h post inoculation (hpi) were analyzed. The downstream analysis identified 8 471 DEGs – due to the high number, an additional filter based on fold change value greater than 1.9 was applied and resulted in 1 802 soybean HDEGs (Highly Differentially Expressed Genes). From this narrowed pool, 203 and 57 DEGs were identified in response to the non-pathogenic isolate, and 1 659 and 151 DEGs in response to the pathogenic isolate at 72 and 96 hpi, respectively. As one may expect, more drastic changes were observed in response to the pathogenic isolate. Furthermore, not only the number of genes, but also the magnitude of induction was much greater in response to the pathogenic fungus. This response included a stronger activation of many well-known defense-related genes, several genes involved in the ethylene biosynthesis and signaling, TFs, secondary and sugar metabolism.

Cyst Nematode

The soybean cyst nematode (SCN), *Heterodera glycines Ichinohe*, is another cause of biotic stress in soybean, especially in *Glycine max* cultivation. The US alone suffers over one billion dollars of loss per year due to damage inflicted by this endoparasite. SCN reside in the roots, forming cysts during their life cycle that impair the plant productive qualities via chlorosis of the above ground parts and the root necrosis. Present control approaches are severely limited and include application of nematocides, crop rotation and plantation of resistant cultivars. Therefore, investigation of the nematode virulence can lead to designing novel SCN control strategies. Studies report plants coping with the SCN-induced stress by reprogramming gene expression, which is said to be driven by small RNAs (Sunkar, 2010).

High-throughput RNA sequencing was used to compare miRNA accumulation levels in the roots of two soybean cultivars that varied in susceptibility to SCN (Li *et al.*, 2012b). *Glycine max*, cultivar Harbin xiaoheidou (HB) was chosen as a resistant standard, and Liaodou 10 (L10) was used as the SCN sensitive standard. The soybean plants were grown in a greenhouse in an SCN infected soil. 30 days after emergence of seedlings, root samples were collected. Illumina Genome Analyzer II was used to sequence small RNAs isolated from the roots. Majority of the differentially expressed miRNAs were down-regulated in response to the SCN infection, whereas only 6 miRNAs were up-regulated, suggesting that reduction in miRNAs levels plays an important role in the soybean's interaction with SCN. For both genotypes, miR171c and miR319 were highly induced by the stress conditions; miR390b was up-regulated in HB specifically, while miR862, miR5372 and four members of miR169 were SCN induced only in L10. In both cultivars, 16 miRNA families were regulated in common, with 8 families (miR156, miR162, miR166a, miR167, miR319, miR397, miR398, miR408) conserved between the plants, 2 families (miR2119, miR3522) specific for *Fabaceae* and 6 miRNA families (miR1520, miR4365, miR4387, miR4413, miR4996, miR5671) were soybean specific. The obtained results suggest that both, the conserved and soybean specific miRNAs are engaged in the SCN defense (Li *et al.*, 2012b).

Soybean mosaic virus

Another considerable threat for crop yield and food supply security are plant pathogens. Viruses are obligate intracellular parasites that adapt the host cells to provide a molecular apparatus in order to produce new viruses. Among them, the soybean mosaic virus (SMV) causes yield loss and lowers seed quality wherever the soybean is widely cultivated. Genetic resistance against plant pathogens is the most effective method of the virus control. Soybean possesses many sources of SMV resistance, most of which are regulated by a single dominant gene, but not for all the SMV strains. There are three independent loci for SMV resistance: Rsv1, Rsv3, and Rsv4 (Ma *et al.*, 2004). Constant search for targets regulated during the SMV infection is very important for gaining knowledge about the soybean pathosystem.

Soybean *Glycine max* Nannong 1138-2 variety was analyzed using direct sequencing and this analysis was further confirmed by stem-loop qRT-PCR (Yin *et al.*, 2013). Plants were grown in a greenhouse for 10 days, followed by inoculation with SMV by rubbing the unifoliates. For control samples, the inoculum was substituted with sodium phosphate buffer. Leaves from both treatments were collected for RNA isolation 4 h after inoculation. Fraction of small RNAs was sequenced using Illumina 1 G genome Analyzer. The SMV infection significantly increased expression levels of 11 miRNA families (miR3522, miR2118-3p, miR171, miR530, miR1514, miR160, miR408, miR1510-3p, miR399, miR482-3p, miR1535). On the other hand, 3 miRNA families (miR390, miR4416, miR1524) were down-regulated by the SMV biotic stress. qRT-PCR expression pattern verification indicated that expression levels of the analyzed miRNAs (miR160, miR393, miR1510, miR1535, miR1514, miR2109) were in agreement with direct sequencing results, with exception of miR164, which was up-regulated according to qRT-PCR (conversely to results obtained by direct sequencing). Most of the conserved miRNA families (miR156, miR159/319, miR160, miR164, miR166, miR167, miR169, miR171, miR172 and miR394) were said to control transcription factors that are crucial for developmental processes. Contrary to that, non-conserved miRNAs target genes that vary in function. Few examples of the target enzymes are: isopentenyl transferase, alcohol dehydrogenase, glycosyl hydrolase, and polyphenol oxidase, which are involved in growth, development and the environmental stress response (Yin *et al.*, 2013).

PERSPECTIVES

As can be seen, large scale RNA expression profiles and protein analyses give us an enormous volume of information. Moreover, with the advent of new techniques we can generate the experimental research data even quicker than perform its detailed analysis. miRNAs profiles, transcriptomic and proteomic analyses from different plant tissues, developmental stages or stress responses help us to discover and fully appreciate the rich landscapes of alterations in the expression of genetic information. The next step – validation of the identified changes can lead to elucidation of molecular markers and, in the long term, to introduce valuable and desirable traits into plant varieties.

The scientific world focused on the plant cultivation still pursuits new crop varieties and landraces characterized by higher tolerance to adverse conditions, both biotic and abiotic, and by greater quality and yield. In the face of global climate changes and constantly growing human population, the pressure for finding new, better adopted crops is even greater than before, so we are incessantly shaping the plant genomes with conventional breeding techniques or with methods discovered with the rise of molecular biology. The soybean plasticity allowed to broaden its cultivation far beyond its place of origin, and thanks to its unique properties it became a base for nowadays agriculture industry. The above described examples of experiments and research show how highly complicated the mutual relationship between the plants and the environment is. Elucidation of the basis of molecular mechanisms involved in the stress responses in such an economically important species should therefore become one of the priority tasks for the research community. Advances based on the proteomics analysis, transcriptome characterization and miRNA controlled gene expression give us deeper insights into the plants' inner cellular life. Knowledge from these lessons should be fully utilized to truly mine the mechanisms of stress tolerance and adaptation.

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