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Identification and characterization of changes in soybean miRNA biosynthesis in response to low temperature stress

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TABLE OF CONTENTS

LIST OF PUBLICATIONS CONSTITUTING THE DOCTORAL THESIS

1. Tyczewska A, Gracz J, Kuczyński J, Twardowski T
   Deciphering the soybean molecular stress response via high-throughput approaches
   Acta Biochimica Polonica, 2016, 63(4): 631-643 (IF 2016 = 1.159)

2. Kuczyński J, Twardowski T, Nawracała J, Gracz-Bernaciak J, Tyczewska A
   Chilling stress tolerance of two soya bean cultivars: Phenotypic and molecular responses
   Journal of Agronomy and Crop Science, 2020, 206: 759-772 (IF 2020 = 3.473)

3. Kuczyński J, Gracz-Bernaciak J, Twardowski T, Karłowski WM, Tyczewska A
   Cold stress-induced miRNA and degradome changes in four soybean varieties differing in chilling resistance
   Journal of Agronomy and Crop Science, 2022, 208: 777-794 (IF 2022 = 3.5)

STRESZCZENIE

Soja [*Glycine max* (L.) Merr.] jest istotną rośliną oleistą i wysoko cenioną na całym świecie jadalną rośliną strączkową. Soja jest bogatym źródłem tłuszczów i jednym z najlepszych roślinnych źródeł białka, ponieważ zawiera wszystkie aminokwasy niezbędne w diecie człowieka oraz skarmianiu zwierząt gospodarskich. W Europie uprawa soi jest ograniczona ze względu na niesprzyjający klimat. Popyt na soję w Europie, częściowo spowodowany ciągłym wzrostem liczby ludności, jest znacznie większy niż rzeczywista produkcja, dlatego Europa jest w dużym stopniu uzależniona od importu soi, głównie z obu Ameryk. Ciągłe doskonalenie jakości i plonowania odmian roślin uprawnych jest niezbędne dla rozwoju rolnictwa, zwłaszcza w obliczu zmieniających się oraz trudnych warunków środowiskowych. Wykazano, że wiele mikroRNA (miRNA) pełni ważne funkcje regulacyjne we wzroście, rozwoju i odpowiedzi roślin na stres. Opracowanie technologii sekwencjonowania o dużej przepustowości umożliwiło opisanie wielu roślinnych miRNA. Ponadto pomyślnie zidentyfikowano dużą pulę genów kotrolowanych przez miRNA, co umożliwiło naukowcom uzyskanie wglądu w powiązania regulacyjne między miRNA, a ich genami docelowymi. Celem pracy było wyjaśnienie molekularnych podstaw odporności soi na stres zimna, ze szczególnym uwzględnieniem miRNA. Na początkowych etapach pracy przeprowadzono fenotypowanie dwóch odmian soi poddanych stresowi zimna w celu zbadania jego wpływu na produktywność soi. Wyniki doświadczeń polowych i pomiarów wykonanych podczas wzrostu soi w kontrolowanych warunkach chłodu wykazały, że odmiany Augusta i Fiskeby V różnie reagowały na niskie temperatury. Pomimo wpływu niskich temperatur na wzrost generatywny obu odmian, liczba produkowanych nasion utrzymywała się na niezmienionym poziomie. Różnicową ekspresję pięciu miRNA w warunkach stresu zimna wykryto za pomocą ddPCR. Zaobserwowano, że miRNA w odmianach Augusta i Fiskeby V poddanych stresowi w fazie reprodukcyjnej wykazywały odmienne profile ekspresji. Odkryto ujemną korelację między ekspresją miR169, miR319 i miR398, a ich genami docelowymi w korzeniach odmian Augusta i Fiskeby V. W kolejnej fazie przeanalizowano rolę miRNA i ich docelowych genów w tolerancji na zimno czterech różnych odmian soi (Augusta, Fiskeby V, Toyomusume i *Glycine soja* [*G. soja*]) o różnym poziomie odporności na stres. Wysokowydajna analiza sekwencjonowania krótkich niekodujących RNA tych czterech odmian soi w warunkach stresowych, w porównaniu do kontrolnych ujawniła zróżnicowaną ekspresję 162 znanych miRNA, jak również 18 nowych miRNA. Analiza degradomu umożliwiła przypisanie miRNA o zróżnicowanej ekspresji do ich potencjalnych genów docelowych. Na podstawie anotacji w bazach Gene Ontology (GO) i Kyoto Encyclopedia of Genes and Genomes (KEGG) odkryto, że w ramach odpowiedzi na stres abiotyczny zaangażowane są one w procesy takie jak: metabolizm reaktywnych form tlenu, synteza flawonoidów i kontrolowanie potencjału osmotycznego. Wyniki tej rozprawy doktorskiej zostały opublikowane w dwóch recenzowanych artykułach eksperymentalnych. Zagadnienia dotyczące wysokowydajnych technik stosowanych w analizie odpowiedzi soi na stres zostały opisane i podsumowane w pracy przeglądowej, która również jest częścią niniejszej rozprawy doktorskiej.

ABSTRACT

Soybean [*Glycine max* (L.) Merr.] is a significant oil crop and a highly valued food legume around the world. Soybeans are a rich source of oils and are one of the best plant-based sources of protein, as they contain all the essential amino acids necessary for human and animal nutrition. In Europe, soybean cultivation is limited because of the unfavorable climate. The demand for soybean in Europe, partly caused by the continual rise in population, is much greater than the actual amount produced, therefore Europe relies heavily on the imports of soybean mainly from Americas. Continuous improvement in the quality and yield of crop varieties is necessary for the advancement of agriculture, especially in the face of changing or difficult conditions. Recently, many miRNAs have been demonstrated to have important regulatory functions in plant growth, development, and stress responses. The emergence of high-throughput sequencing technology has enabled the discovery and analysis of many miRNAs in plants. Additionally, a great number of miRNA–mRNA target pairs have been successfully identified, enabling researchers to gain insight into the regulatory connections between miRNAs and their target genes. The aim of this study was to explain the molecular basis of the cold stress resistance in soybean, with the emphasis on miRNA. At the initial steps of work, the phenotyping of two soybean cultivars under chilling stress was conducted to investigate the impact on soybean productivity. The results of field trials and measurements taken during the growth of soybean in cold conditions showed that Augusta and Fiskeby V cultivars had different reactions to low temperatures. Despite the low temperatures having impact on the reproductive growth of both varieties, the number of seeds produced stayed the same. The differential expression of five candidate miRNAs under cold stress was detected using ddPCR. miRNAs that showed contrasting expression patterns in Augusta and Fiskeby V chilled in the reproductive stage have been identified. Based on the Gene Ontology analysis, a group of potential target genes was identified. A negative correlation between the expression of miR169, miR319 and miR398 and their targets was found in the roots of Augusta and Fiskeby V. In the next step the role of miRNAs and their target genes in the cold tolerance was analyzed in four different soybean varieties (Augusta, Fiskeby V, Toyomusume and *Glycine soja* [*G. soja*]) characterized by varying levels of stress resistance. High-throughput sequencing analysis of these four cultivars revealed the differential expression of 162 known miRNAs, as well as 18 novel miRNAs, under stress conditions. Analysis of the degradome enabled the assignment of differentially expressed miRNAs to their potential target genes. Based on the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations, they were discovered to be connected to plant abiotic stress response processes such as scavenging reactive oxygen species, synthesizing flavonoids, and controlling osmotic potential. The findings from this doctoral dissertation were published in two peer-reviewed experimental articles. Issues regarding high-throughput techniques used in the soybean stress response analysis were described and summarized in the review publication, which is also a part of this doctoral dissertation.

INTRODUCTION

Soybean

Soybean [*Glycine max* (L.) Merr.] is the most economically important legume crop globally. Considered in terms of global production (371.7 mio. tonnes) and acreage (129.5 mio. hectares), it ranks 6th and 4th, respectively, amongst all crops, and is first by far amongst legumes alone (FAOSTAT, 2021). Soybeans are greatly valued for their high nutritional value, which is attributed to their high levels of unsaturated fatty acids (approx. 18-20%), proteins (approx. 35-40%), and micronutrients. This makes them essential in the human food and animal feed industry [1]. It is consumed widely in traditional Asian cuisines as a variety of fresh (edamame, soybean sprouts) and fermented (tofu, natto, soy sauce) products. Its origins trace back to China 5,000 years ago, before introduction to Japan and Korea 2,000 years ago [2]–[4]. Furthermore, soybean has the capacity to form a symbiotic relationship with *Bradyrhizobium japonicum* through the formation of rhizobia, and proves to be an efficient nitrogen-fixing legume [5]. Consequently, soybean cultivation requires reduced N-fertilizer applications. Therefore, soybean ensures an increased nutrient availability, which makes it a valuable component of crop rotation cycles [6].

Soybean in Europe

Most of the soybeans harvested globally come from the Americas (87.2 %, FAOSTAT 2021, see website sources). Europe's global production share of 3.1% is comparatively low, of which only 23.4% are soybeans grown on soils of the European Union (FAOSTAT 2021). This means that Europe is heavily reliant on imports from the Americas for its soybean supply. Politicians in the European Union have been taking notice of the protein deficiency, prompting the idea to enlarge the acreage of leguminous crops as a way to increase protein production [7]. Europe is increasingly utilizing soybeans as a source of protein for both animal feed and human consumption, partly due to the growing popularity of vegetarian or vegan lifestyles. Over the past two decades, the acreage of soybean planting has been growing, leading to a substantial increase in European soybean production. For example, in Germany, the soybean acreage grew almost five-fold from 5000 ha in 2013 to 34000 ha in 2021. In 2021, the EU member states produced 2.7 million tons of soybeans. Despite this, the import quantity of soybean in Europe has not decreased, and there has even been a slight increase in the past decade (27 million tons in 2013; 33,5 million tons in 2021, EU Commission 2021, see website sources), showing that Europe still depends heavily on soybean imports. Currently, most of the soybean production in

Europe is centered in a few nations, such as Ukraine, Italy, Serbia, France, Romania, and Croatia (FAOSTAT 2021). This indicates that Southern Europe is the predominant soybean producing area in EU at this time. The cultivation of legumes such as soybeans may have been boosted by various EU initiatives, including EUROLEGUME, LEGATO, and Legume Futures, as well as domestic and international networks and organizations like Donau Soja, Europe Soya and Soja-Förderring (see website sources). Fifteen nations that are part of the European Union have agreed to the European Soy Declaration in an effort to boost the growth of legumes, which is included in their respective domestic crop plans [8]. Despite the increase in European cultivation of soybean, the demand for soy-based products is still immense, resulting in a huge gap between the production and its requirements. In 2021, the EU's total production of 2.7 million tons of soybeans was dwarfed by the 33.5 million tons imported (15.9 million tons of soybean seeds and 17.6 million tons of soybean meal, EU Commission 2021, ec.europa.eu). In order to become more independent and environmentally friendly in terms of soybean production, it is essential to increase crop yields and expand the area of land used for growing soybeans. This is not a simple task, as it involves a number of difficulties, such as the adaptation of a plant from the subtropics to short-day conditions and cold temperatures.

Cold stress

Abiotic stresses, including drought, cold and high salinity, are very common in modern farming systems. Because plants are unable to move, they must be able to withstand these difficult conditions, which can impede growth and reduce yield [9]. Soybeans are native to warm, subtropical climates, and can be adversely affected by cold weather, resulting in slower growth and the loss of flowers and pods, thereby leading to a decrease in productivity [10]–[13]. Just like in case of overcoming the photoperiod sensitivity, soybeans have also gone beyond a specific temperature range and are now being cultivated in Canada, Scandinavia, and Hokkaido, which are all much further away from the tropics[14]. Some decades ago it was noticed that there was a variation in cold temperatures tolerance in two distinct stages of soybean development: when the seeds were germinating and emerging [15] and during the flowering and early stages of pod formation [15], [16]. When attempting to grow soybeans in higher latitudes, one must be conscious of the potential occurrence of freezing temperatures after planting or chilly nights with temperatures of less than 15°C during the early stages of growth. To enable the cultivation of soybeans in northern regions with shorter growing seasons, soybean varieties that can withstand cold temperatures, particularly during the early stages of development when soybeans are most vulnerable to cold, must be developed [17]. The exact

source of soybean's capacity to endure cold temperatures has not been determined, but research has demonstrated a connection between certain genetic markers, such as major maturity alleles, the pubescence color T locus, and the plant height Dt1 locus [11], [18]. Two independent groups found that the genotypes that were most tolerant of cold temperatures in their studies came from varieties which included either Fiskeby V or Fiskeby 840-7-3, which were developed by dr. Sven Holmberg in Sweden [15], [19]. Crops have developed intricate and sophisticated reactions to abiotic stresses, including altering gene expression, regulating metabolism, adjusting water management, and protecting cell components from the effects of stress [20]. Gene expression in plants is controlled over time and space through regulation at the transcriptional, post-transcriptional, and translational stages. miRNAs are an important part of the stress response system because they suppress the production of target genes that code for transcription factors, which control the expression of numerous other genes. In addition, miRNA genes are controlled by upstream transcription factors [21]. This makes it essential to identify miRNAs that are regulated by stress in key crops, which will help us to comprehend how plants resist adversities and to uncover the interactions between miRNA and their target genes. Research in plant physiology has demonstrated that even closely related crop species have varying degrees of tolerance to stress. As an example, maize, rice and sorghum are all evolutionarily related crops. Rice and maize, as well as sorghum, branched out from a concestor approximately 50 million years ago (mya) [22]. Despite this, they have vastly different abilities to withstand drought. Rice requires ample moisture in order to survive, while both maize and sorghum can thrive in drier climates. Though maize and sorghum are closely related species, which diverged from each other 11.9 million years ago [22], they have different levels of stress tolerance. Research has demonstrated that sorghum is much more resistant to drought conditions than maize [23]. Therefore, it is worth making an effort to gain a deeper understanding of the molecular mechanisms and pathways associated with cold tolerance in already cultivated soybean material.

miRNA biogenesis in plants

In order to continue to be able to adapt to their environment, plants must develop more intricate internal systems for reacting to and avoiding external stresses through phenotypic plasticity [24]. In 1993, the first miRNA was identified in *Caenorhabditis elegans*, organism which lives in temperate climate [25]. It was not until 2001 that miRNAs were acknowledged as a distinct type of RNA. Subsequently, researchers started looking for miRNA in plants due to the abundance of miRNA in animals. In 2002, various research teams discovered over one

hundred miRNAs from *Arabidopsis thaliana* by employing a cloning technique [26], [27]. Hundreds of microRNAs have been discovered in both plant and animal species since then [28], [29]. miRNAs are non-coding endogenous posttranscriptional gene regulators, that function either by targeting mRNAs for cleavage or by blocking their translation [30]. miRNAs in plants tend to be around 20-22 nucleotides long, and many of them have remained highly conserved for roughly 125 million years, since monocotyledons, eudicots and mosses diverged from each other [31], [32]. microRNAs that are highly conserved are believed to mainly control the expression of transcription factors, while those that are not conserved are assumed to be involved in more specific biological activities within the organism. These microRNAs are seen as the key gene regulators within the cell [33]. microRNAs are involved in regulating various metabolic and biological processes, such as the development of organs, cell growth, hormone signaling, the transition from vegetative to reproductive development, and how organisms respond to adverse conditions [34]. Transcription of plant miRNAs is mainly conducted by DNA-dependent RNA polymerase II, and they are mostly found in intergenic regions in the genomes [35]. microRNA gene (MIR) transcription product is called a primary miRNA (pri-miRNA). It is treated the same way as the rest of RNA molecules, by a 5'cap and a 3'polyadenylated tail addition. The interaction of the RNA-binding protein, DAWDLE (DDL), with Dicer like 1 protein stabilizes an imperfect looped structure of pri-miRNAs [36]. The enzyme Dicer-like 1 is responsible for transforming pri-miRNAs into a precursor, otherwise known as pre-miRNA, which has a stem-loop structure. Depending on the particular family member, the Dicer-like protein process pri-miRNAs into sRNA strands that range from 18 to 24 nucleotides [36]. Collaboration between Dicer-like 1, the C2H2-zinc finger protein SERRATE (SE), the double-stranded RNA-binding protein HYPONASTIC LEAVES1 (HYL1), and the nuclear cap-binding complex (CBC) occurs in miRNA processing areas called D-bodies in order to facilitate transformation of pri-miRNA into pre-miRNA [37]. HEN1, a S-adenosyl methionine-dependent methyltransferase, then stabilizes the mature miRNA duplex (miRNA:miRNA*) generated by the Dicer-like 1 enzyme cleavage by methylating the duplex. This prevents the duplex from being destroyed by nucleases in the cytoplasm when it is exported from the nucleus. Once methylation has occurred, the plant homolog of exportin-5, HASTY, transports the fully developed miRNA duplex out of the nucleus [38]. A miRNA duplex consists of a miR or guide strand (miRNA), and the miR* or passenger strand (miRNA*), which subsequently gets degraded. The functional mature miRNA associates with an RNA-Induced Silencing Complex (RISC) which comprises a protein from the ARGONAUTE (AGO) family. Furthermore, the RISC complex is composed of a PIWI domain and a sRNA- binding PAZ

domain [36]. Newly assembled RISC complex scans for mRNA molecules that have either an exact or very similar match, and attaches to them. In case of the perfect complementarity between miRNA and its mRNA target, usually there is an endonucleolytic cleavage carried out by the RISC complex. This is the most prevalent way to control gene expression post-transcriptionally in plants, as their miRNAs usually are more perfectly complimentary with mRNA targets then their animal counterparts. Products of the cleavage of target RNAs can be identified by RNA-dependent RNA polymerase (RDR) proteins and cause the production of new dsRNA molecules, which further increases the posttranscriptional gene silencing. DICER-LIKE proteins (DCLs) then handle these molecules and process them into secondary small RNAs. Accumulation of these secondary small RNAs in the cytoplasm and their subsequent distribution to other cells through plasmodesmata leads to the systemic silencing signal [39]. In contrast, if the complementarity between miRNA and the targe mRNA is not perfect, the RISC complex blocks the translation of mRNA [40].

miRNA in cold stress

Abiotic stresses, such as drought, salinity, cold, heat, exposure to heavy metals, lack of nutrients, and intense light, in addition to biotic stresses like bacteria, fungi, viruses and pests, are critical impediments to agriculture around the world. Over the course of their evolution, plants have come up with ways to react to and adjust to challenging circumstances, including biological cascades where miRNAs are essential for increasing resistance to stress [41]. Both abiotic and biotic stresses result in the alteration of MIR genes' expression in a variety of plants, such as soybean, maize, rice, sugarcane, tomato and wheat [42], [43]. Research into how these miRNAs are expressed and how they accumulate has yielded a lot of data that can help us understand the systems that help protect against different kinds of stress. The results of such studies are being utilized to develop biotechnological techniques for optimizing these systems and making crops more resilient to stress. Evidence of miRNAs being involved in plant reaction to stress has been revealed through the up- or down-regulation, overexpression, or knock-in of transcribed MIR gene sequences in various species. Thus, the amplification of genes encoding miRNAs using constitutive promoters (like U6 Promoter, cytomegalovirus (CMV) promoter, cauliflower mosaic virus (CaMV) 35S) has been successful in conferring desirable agricultural qualities, including resistance to heat, cold, drought, salinity, and pathogens [44]. Overexpression of miRNAs can be found in other patented inventions, such as increased cyst nematodes resistance (miR164 and miR396), improved tolerance to salinity (miR398) and drought (miR166), as well as target mimicry and engineered MIR genes [44]. Despite the

potential benefits of overexpressing miRNAs, it can often lead to undesired side effects due to involvement of certain miRNAs in the expression control of other miRNAs [45]. An excessive number of specific miRNAs can lead to undesired physical characteristics of plants, due to the modification of the expression of key target genes associated with plant growth [46]. It is possible to achieve greater specificity with the target of overexpression by using promoters that are specific to the chosen tissue [47] or the ones are induced by stress [48]. Other approaches involve expressing engineered targets which can offset the impact of undesired activity of endogenous miRNA [49], the production of MIR genes tailored to only target appropriate mRNAs [50], and the up-regulation of mRNAs that are not affected by particular miRNAs [51]. A novel technique for either increasing or decreasing the amount of miRNA present in a sample using a vector derived from barley stripe mosaic virus has been developed [52]. This vector can be employed to study the effects of miRNAs. Lately, further understanding of how miRNA can be precisely adjusted has been gained using the CRISPR/Cas9 or CRISPR/Cpf1 system. This system has been employed to edit [53] or control transcription [54], [55] of genes encoding miRNAs.

OBJECTIVES

miRNAs are acknowledged as major determinants of post-transcriptional regulation and gene regulatory networks. Chilling tolerance and acclimation are intricate processes that involve numerous transcription factors, miRNAs and genes. The exact way in which microRNAs contribute to low-temperature tolerance has yet to be thoroughly investigated. Soybean is known to be a warm climate crop species, and as a result, its growth and yield are highly affected by chilling stress. Examining soybean varieties with different levels of cold stress resistance, will facilitate the development of new chilling resistant varieties, and thereby reduce the need for imports and increase domestic soybean production.

The aim of the doctoral thesis was to determine changes in soybean miRNA biosynthesis in response to low temperature stress. Achieving this goal required the implementation of the following tasks:

(I) Determination of the effect of cold stress on the phenotype of soybean under field and laboratory conditions.

(II) Identification of miRNAs involved in soybean response to cold stress.

(III) Identification of miRNA target genes involved in soybean response to cold stress.

PLANT MATERIAL

In order to enrich the molecular and phenotypical analysis we used four cultivars (Augusta, Fiskeby V, Toyomusume and *G. soja*) that differ in their sensitivity to chilling stress. Fiskeby V was bred by dr. Sven A. Holmberg in Sweden, near Norrkoping (58°30′N). Augusta was selected from two crosses: in the first step, line 104 was obtained from a cross between Fiskeby V and line PI 194643, and; in the second step, line 104 was crossed with line 11 (*G. soja* wild species). Line 11 of *G. soja* grows in the natural environment of far Eastern Russia at latitudes similar to those of Poland and has a long-day tolerant genotype. Therefore, chilling tolerance of Augusta was acquired from Fiskeby V, and the variety had two sources of photoperiod insensitivity. The seeds of the Augusta and Fiskeby V soybean cultivars were provided by prof. J. Nawracała from the Poznan University of Life Sciences, Poland. *G. soja* is a wild soybean annual species native to China, Japan, Russia and parts of Korea and is a wild progenitor of the cultivated species *Glycine max* (*G. max*). *G. soja* accession PI 538411A was collected over Amur River (Far East of Russia) on latitude: 52°58′39″N and longitude: 127°21′44″E. Toyomusume was chosen as a chilling-sensitive genotype. It is a Japanese variety from Hokkaido Island, where it is grown mainly for the production of tofu.

Table 1. Scheme of the chilling stress treatment.

Stage of soybean growth	Optimal growth temperature	Stress temperature (day/night)	Duration of stress conditions
VE – seedlings	20°C	4°C	48 h
V1 – vegetative	20°C	8°C	120 h (5 days)
R1 – reproductive	20°C	14°C /7°C	168 h (7 days)

Abbreviations: VE – seedling emergence stage, V1 – first trifoliate stage , R1 – the beginning of flowering.

Plants were grown under controlled environmental conditions in a phytotron at a temperature of 20°C with a relative humidity of 60% and a 16:8 hr-light:dark photoperiod prior to stressing treatments. Plants intended for RNA extraction were divided into three groups, and each group included plants subjected to chilling treatment at a different developmental stage, as well as control plants (Table 1). The first batch of plants was stressed at the VE stage (emerging seedlings) by keeping them at 4°C for 48 hr in Percival chambers. The next set of plants was exposed to 8°C for 120 hr (5 days) at the V1 growth stage (first trifoliate). The last group was exposed to 14°C during the day and 7°C at night for 168 hr (7 days) at the R1 growth stage (the beginning of flowering). In order to simulate the detrimental conditions of temperate

climate in field cultivations of soybean, this experiment design was based on the expertise of soybean breeders. In the control and treated groups, between 20 and 30 soybean plants were cultivated. The plants designated for phenotyping were cultured in the same manner as described above; however, their cultivation continued after each chilling treatment in optimal conditions until full maturity (R8 stage).

The field experiment was conducted by prof. J. Nawracała at the Agricultural Research Station Dłoń, Poznań University of Life Sciences, Poland (51°41′37″N, 17°04′06″E), during the 2016 and 2017 growing seasons.

For nucleic acids extraction the radicals and leaflets were collected from seedlings, and the first pair of leaves and trifoliates were collected separately from plants at the V1 stage. Trifoliates were collected from plants at the R1 stage. All samples were harvested from stressed and non-stressed plants immediately after each treatment, flash-frozen and stored at -80°C until the isolation of nucleic acids.

SHORT DESCRIPTION OF THE PUBLICATIONS CONSTITUTING THE DOCTORAL THESIS

1. **Tyczewska A, Gracz J, Kuczyński J, Twardowski T**
   Deciphering the soybean molecular stress response via high-throughput approaches
   Acta Biochimica Polonica, 2016, 63(4): 631-643

We started our work on soybean stress resistance with a literature review of the broad-spectrum research dealing with both abiotic and biotic adverse conditions. We managed to compile a detailed body of knowledge focused on three facets of soybean stress resistance, transcriptome, miRNA and proteome. In this review, we presented classical, as well as modern approaches to study said aspects of plants biochemistry. Moreover, we discussed recent achievements and discoveries in these areas, focusing on abiotic stresses such as drought, flooding, cold stress and nutritional depravation, as well as viral, fungal and nematode related biotic stresses. While high-throughput analyses of RNA expression profiles and proteins provide huge amount of data, there still remains the challenge of identifying true and meaningful patterns and molecules. Advances in large volume techniques allow for more in-depth research, such as comparison of miRNAs profiles, transcriptomic and proteomic analyses from different plant tissues and developmental stages. This can also encourage interest in thus far less explored areas of stress tolerance and adaptation. This paper highlighted the capability of high-throughput techniques to generate big data that for the most part needs further refinement and confirmation. Nevertheless, these data may allow the agriculture industry to meet the demands of ever-growing population, by facilitating the breeding of new and improved crop varieties.

2. **Kuczyński J, Twardowski T, Nawracała J, Gracz-Bernaciak J, Tyczewska A**
   Chilling stress tolerance of two soya bean cultivars: Phenotypic and molecular responses
   Journal of Agronomy and Crop Science, 2020, 206: 759-772

In this publication, we focused on the comparative analysis of phenotype and miRNA expression patterns in two cold-resistant soybean cultivars subjected to chilling stress. Phenotyping of Augusta and Fiskeby V cultivars was conducted both in the field and in controlled conditions in phytotrons. The degree of the chilling tolerance of the soybean varieties was estimated by measuring the rate of growth, the flowering efficiency, the number of seeds and pods per plant and chilling tolerance index of the three groups of plants subjected to stress at different growth stages (see Plant material section here, and Tables 2 and 3 in the

publication). Although chilling stress treatment altered the growth and development of two cold-resistant soybean cultivars, Augusta and Fiskeby V, their overall seed output did not suffer significantly. Both cultivars presented different phenotypic responses to chilling in each of the three growth stages tested. Overall, both cultivars sustained their seed production capability during stress treatment at levels comparable to those of control plants, emphasizing their chilling tolerance. Performing the experiments over two growing seasons allowed for the comparison of soybean responses to different conditions in terms of production circumstances in the region of interest (Poland). Plants of both varieties cultivated during the 2017 season displayed decreased vegetative growth compared to those cultivated during the 2016 season, as demonstrated by the plant height measurements. Additionally, the reproductive growth parameters, such as the seed weight per plant and the 1,000 seed weight, indicated the inferior performance of Augusta and Fiskeby V in 2017 (Table 5 in the publication). The continuous measurements of temperature at the Agricultural Research Station Dłoń, Poznań University of Life Sciences indicated the warming tendencies in this region. Nonetheless, extreme weather events occur unexpectedly and may influence the cultivation of soybean. Despite the weather conditions, Augusta exhibited better characteristics in terms of seed output than Fiskeby V in the field conditions (Table 5 in the publication). The expression profiles of the five miRNAs, chosen based on the previous literature review, were investigated in Augusta and Fiskeby V plants subjected to chilling treatment by ddPCR (Figures 1 and 2 in the publication). To understand the roles of the selected miRNAs (miR169, miR319, miR397, miR398) in chilling stress responses, the expression profiles of their putative target genes (NF-YA, GAMYB, Laccase and CCS, respectively), which were chosen based on the gene ontology analysis, were investigated. Four miRNAs and their 85 targets were classified into 48 molecular functions, 89 biological processes and 21 cellular components. Additionally, we described the expression profiles of several miRNAs involved in the chilling stress response. The differential expression of selected miRNAs in response to low temperature stress was detected primarily in roots and trifoliates of plants treated at the VE and R1 stages, respectively. Moreover, the genes that may have been targeted by the analyzed miRNAs were identified and studied. The analysis of the miRNA and target gene expression profiles further elucidated the differences between the two varieties and confirmed that miRNAs may play a role in soybean chilling stress responses.

In summary, we described the impact of chilling stress on the soybean phenotype at different growth stages. We managed to validate the expression patterns of previously identified stress associated miRNAs in soybean. Lastly, we identified the potential genes targeted by these

miRNAs. These results highlight the importance of miRNA involvement in the chilling stress response in plants.

3. **Kuczyński J, Gracz-Bernaciak J, Twardowski T, Karłowski WM, Tyczewska A**
Cold stress-induced miRNA and degradome changes in four soybean varieties differing in chilling resistance
Journal of Agronomy and Crop Science, 2021, 208: 777-794

The aim of this study was to identify cold stress related miRNAs and their target genes based on the comparison of four differentially cold-resistant soybean cultivars. To identify the miRNAs involved in the chilling stress response, 72 sRNA libraries from both chilled and control seedling roots and cotyledons as well as V1 stage shoots, all performed in triplicate, were constructed for 4 soybean varieties that were sequenced by Illumina Technology. The filtered reads from 72 sRNA libraries were compared with the collection of mature (and precursor) soybean miRNAs from miRBase release 22.1. A total of 321 known soybean miRNA families were identified in all four cultivars (Table S2 in the publication). Additionally, 348 novel miRNAs were found in the four studied cultivars (213 miRNAs in Augusta, 220 in Fiskeby V, 224 in Toyomusume and 218 in *G. soja*) (Table S3 in the publication), among which 129 miRNAs were common for all four cultivars. A total of 162 differentially expressed miRNAs belonging to 137 miRNA families were identified between chilled and control samples of the four soybean cultivars. Among these differentially expressed miRNAs in all the tested cultivars, 93 were downregulated in chilled samples as compared to those in controls in at least one cultivar. On the other hand, 137 miRNAs were upregulated in samples from stressed plants compared to those in controls in at least one cultivar. Amid the plethora of miRNAs that exhibited differential expression between the four studied cultivars, a group of miRNAs, consisting of both conserved miRNAs (miR159, miR2111, miR396 and miR482) and legume-specific miRNAs (miR10197 and miR1509), with a common expression pattern in all the analyzed varieties caught our immediate attention. Among these, miR1509 and miR396 were found to be upregulated in trifoliates of chilled soybean plants at V1 stage, whereas miR10197, miR159, miR2111 and miR482 were downregulated. In the analysis of the degradome libraries, genes coding pyruvate dehydrogenase, auxin signaling F-box 2, aspartyl protease, TIM21-like protein, tobacco mosaic virus (TMV) resistance protein N and vegetative storage protein were found to be the potential targets of these miRNAs. Expression profiles of some miRNAs showed specificity towards a particular tissue, in that they were found only in radicles, such as miR10190 and miR862, or in trifoliates, such as miR1511, miR168 and miR391. Interestingly,

expressional patterns of other miRNAs such as miR1509, miR10440 or miR171 proved that chilling stress can cause one miRNA to be upregulated in one tissue but to be downregulated in another tissue.

To verify the reliability of sequencing results, the expression levels of four miRNAs (miR169, miR408, miR2109 and miR5770) responsive to chilling stress were evaluated by ddPCR (Figure 6 in the publication). The ddPCR results showed that the expression patterns of four selected miRNAs were mostly in accordance with the assessment of sRNA sequencing (except for miR169 in Augusta and Fiskeby V, and miR408 in Toyomusume). On the basis of the degradome sequencing and subsequent CleaveLand program analysis, potential targets of soybean miRNAs that are involved in the chilling stress responses were identified. Degradome analysis, as well as GO and KEGG annotations, allowed to assign potential target genes to the differentially expressed miRNAs. Many of these genes were found to be related to plant abiotic stress response mechanisms such as reactive oxygen species (ROS) scavenging, flavonoid biosynthesis and regulation of osmotic potential. Three genes were chosen to be studied for any alterations in gene expression level due to chilling in the roots and cotyledons of four soybean varieties.: *G. max* putative phytocyanin (Phyt), which functions as an electron transporter, *G. max* malate dehydrogenase (GmMDH), which catalyzes the reversible interconversion of malate and oxaloacetate using NAD(H)/NADP(H) as a cofactor and regulates plant development and abiotic stress tolerance, and *G. max* transcription factor NAC-19. The analysis of expression levels of selected genes showed changes caused by cold stress. The differential expression profiles of genes encoding enzymes involved in redox homeostasis and electron transport were observed, which may be responsible, to some extent, for increased/ decreased susceptibility to abiotic stresses.

In summary, the cold responsive miRNAs in four soybean cultivars varying in cold stress resistance using high-throughput sequencing have been identified. Putative, stress tolerance related, target genes were found and their involvement in chilling stress was investigated. Overall, these findings elucidate the important role of miRNAs in soybean chilling stress response.

CONCLUSIONS

The phenotypical and molecular analyses that have been carried out broaden the knowledge on the molecular mechanism of low temperature response in soybean. Crucially, molecular comparison of varieties differing in the chilling tolerance helped to distinguish significant variations in miRNA expression levels and target gene degradation patterns. Experiments described in this thesis allowed to draw the following conclusions:

1. The growth and development of Augusta and Fiskeby V soybean varieties was affected in response to chilling stress. These two cold-resistant varieties were most susceptible to chilling stress at the flowering stage (R1). Nonetheless, the yield of Augusta was the lowest in plants chilled at the seedling stage (VE). The results of the field experiment showed that Augusta variety, compared to Fiskeby V, was more suitable for Polish growth conditions due to its better performance. The distinct phenotypes exhibited by Augusta and Fiskeby V may be explained by the dissimilarities between these cultivars at the molecular level.

2. The analysis of the miRNA and target gene expression profiles further elucidated the differences between tested varieties and confirmed that miRNAs are key regulators in soybean chilling stress responses. Several miRNAs such as miR156, miR169 and miR5770 had similar expression patterns in Augusta, Fiskeby V and *G. soja*, as opposed to cold-sensitive Toyomusume variety, firmly implying their role in low temperature tolerance.

3. Degradome analysis as well as GO and KEGG annotations allowed to assign potential target genes to the differentially expressed miRNAs. Many of these genes were found to be related to plant abiotic stress response mechanisms such as ROS scavenging, flavonoid biosynthesis and regulation of osmotic potential.

In summary, the presented results offer important information about the role of miRNAs in soybean chilling resistance and may be essential for the creation of new cultivars.

ABBREVIATIONS

AGO	– ARGONAUTE gene family
BSMV	– barley stripe mosaic virus
CaMV	– cauliflower mosaic virus
CBC	– the nuclear cap –binding complex
CMV	– cytomegalovirus
DCL	– DICER –LIKE protein
ddPCR	– droplet –digital PCR
DDL	– DAWDLE protein
GO	– Gene Ontology
HEN1	– Small RNA 2' –O –methyltransferase
HYL1	– double –stranded RNA –binding protein HYPONASTIC LEAVES1
KEGG	– Kyoto Encyclopedia of Genes and Genomes
MIR	– microRNA gene
miRNA	– microRNA
miRNA*	– passenger strand
RDR	– RNA –dependent RNA polymerase
RISC	– RNA –Induced Silencing Complex
ROS	– reactive oxygen species
SE	– C2H2 –zinc finger protein SERRATE
TMV	– tobacco mosaic virus

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APPENDIX

1. CO-AUTHOR STATEMENTS
2. PUBLICATION No. 1

   Deciphering the soybean molecular stress response via high-throughput approaches

3. PUBLICATION No. 2

   Chilling stress tolerance of two soya bean cultivars: Phenotypic and molecular responses

4. PUBLICATION No. 3

   Chilling stress tolerance of two soya bean cultivars: Phenotypic and molecular responses

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STATEMENT

I hereby declare that I am aware that the work: *Tyczewska A, Gracz J, Kuczyński J, Twardowski T, Deciphering the soybean molecular stress response via high-throughput approaches, Acta Biochimica Polonica, 2016, 63(4): 631-643, doi: 10.18388/abp.2016_1340*, of which I am a co-author, has been included in the doctoral thesis of Jakub Kuczyński. I contributed to: conceptualization, formal analysis, editing the drafts of the manuscript.

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STATEMENT

I hereby declare that I am aware that the work*: Tyczewska A, Gracz J, Kuczyński J, Twardowski T, Deciphering the soybean molecular stress response via high-throughput approaches, Acta Biochimica Polonica, 2016, 63(4): 631-643, doi: 10.18388/abp.2016_1340*, of which I am a co-author, has been included in the doctoral thesis of Jakub Kuczyński. I contributed to: conceptualization, writing the draft of the manuscript, editing the subsequent drafts of the manuscript.

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STATEMENT

I hereby declare that I am aware that the work*: Tyczewska A, Gracz J, Kuczyński J, Twardowski T, Deciphering the soybean molecular stress response via high-throughput approaches, Acta Biochimica Polonica, 2016, 63(4): 631-643, doi: 10.18388/abp.2016_1340*, of which I am a co-author, has been included in the doctoral thesis of Jakub Kuczyński. I contributed to: conceptualization, writing the draft of the manuscript, editing the subsequent drafts of the manuscript.

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STATEMENT

I hereby declare that I am aware that the work: *Kuczyński J, Twardowski T, Nawracała J, Gracz-Bernaciak J, Tyczewska A, Chilling stress tolerance of two soya bean cultivars: Phenotypic and molecular responses, Journal of Agronomy and Crop Science, 2020, 206: 759-772, doi:10.1111/jac.12431*, of which I am a co-author, has been included in the doctoral thesis of Jakub Kuczyński. I contributed to: providing ideas and designing the research, formal analysis, procuring resources, editing the drafts of the manuscript, funding acquisition.

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STATEMENT

I hereby declare that I am aware that the work: *Kuczyński J, Twardowski T, Nawracała J, Gracz-Bernaciak J, Tyczewska A, Chilling stress tolerance of two soya bean cultivars: Phenotypic and molecular responses, Journal of Agronomy and Crop Science, 2020, 206: 759-772, doi:10.1111/jac.12431*, of which I am a co-author, has been included in the doctoral thesis of Jakub Kuczyński. I contributed to: providing ideas and designing the research, performing the experiments and analyzing the data, editing the drafts of the manuscript.

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Poznań, 11.09.2023

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STATEMENT

I hereby declare that I am aware that the work*: Kuczyński J, Twardowski T, Nawracała J, Gracz-Bernaciak J, Tyczewska A, Chilling stress tolerance of two soya bean cultivars: Phenotypic and molecular responses, Journal of Agronomy and Crop Science, 2020, 206: 759-772, doi:10.1111/jac.12431*, of which I am a co-author, has been included in the doctoral thesis of Jakub Kuczyński. I contributed to: providing ideas and designing the research, performing the experiments and analyzing the data.

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I hereby declare that I am aware that the work: *Kuczyński J, Gracz-Bernaciak J, Twardowski T, Karłowski WM, Tyczewska A, Cold stress-induced miRNA and degradome changes in four soybean varieties differing in chilling resistance Journal of Agronomy and Crop Science, 2022, 208: 777-794, doi:10.1111/jac.12557*, of which I am a co-author, has been included in the doctoral thesis of Jakub Kuczyński. I contributed to: conceptualization, formal analysis, procuring resources, editing the drafts of the manuscript, funding acquisition.

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I hereby declare that I am aware that the work*: Kuczyński J, Gracz-Bernaciak J, Twardowski T, Karłowski WM, Tyczewska A, Cold stress-induced miRNA and degradome changes in four soybean varieties differing in chilling resistance Journal of Agronomy and Crop Science, 2022, 208: 777-794, doi:10.1111/jac.12557*, of which I am a co-author, has been included in the doctoral thesis of Jakub Kuczyński. I contributed to: conceptualization, providing methodology, validation, performing the experiments and analyzing the data, editing the drafts of the manuscript, supervision and project administration.

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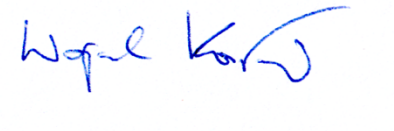

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Review

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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Chilling stress tolerance of two soya bean cultivars: Phenotypic and molecular responses

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Abstract

Chilling stress is a major factor limiting the yield of soya bean [*Glycine max* (L.) Merr.] on a global scale. However, the regulatory network that controls the chilling response of soya bean remains unclear. In the present study, phenotyping and quantitative analyses of miRNAs in soya bean under chilling stress were carried out to determine the impact of environmental constraints on soya bean productivity. Measurements done during soya bean growth in chilling along with the results of field trials indicated that the cultivars Augusta and Fiskeby V responded differently to low temperatures. Although chilling affected the reproductive development of both cultivars, the final seed output remained unchanged. The differential expression of miR169, miR319, miR397 and miR398 under cold stress was detected using ddPCR. Upon chilling in the reproductive stage, we found that these miRNAs had contrasting expression profiles in Augusta and Fiskeby V. A set of candidate target genes was predicted based on degradome sequencing data. A negative correlation was found between the expression of miR169, miR319 and miR398 and their targets in the roots of both cultivars. Our work elucidates the impact of chilling stress on the productivity of two soya bean cultivars and reveals the importance of miRNA involvement in the low temperature response.

KEYWORDS

chilling stress, differential expression, microRNA, phenotyping, soya bean

1 | INTRODUCTION

Soya bean [*Glycine max* (L.) Merr.] is an important legume crop with a significant worldwide production (Govindasamy et al., 2017), accounting for 340 million metric tons (mmt) in 2017. Exceptionally high protein (40%) and oil (20%) content make soya bean an outstanding source of nutrition that is used in a number of food products for humans, as well as in animal feeds. In addition, oil extracted from soya bean seeds can be used in the production of biofuels (Zhang, Pan, & Stellwag, 2008). All these benefits can be attributed to the symbiotic interaction of soya bean with *Bradyrhizobium japonicum* occurring in root nodules, which results in the fixation of atmospheric nitrogen (Zhang, Wang, et al., 2014). This cooperation benefits the environment by decreasing the need for the application of fertilizers and improving soil quality for subsequent crops, such as wheat or maize. In 2017, the production of soya bean in the European Union accounted for 2.74 mmt, which represented ~8% of the EU demand for soya bean seed, oil and meal. Simultaneously, the thriving animal production industry required the European Union to import over 33 mmt of soya bean products, mainly from Argentina and Brazil (https://www.idhsustainabletrade.com/uploaded/2019/04/European-Soy-Monitor.pdf). The high demand for soya bean-derived products and difficult environmental conditions in temperate climates of the Northern and Central Europe, due to cold springs and early summer droughts,

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necessitate the development of new cultivars with better yields and improved resistance to abiotic stresses.

Cold stress is one of the factors influencing plant development in many regions of the world, including Poland (Janská, Maršík, Zelenková, & Ovesná, 2010). Under cold stress, plants exhibit a variety of phenotypic changes, including reduced leaf development and wilting, the sterility of flowers, delayed seeding and suboptimal germination (Suzuki, Nagasuga, & Okada, 2008; Wen, Oono, & Imai, 2002). On the molecular level, cold stress negatively affects enzyme catalytic properties and the plasma membrane liquid/crystalline physical structure (Yadav, 2009). Stress response is a complex process that involves physiological and biochemical changes driven by the reprogramming of gene expression. Previously, the involvement of microRNA (miRNA) in plant stress responses was shown in sugarcane (Thiebaut et al., 2012), rice (Jian et al., 2010; Wang, Sun, Hoshino, et al., 2014; Yang et al., 2013; Zhou et al., 2013), *Arabidopsis thaliana* (Dong & Pei, 2014; Xu et al., 2014), *Prunus persica* (Barakat et al., 2012) and trifoliate orange (Zhang, Li, & Liu, 2014). miRNAs are endogenous small non-coding RNAs that are significant factors involved in the regulation of gene expression (Bartel, 2004). These molecules have been shown to facilitate the inhibition of gene expression at the transcriptional and post-transcriptional levels via either the near-perfect complementarity-based cleavage of transcripts or the restriction of the translation process (Reinhart, Weinstein, Rhoades, Bartel, & Bartel, 2002; Rhoades et al., 2002). Thus far, a number of groups have shown that miRNAs play a role in the response of soya bean to abiotic and biotic stressors (reviewed in Tyczewska, Gracz, Kuczyński, & Twardowski, 2016), such as heavy metals (Qiao-Ying et al., 2012), drought, salinity, alkalinity (Liu et al., 2016), phosphate starvation (Xu et al., 2013), and *Phytophthora sojae* (Wong et al., 2014) and cyst nematode infection (Li et al., 2012). In vegetable soya bean (Xu et al., 2016) and in nodules of *G. max* (Zhang, Wang, et al., 2014), the expression of several miRNAs has been identified to be significantly changed during chilling stress. Nevertheless, our knowledge of miRNA function under chilling stress conditions is limited, especially when comparing two production grade cultivars.

In this study, we compared the responses to chilling stress of two cold-resistant soya bean cultivars (Augusta and Fiskeby V) at the phenotypic and molecular levels. The application of cold stress in three separate growth stages of soya bean allowed for the thorough investigation of stress responses in the tested plants. Field trials were used to validate the data collected from plants cultivated in a phytotron. Furthermore, digital droplet PCR (ddPCR) was used to assess the expression patterns of both miRNAs and their putative target genes during chilling stress. The aim of this study was to determine the responses of two soya bean cultivars to chilling at the phenotypic level as well as the role of specific miRNAs in soya bean defence mechanisms. Our results provide insight into cold stress response mechanisms and the regulatory roles of miRNAs in production-grade soya bean.

2 | MATERIALS AND METHODS

2.1 | Plant material

Two soya bean varieties that were photoperiod-insensitive and tolerant of chilling stress were chosen: Fiskeby V and Augusta. Fiskeby V was bred by Dr. Sven A. Holmberg in Sweden, near the city of Norrkoping (58°30′N). The chilling tolerance of the Swedish cultivar Fiskeby V is presumed to be derived from the Sakhalin landrace Namikawa. Augusta was selected from two crosses: in the first, a cross between Fiskeby V and line PI 194643 was made, and line 104 was obtained; in the second, line 104 was crossed with line 11 (*Glycine soja* wild species). Line 11 of *G. soja* grows in the natural environment of far Eastern Russia at latitudes similar to those of Poland and has a long-day tolerant genotype. Therefore, Augusta has two sources of photoperiod insensitivity, and its chilling tolerance is derived from Fiskeby V. The seeds of the Augusta and Fiskeby V soya bean cultivars were supplied by Prof. J. Nawracała from the Poznań University of Life Sciences, Poland. Prior to sowing, the soya bean seeds were inoculated with *Bradyrhizobium japonicum* (HiStick® Soy) to induce nodule formation. The soya bean varieties Augusta and Fiskeby V were planted in pots filled with a mixture of all-purpose potting soil and sand at a 3:1 ratio. Plants were grown under controlled environmental conditions in a phytotron at a temperature of 20°C with a relative humidity of 60% and a 16:8 hr-light:dark photoperiod prior to stressing treatments.

Plants intended for RNA extraction were divided into three groups, and each group included plants subjected to chilling treatment at a different developmental stage, as well as control plants (Table 1). The first batch of plants was stressed at the VE stage (emerging seedlings) by keeping them at 4°C for 48 hr in Percival chambers. The next set of plants was exposed to 8°C for 120 hr (5 days) at the V1 growth stage (first trifoliate). The last group was exposed to 14°C during the day and 7°C at night for 168 hr (7 days) at the R1 growth stage (the beginning of flowering). In order to simulate

TABLE 1 Scheme of the chilling stress treatment

Stage of soya bean growth	Optimal growth temperature	Stress temperature (day/night)	Duration of stress conditions
VE—seedlings	20°C	4°C	48 hr
V1—vegetative	20°C	8°C	120 hr (5 days)
R1—reproductive	20°C	14°C/7°C	168 hr (7 days)

Abbreviations: V1, first trifoliate stage, R1—the beginning of flowering; VE, seedling emergence stage.

the detrimental conditions of temperate climate in field cultivations of soya bean, this experiment design was based on the expertise of breeders in soya bean breeding. In the control and treated groups, between 20 and 30 soya bean plants were cultivated.

The plants designated for phenotyping were cultured in the same manner as described above; however, their cultivation continued after each chilling treatment in optimal conditions until full maturity (R8 stage).

2.2 | Sample preparation and small RNA extraction

The radicals and leaflets were collected from seedlings, and the first pair of leaves and trifoliates were collected separately from plants at the V1 stage. Trifoliates were collected from plants at the R1 stage. All samples were harvested from chilled and non-treated plants immediately after each treatment, flash-frozen and stored at −80°C until the isolation of nucleic acids. The soya bean tissue samples were ground to a fine powder with a TissueLyser II (Qiagen).

Total RNA samples were extracted with the miRVana kit (Thermo Fisher) according to the manufacturer's instructions. Small RNAs were extracted using the miRVana kit (Thermo Fisher) according to the manufacturer's instructions for small RNA-enriched isolation. The small and total RNA quantities were determined with a Qubit 4 Fluorometer (Thermo Fisher).

2.3 | Phenotyping of plants grown in the phytotron

The assessment of the impact of chilling stress on the soya bean phenotype was performed by measuring several important parameters. Vegetative growth was determined by measuring the height of plants at the V1 and R1 stages and calculating the number of days between these stages. The growth rate was then calculated as the difference in height between the R1 and V1 plants divided by the number of days that it took for the plants to grow from the V1 to the R1 stage. During flowering, several parameters were measured, including the number of flowers on each plant, the number of pods on each plant and the number of seeds in each pod. Finally, after harvesting, the seeds from the stressed and control groups were weighed and counted. Based on these data, we calculated the effectiveness of flowering by comparing the number of pods per plant to the number of flowers on each plant. The average number of seeds in a pod was quantified by dividing the number of seeds per plant by the number of pods per plant. Finally, the chilling tolerance index was calculated as the mean weight of the seeds from stressed plants divided by the mean weight of the seeds from control plants.

2.4 | Phenotyping of plants grown in the field

The field experiment was conducted at the Agricultural Research Station Dłoń, Poznań University of Life Sciences, Poland (51°41′37″N, 17°04′06″E), during the 2016 and 2017 growing seasons. The experimental design utilized a latin square plot arrangement with four replicates. The soil was haplic luvisol, and the crop grown previous to the experiment was wheat.

The plots contained four rows 1.5 m long and 50 cm apart. Sixty seeds per plot of each cultivar were sown on 25 April 2016 and 26 April 2017. Just after this planting, a pre-emergence herbicide that contained linuron (0.1 g/m^2) and S-metolachlor (0.14 g/m^2) was applied. The fertilizer was applied in accordance with conventional farming practices in this area (N – 30 kg/ha, P – 80 kg/ha, K – 120 kg/ha).

The dates of the beginning of flowering and the maturity of the plants were recorded. At physiological maturity, 10 single plants from each plot were harvested manually. The morphological and yield component traits, including the plant height, number of branches, number of pods and seeds, seed number in each pod, the 1,000 seed weight and the weight of seeds per plant, were measured.

The rainfall and air temperatures were measured with a Vantage VUE 6357EU (Davis Instruments) that was located ~400 m away from the experimental field.

2.5 | Analysis of the expression of miRNAs and their targets

Five miRNAs (miR159, miR169, miR319, miR397 and miR398) with putative roles in chilling stress responses were selected for expression level analysis based on a literature review. The candidate miRNA target genes were determined based on the results of the GO analysis. A set amount of extracted RNA (1 μg sRNA and 1.5 μg of total RNA) was reverse-transcribed using SuperScript IV Reverse Transcriptase (Thermo Fisher) as described in (Varkonyi-Gasic, Wu, Wood, Walton, & Hellens, 2007). Stem-loop primers were designed for the miRNA reverse transcription reactions, whereas reverse PCR primers were used for the miRNA target genes. The RT and PCR primers are shown in Table S3. To quantify the number of miRNA molecules in the plant samples, a ddPCR mixture composed of 10 μl of ddPCR Super Mix Eva Green, primers (the final concentration of each primer was 200 nM), template (reverse-transcribed, elongated miRNA) and RNase-free H_2O was used. A 20 μl reaction mixture was used to generate the droplets in an 8-well cartridge using a QX100 droplet generator (Bio-Rad). The droplets were carefully transferred to a 96-well ddPCR plate and heat-sealed with foil (Bio-Rad). The cDNA was then amplified in a T100 PCR thermal cycler (Bio-Rad) under the following cycling conditions: 5 min of denaturation at 95°C, followed by 40 cycles with a three-step thermal profile of 30 s of denaturation at 95°C, 30 s of annealing at 55°C and 45 s of extension at 72°C. After that, the products were kept at 72°C for 2 min for the final extension. After amplification, the products were cooled to 4°C for 5 min and then heated to 90°C for 5 min and finally cooled again to 12°C. The droplets were quantified in a QX100 droplet reader (Bio-Rad). Data acquisition and analysis were performed using QuantaSoft software (Bio-Rad). The positive droplets

TABLE 2 Plant height, growth rate, flowering efficiency, number of seeds per plant, number of pods per plant, number of seeds per pod, thousand seed weight and chilling tolerance index in the Augusta cultivar under control and chilling conditions

	Plant height (R1) [cm]	Growth rate [cm/day]	Flowering efficiency [%]	Number of seeds per plant	Number of pods per plant	Number of seeds per pod	Thousand seed weight [g]	Chilling tolerance index [%]
Seedling								
Control	50.0.31 ± 8.13[a]	2.22 ± 0.67	44.68 ± 8.76[a]	21.37 ± 7.83	15.41 ± 5.62	1.40 ± 0.32	102.79	92.87
Stress	45.36 ± 10.44[b]	2.07 ± 0.49	30.85 ± 13.16[b]	19.46 ± 6.48	15.73 ± 4.26	1.26 ± 0.29	104.84	
V1								
Control	54.78 ± 15.72[a]	2.33 ± 0.86[a]	44.40 ± 8.84[a]	16.85 ± 6.91	14.17 ± 4.94	1.19 ± 0.29[a]	106.18	111.07
Stress	38.39 ± 7.53[b]	1.27 ± 0.39[b]	49.60 ± 9.16[b]	19.60 ± 8.21	13.78 ± 5.89	1.45 ± 0.32[b]	101.40	
R1								
Control	61.37 ± 12.90	2.66 ± 0.78	37.29 ± 10.94[a]	12.95 ± 3.99[a]	10.62 ± 2.81[a]	1.22 ± 0.25[a]	67.89	102.43
Stress	60.73 ± 17.80	2.57 ± 1.04	24.60 ± 5.42[b]	10.34 ± 4.25[b]	7.30 ± 2.60[b]	1.43 ± 0.33[b]	87.08	

Note: Data represent the mean (n = 23–28) and standard deviation. Different letters denote statistically significant differences between the control and chilling treatment samples at $p < .05$ after Student's t test.

Abbreviations: R1, the beginning of flowering; V1, first trifoliate stage.

TABLE 3 Plant height, growth rate, flowering efficiency, number of seeds per plant, number of pods per plant, number of seeds per pod, thousand seed weight and chilling tolerance index in the Fiske by V cultivar under control and chilling conditions

	Plant height (R1) [cm]	Growth rate [cm/day]	Flowering efficiency [%]	Number of seeds per plant	Number of pods per plant	Number of seeds per pod	Thousand seed weight [g]	Chilling tolerance index [%]
Seedling								
Control	62.95 ± 18.12	2.05 ± 0.78	56.38 ± 15.31	13.70 ± 5.22	7.76 ± 2.57	1.77 ± 0.34	198.38	116.63
Stress	70.00 ± 10.98	2.42 ± 0.50	54.83 ± 9.17	15.50 ± 3.18	8.36 ± 2.11	1.88 ± 0.43	204.80	
V1								
Control	54.40 ± 13.03	1.54 ± 0.56	47.97 ± 11.64[a]	11.68 ± 4.65	6.81 ± 2.30	1.69 ± 0.40	189.54	112.94
Stress	54.40 ± 11.75	1.67 ± 0.49	55.43 ± 12.98[b]	12.16 ± 5.09	7.45 ± 3.23	1.66 ± 0.45	205.55	
R1								
Control	60.95 ± 9.24	1.87 ± 0.39	56.08 ± 11.90[a]	12.60 ± 4.69[a]	7.17 ± 2.69	1.79 ± 0.39[a]	155.70	92.60
Stress	58.75 ± 14.27	1.66 ± 0.55	46.73 ± 13.38[b]	8.90 ± 3.18[b]	8.45 ± 3.80	1.15 ± 0.42[b]	204.26	

Note: Data represent the mean (n = 20–24) and standard deviation. Different letters denote statistically significant differences between the control and chilling treatment samples at $p < .05$ after Student's t test.

Abbreviations: R1, the beginning of flowering; V1, first trifoliate stage.

containing the amplification products were discriminated from the negative droplets by setting the fluorescence amplitude threshold to the lowest value of the positive droplet cluster.

Yeast tRNA-Thr(TGT) molecules with a scrambled sequence were added to each RT reaction as an internal control.

2.6 | GO analysis

All of the selected miRNAs were annotated along with their respective target genes based on the RNA–RNA interaction data and searches of the Phytozome degradome database. Then, all putative target genes were searched in the AmiGo database to investigate the gene ontology.

2.7 | Statistical analysis

The differences between the control and chilling treatments among Augusta and Fiskeby V were evaluated with Student's *t* test. All data were tested for normality (Shapiro–Wilk test). When this approach failed to meet the normality assumptions, the data were analysed using the non-parametric Wilcoxon signed-rank test. The differences between the control and chilling treatments were evaluated with ANOVA test considering variety and treatment. The homogeneity of variances was tested with Levene's test. When this failed to meet the ANOVA assumptions, the data were analysed using aligned ranks transformation ANOVA. The resulting *p*-values were considered to be statistically significant at $p < .05$. The statistical analyses were performed in R for Windows, version 3.6.0. Asterisks indicate the significant differences: $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

3 | RESULTS

3.1 | The influence of chilling stress on soya bean phenotype in controlled growth conditions

The degree of the chilling tolerance of the soya bean varieties was estimated by measuring the rate of growth, the flowering efficiency, the number of seeds and pods per plant and chilling tolerance index of the three groups of plants subjected to stress at different growth stages (Tables 2 and 3). The vegetative development, as represented by the growth rate, was profoundly affected in Augusta plants stressed at the V1 stage, which grew 45% slower than plants in the control group (Table 2). Moreover, the analysis of variance confirmed the interaction between variety and chilling treatment, which affected the growth rate of Augusta and Fiskeby V plants stressed at the V1 stage (Table 4). The flowering efficiency was changed in plants chilled during the R1 stage for both cultivars. The effectiveness of pod formation dropped by 34% and 17% in Augusta and Fiskeby V, respectively (Tables 2 and 3). Additionally, chilling stress applied to seedlings of the Augusta cultivar resulted in a 31% decrease in flowering efficiency (Table 2). Surprisingly, the number of pods was negatively affected only in the Augusta cultivar (reduction of 31%) when plants were stressed during the R1 stage (Table 2), which was the effect of the interaction between variety and chilling treatment, as indicated by the analysis

TABLE 4 F-statistics and *p*-values (Sig.) of the two-way analysis of variance (ANOVA) of the effects of variety (Var.) and chilling treatment (Treat.), with their interaction factor (Var. × Treat.) on production traits

	Seedling			V1			R1		
	Variety	Treat.	Var. × Treat.	Variety	Treat.	Var. × Treat.	Variety	Treat.	Var. × Treat.
$F_{plant\ height}$	64.04	0.10	5.83	10.75	16.29	10.40	0.11	0.48	0.02
Sig.	***	ns	*	**	***	**	ns	ns	ns
$F_{growth\ rate}$	0.40	0.45	4.54	2.23	19.82	25.62	23.76	1.12	0.01
Sig.	ns	ns	*	ns	***	***	***	ns	ns
$F_{flowering\ efficiency}$	59.85	13.16	6.95	5.23	8.77	0.27	88.06	26.11	0.99
Sig.	***	***	**	*	**	ns	***	***	ns
$F_{numer\ of\ seeds\ per\ plant}$	21.20	0.13	2.17	23.60	1.80	0.76	0.85	13.17	0.40
Sig.	***	ns	ns	***	ns	ns	ns	***	ns
$F_{number\ of\ pods\ per\ plant}$	111.96	1.13	0.01	71.68	0.30	0.68	3.84	3.22	13.30
Sig.	***	ns	ns	***	ns	ns	ns	ns	***
$F_{number\ of\ seeds\ per\ pod}$	53.98	0.16	3.59	23.31	3.33	4.00	5.48	6.99	33.38
Sig.	***	ns	ns	***	ns	*	*	**	***

Abbreviations: ns, not significant;R1, the beginning of flowering; V1, first trifoliate stage.

Asterisks indicate significant differences

$^{*}p < .05$.

$^{**}p < .01$.

$^{***}p < .001$.

of variance (Table 4). Interestingly, further observations showed that the number of seeds per plant of both cultivars was affected after chilling in the R1 stage and showed a 20% and 29% reduction in Augusta and Fiskeby V, respectively (Tables 2 and 3). In the case of Fiskeby V, a lower average number of seeds per pod resulted from a reduction in the number of seeds per plant coupled with an increase in the number of pods per plant. Conversely, in Augusta, the number of seeds per pod did not change significantly as a result of chilling stress because the number of pods and seeds per plant were reduced simultaneously. The final parameter used to determine the overall yield potential of the studied plants was the chilling tolerance index. Remarkably, Augusta and Fiskeby V plants stressed at the seedling and vegetative growth stages showed indexes that were increased by ~10% compared to those of control plants (Tables 2 and 3). Lowered seed output was observed in the Fiskeby V cultivar subjected to stress at the R1 stage (reduction of 7.4%) and Augusta plants chilled at the VE stage (reduction of 7.13%; Tables 2 and 3).

3.2 | The phenotyping of soya bean in field conditions

In addition to phenotype analysis under controlled conditions, a field experiment was carried out to compare the production potential of Augusta and Fiskeby V. Performing the experiments over two growing seasons allowed for the comparison of soya bean responses to different conditions in terms of production circumstances in the region of interest (Poland). Plants of both varieties cultivated during the 2017 season displayed decreased vegetative growth compared to those cultivated during the 2016 season, as demonstrated by the plant height measurements. Additionally, the reproductive growth parameters, such as the seed weight per plant and the 1,000 seed weight, indicate the inferior performance of Augusta and Fiskeby V in 2017 (Table 5). As indicated by ANOVA, the number of seeds per pod was influenced by the interaction between variety and seasonal changes (Table 6). The Polish cultivar showed yields that were significantly worse in the 2017 season, during which its seed production per plant dropped to 13 from 16.2 g in the previous year, and its 1,000 seed weight decreased from 18.2 to 13.2 g (Table 5). Fiskeby V suffered a loss in terms of the weight of seeds per plant, which decreased from 13 g in the 2016 season to 9.5 g in the next season and the 1,000 seed weight decreased from 26.4 to 23.7 g (Table 5). Unfortunately, these differences in soya bean growth between the two years of the experiment were due to meteorological anomalies such as hail and flooding in 2017.

3.3 | miRNA differential expression in soya bean in the three developmental stages

The expression profiles of the five miRNAs were investigated in Augusta and Fiskeby V plants subjected to chilling treatment by

TABLE 5 Plant height, number of seeds per plant, number of pods per plant, number of seeds per pod, thousand seed weight, weight of seeds per plant and number of days from planting to flowering in Augusta and Fiskeby V cultivars under field conditions

Cultivar	Growing season	Plant height [cm]	Number of seeds per plant	Number of pods per plant	Number of seeds per pod	Thousand seed weight [g]	Weight of seeds per plant	Number of days from planting to flowering
Augusta	2016	55.7 ± 5.10[a]	88.9 ± 20.51	47.4 ± 10.23	1.87 ± 0.05	182.0 ± 1.12	16.2 ± 3.65[a]	57
	2017	45.7 ± 1.98[b]	76.5 ± 16.40	32.9 ± 7.29	2.33 ± 0.08	132.0 ± 13.9	10.0 ± 2.03[b]	57
Fiskeby V	2016	34.5 ± 3.78	49.1 ± 3.97	25.3 ± 0.77[a]	1.94 ± 0.11	264.0 ± 1.95[a]	13.0 ± 1.11	51
	2017	30.6 ± 2.16	45.8 ± 6.40	24.3 ± 4.18[b]	1.88 ± 0.09	209.0 ± 6.23[b]	9.5 ± 1.30	50

Note: Data represent the mean ($n = 10$) and standard deviation. Different letters denote statistically significant differences between seasons at $p < .05$ after Student's t test.

TABLE 6 F-statistics and *p*-values (Sig.) of the two-way analysis of variance (ANOVA) of the effects of variety (Var.) and season, with their interaction factor (Var. × Season) on production traits

	Variety	Season	Var. × Season
$F_{plant\ height}$	80.93	11.73	2.22
Sig.	***	**	ns
$F_{number\ of\ seeds\ per\ plant}$	36.79	0.78	0.23
Sig.	***	ns	ns
$F_{number\ of\ pods\ per\ plant}$	37.01	3.90	2.98
Sig.	***	ns	ns
$F_{number\ of\ seeds\ per\ pod}$	14.70	18.87	27.84
Sig.	**	***	***
$F_{thousand\ seed\ weight}$	36.57	36.57	0.45
Sig.	***	***	ns
$F_{weight\ of\ seeds\ per\ plant}$	0.61	14.25	0.97
Sig.	ns	**	ns

Abbreviation: ns, not significant.
Asterisks indicate significant differences
*$p < .05$.
**$p < .01$.
***$p < .001$.

ddPCR (Figures 1 and 2). The expression of four miRNAs in non-stressed plants was ascertained in trifoliates, unifoliates, leaflets and roots from both cultivars grown in control conditions, whereas miR159 was not detected at all. Both miR397 and miR398 showed similar trends, as they were ~154- and 129-fold more abundant in leaves (unifoliates and trifoliates) from Fiskeby V sampled at the V1 and R1 stages in comparison with their abundances in seedling tissues (Figure 1). In Augusta, the expression levels were ~94-fold higher for miR397 and 57-fold higher for miR398 (Figure 1). Likewise, the miR169 expression level was 5.5-fold higher in all of the above-ground samples of Fiskeby V compared to that in roots and was 3.4-fold higher in Augusta (Figure 1a). Conversely, we detected increased amounts of miR319 (13.4-fold higher in Augusta; 4.9-fold higher in Fiskeby V) in seedlings compared to the amounts in unifoliates and trifoliates from the V1 and R1 stages (Figure 1b). The relative expression of the analysed miRNAs in stressed plants differed between the studied cultivars (Figure 2). In plants chilled during flowering development (R1), Augusta and Fiskeby V exhibited contrasting expression patterns for miR169, miR319, miR397 and miR398, and the most prominent contrast was observed for miR397, which showed 80% downregulation in the former and a 250% increase in the latter (Figure 2). Similar trends were observed in plants stressed during vegetative growth (V1), with the exception of miR169, which had relative expression levels in seedling samples that were similar in both cultivars (Figure 2). Remarkably, chilling stress caused considerable changes in the expression of miR169, miR319, miR397 and miR398 in roots compared to that in leaflets, in which miRNA synthesis was stable (Figure 2).

3.4 | Expression levels of miRNAs and predicted target genes

To understand the roles of the selected miRNAs in chilling stress responses, the expression profiles of their putative target genes, which were chosen based on the gene ontology analysis (Appendix S4), were investigated. Four miRNAs and their 85 targets were classified into 48 molecular functions, 89 biological processes and 21 cellular components. The most highly represented cellular component categories were the nucleus and transcription factor complex. Among the biological processes, DNA binding and transcription regulation were the most prominent. The most abundant molecular functions represented by all predicted target genes were DNA and protein binding (Appendix S4). The expression of all target genes was observed in Fiskeby V plants, whereas the expression of the target gene of miR397 was undetectable in Augusta. Interestingly, a negative correlation between the expression levels of miRNAs and their predicted targets was noticed predominantly in seedling roots. Most notably, miR169 overexpression of 0.46-fold and 0.88-fold corresponded with the underexpression by −2.64-fold and −1.11-fold of its target gene, subunit A of nuclear factor Y (NF-YA), in Augusta and Fiskeby V, respectively (Figure 3). In the case of miR319 in Fiskeby V, we observed a 0.7-fold increase in the amount of miR319 and a −1-fold decrease in the amount of its target gene encoding the GAMYB transcription factor (Figure 3). The expression of the laccase gene in Fiskeby V was highly elevated (by ~2.65-fold) in trifoliates and unifoliates from V1 and R1 stages; however, no negative correlation was found between the expression of miR397 and its target gene. A different relationship was found between miR398 and its target gene copper chaperone for superoxide dismutase (CCS) in Augusta seedling roots, where the expression of the first was decreased by −1.73-fold and that of the later increased by 0.33-fold (Figure 3). Such interplay between the expression of small RNAs and important genes could shed light on the molecular mechanisms of the soya bean cold stress response.

4 | DISCUSSION

Nowadays, climate change is among the principal factors causing a decline in agricultural productivity. Drought, salinity, low/high temperatures, flooding, acidic conditions and nutrient starvation are the world's most dominant abiotic stresses, also affecting soya bean yield (Arora & Tewari, 2016; Carrera & Dardanelli, 2016; Jumrani & Bhatia, 2018; Pasley, Huber, Castellano, & Archontoulis, 2020; Pathan et al., 2014). Low temperatures are one of the most important limitations of crop productivity, as it has been shown that temperature and the duration of chilling stress affect the mode of action and intensity of plant responses (Ercoli, Mariotti, Masoni, & Arduini, 2004). In sorghum, the repercussions of stress exposure range from photosynthesis and N-acquisition inhibition to the total termination of growth (Ercoli et al., 2004).

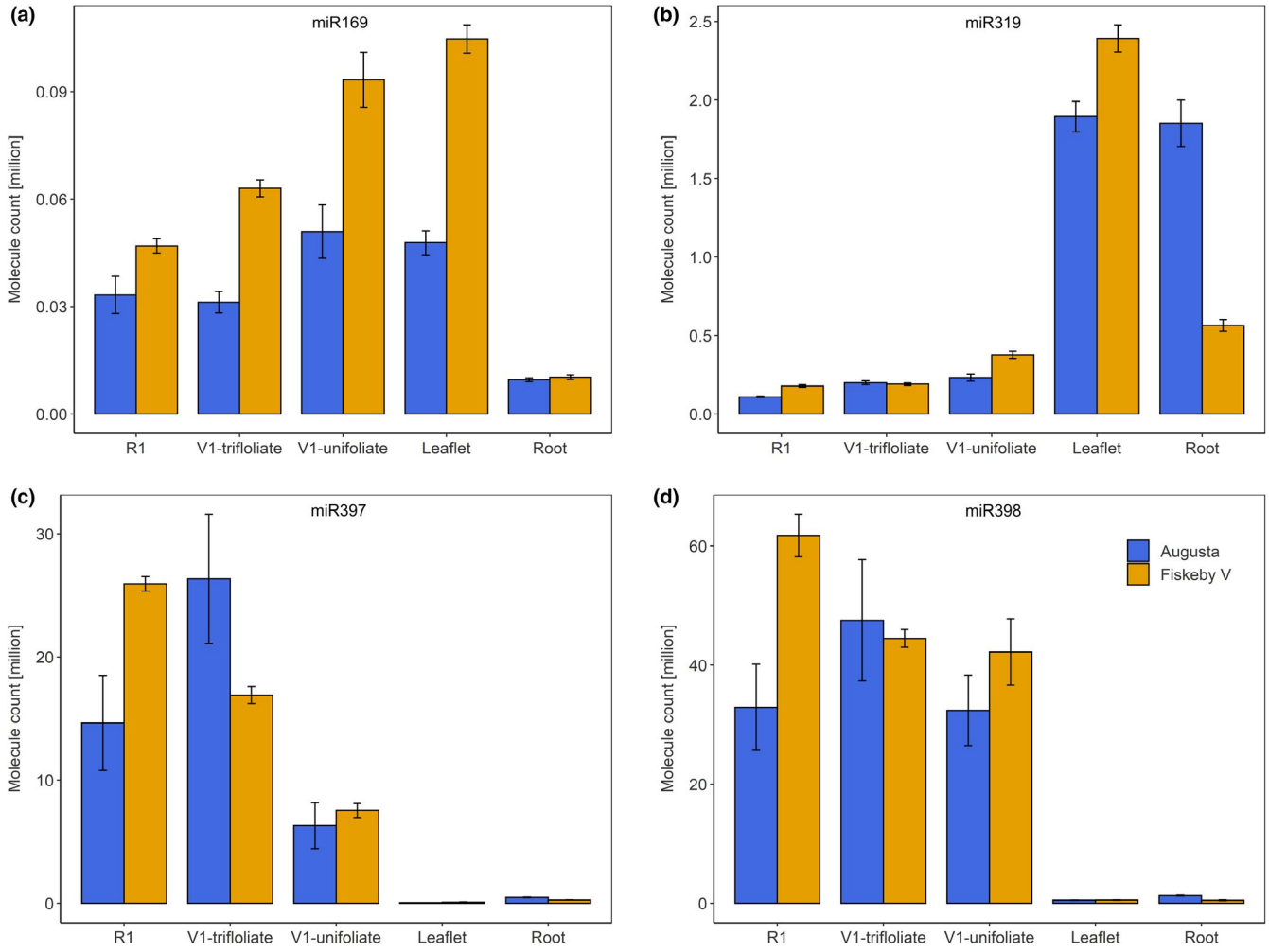


FIGURE 1 ddPCR expression analysis of (a) miR169, (B) miR319, (c) miR397 and (d) miR398 in trifoliates from R1 stage, trifoliates and unifoliates from V1 stage, leaflets and roots from seedlings of Augusta and Fiskeby V soya bean under optimal conditions at different growth stages. Error bars indicate the *SD*. Experiments were performed in triplicate. R1, the beginning of flowering; V1, first trifoliate stage

Although chilling stress treatment altered the growth and development of two cold-resistant soya bean cultivars, Augusta and Fiskeby V, their overall seed output did not suffer significantly. Both cultivars presented different phenotypic responses to chilling in each of the three growth stages (VE, V1, R1) tested. Conversely to Fiskeby V, for Augusta plants subjected to low temperatures during vegetative growth (V1), a decrease in the growth rate (30%) has been observed (Tables 2 and 3). Interestingly, lower flowering efficiency of Augusta plants chilled at the VE stage (by 31%) was not reflected in terms of other parameters, such as the number of seeds per plant, which indicated that to compensate for the unfavourable conditions, the plants produced increased numbers of flowers (Table 2). These data support previous observations that some cultivars are more competent than others in utilizing periods of optimal conditions after chilling to enhance the flowering process (Gass, Schori, Fossati, Soldati, & Stamp, 1996). On the contrary, stressing Augusta at the R1 stage resulted in a reduction in flowering efficiency (by 34%) and that was followed by 20% and 31% decreases in the number of seeds and pods, respectively (Table 2). At the same time, the total yield did not differ significantly from that of the control group, meaning that the increased weight of the average seed rescued the chilling tolerance index of Augusta to an optimal level. Ma et al. (2015) found that *chilling tolerance divergence 1* (*COLD1*) quantitative trait locus (QTL) impacts hormonal pathways essential for proper growth in rice, thereby conferring the plants ability to regain their pre-chilling capacity in terms of seed production. In Fiskeby V chilled at the R1, the flowering efficiency was lowered to a lesser extent (by 17%), but led to a 29% reduction in the number of seeds per plant, while the number of pods per plant increased (by 18%) compared to that in the control, which to some degree compensated the negative side effects of the stress (Table 3). Overall, both cultivars sustained their seed production capability during stress treatment at levels comparable to those of control plants, emphasizing their chilling tolerance. Previously, it has been shown that low temperature stress negatively influenced the shoot, root and physiological parameters of soya bean seedlings (Alsajri et al., 2019), which is in accordance with our results. Interestingly, cold stress that may occur during various stages of life cycle causes more severe damage to soya bean than high temperature stress (George, Bartholomew, & Singleton, 1990; Kurosaki & Yumoto, 2003). It has been shown that heat stress resulted in

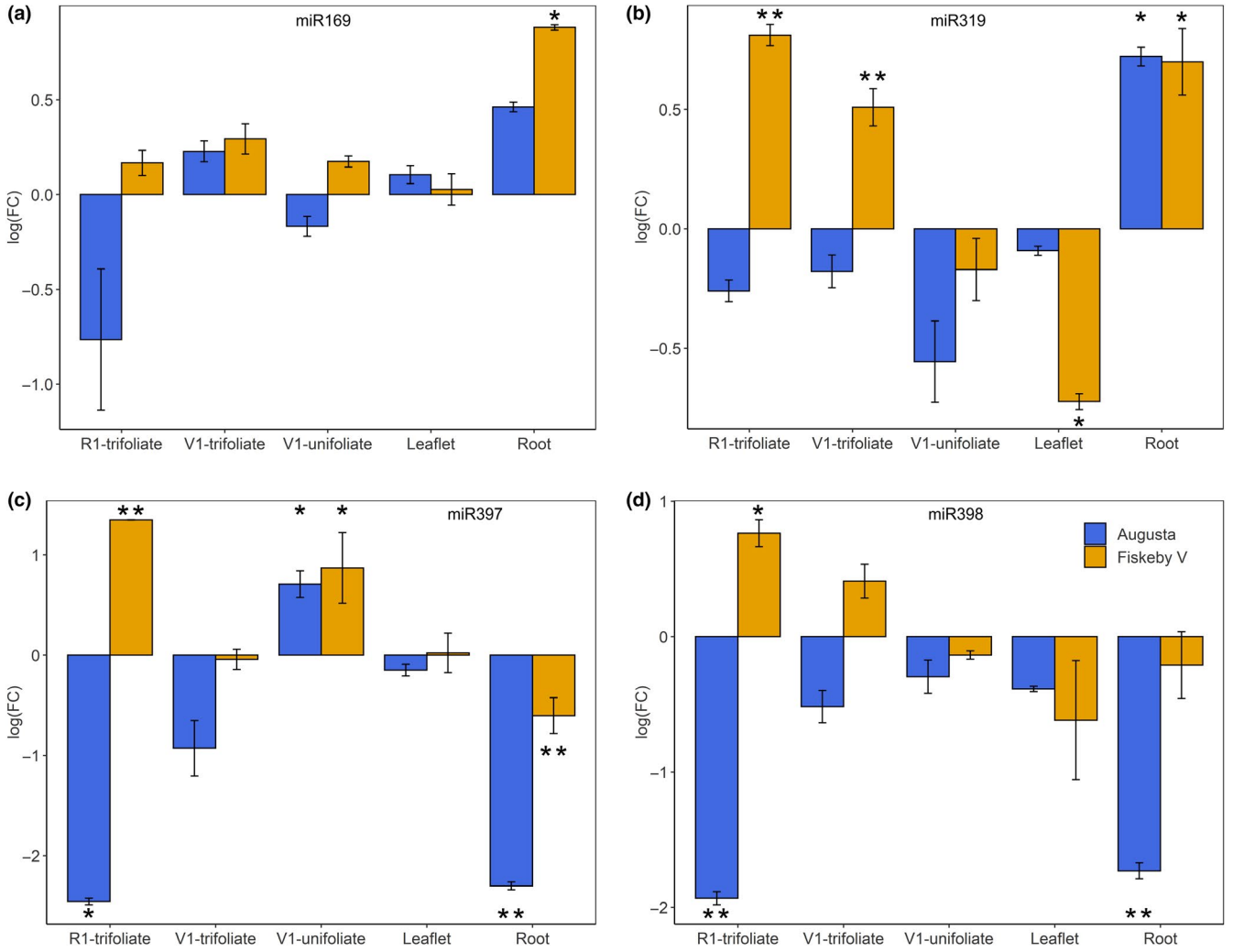
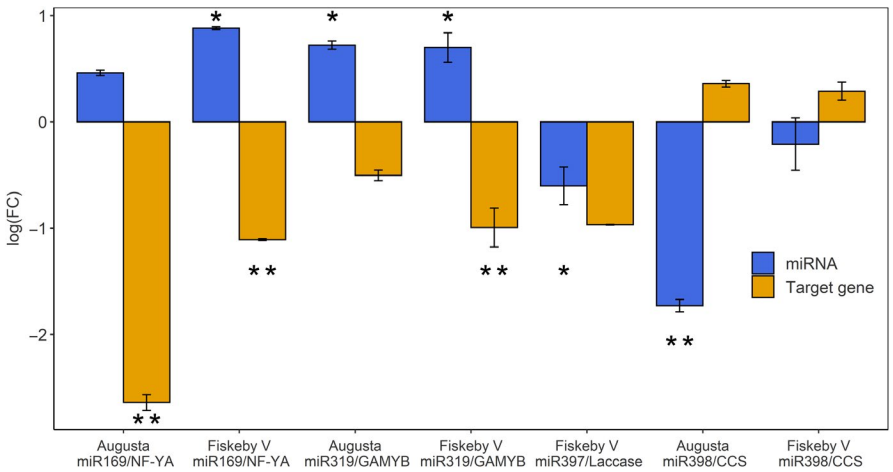


FIGURE 2 Differential expression of (a) miR169, (b) miR319, (c) miR397 and (d) miR398 in trifoliates from R1 stage, trifoliates and unifoliates from V1 stage, leaflets and roots from seedlings of Augusta and Fiskeby V soya bean under chilling stress at different growth stages. Experiments were performed in triplicate. Asterisks indicate significant differences $^{*}p < .05$, $^{**}p < .01$ after Student's *t* test. R1, the beginning of flowering; V1, first trifoliate stage

FIGURE 3 Differential expression of miR169, miR319, miR397, miR398 and their respective target genes (NF-YA, GAMYB, Laccase and CCS) in the seedling roots of Augusta and Fiskeby V cultivars under chilling stress. Experiments were performed in triplicate. Asterisks indicate significant differences between the control and chilling treatment samples $^{*}p < .05$, $^{**}p < .01$ after Student's *t* test



increased flower abscision percentage, decreased pollen viability and pod set percentage in soya bean (Djanaguiraman, Prasad, Boyle, & Schapaugh, 2013). Importantly, as a result of cold stress, the extension of the vegetative growth rate has been observed, as well as increase in the number of axillary branches, the rate of dry weight per plant and pod setting (Skrudlik & Kościelniak, 1996). In Augusta

and Fiskeby V, the chilling tolerance index increased by ~10% as a result of applying cold stress at seedling and vegetative growth stages.

Plants grown in the open field under natural conditions over two vegetation seasons illustrated the impact of Polish climate on the performance of the two chilling-tolerant cultivars. Notably, differences in the growth and development of both cultivars were observed during their adaptation to the environment. In addition to slight fluctuations in temperature, the parameters of the two growing seasons deviated from the average parameters compiled from 1956 to 2009 in terms of rainfall, especially the 2016 season (Tables S1 and S2). The continuous measurements of temperature at Dłoń station indicate the warming tendencies in this region. Nonetheless, extreme weather events occur unexpectedly and may influence the cultivation of soya bean. Despite the weather conditions, Augusta exhibited better characteristics in terms of seed output than Fiskeby V in the field conditions (Table 5).

To reduce the detrimental effects inflicted by environmental stresses, plants utilize various groups of molecules, including miRNAs, as gene regulatory molecules (Sunkar, Li, & Jagadeeswaran, 2012). The identification of altered miRNAs in low temperature-treated plants revealed the critical role of miRNAs in the plant responses to cold stress (An et al., 2014; Chen et al., 2012; Lv et al., 2010; Mantri, Patade, Penna, Ford, & Pang, 2012; Megha, Basu, & Kav, 2018; Sun et al., 2015; Xu et al., 2016). It is therefore of paramount significance to describe the functions of miRNAs in plant adaptation to environmental stresses. This endeavour can be initiated by the identification of miRNAs that are differentially expressed in response to such stresses (López-Galiano et al., 2019). In this study, an attempt was undertaken to characterize the expression patterns of 5 miRNAs and their target genes in 2 chilling-tolerant soya bean cultivars. Four of these were differentially expressed as a result of chilling stress, indicating that miRNAs are important factors in abiotic stress responses in soya bean.

All five chilling-responsive miRNAs tested in this research (miR159, miR169, miR319, miR397, and miR398) are highly conserved in plants and have been associated with cold and other types of stress responses (Sunkar et al., 2012; Tyczewska et al., 2016). As reported in the literature, depending on the plant species, the expression patterns of these miRNAs differed in response to chilling stress. For instance, miR169 was upregulated during cold stress in Arabidopsis, maize and vegetable soya bean (Xu et al., 2014, 2016); conversely, in trifoliate orange and in nodules of soya bean, miR169 was downregulated during cold treatment (Zhang, Wang, et al., 2014; Zhang, Li, et al., 2014). Such findings demonstrate that miRNAs are involved in plant cold responses, but depending on their function, they may be expressed at different levels not only in various plant species but also in different tissues. miR169 has been associated with long-distance signalling, as it was found to be decreased in abundance in phloem sap as a result of nitrogen (N) and phosphorus (P) starvation in Arabidopsis (Pant et al., 2009). Moreover, miR169a has been shown to be involved in the process of N accumulation in Arabidopsis (Zhao, Ding, Zhu, Zhang, & Li, 2011). Interestingly, our results show that the expression of miR169 is tissue-specific in soya bean, it increased (by 39% and 85%) in seedling roots of both Augusta and Fiskeby V stressed at the VE stage, whereas in the leaflets, unifoliates and trifoliates of plants stressed at the VE and V1 stages, its expression was unchanged (Figure 2). Moreover, the expression of miR169 was decreased by 47% in the trifoliates of Augusta plants chilled at R1 stage.

The regulatory roles of miRNAs are manifested via their control of the biosynthesis processes of various proteins encoded by their target genes, which play roles in proliferation, growth and stress response (Voinnet, 2009). These include numerous transcription factors, enzymes and transporters. The bioinformatics prediction suggested a number of candidate target genes for each miRNA that coordinated many biological processes, such as nodulation, anther formation and reactive oxygen species (ROS) scavenging (Appendix S4). In accordance with our analysis, several groups have found that miR169 regulates the expression of the NF-YA transcription factor (Ni, Hu, Jiang, & Zhang, 2013; Sorin et al., 2014; Xu et al., 2014; B. Zhang et al., 2008), which is a part of NF-Y heterotrimeric complex comprising the NF-YA, NF-YB and NF-YC subunits. These subunits facilitate interactions with proteins and DNA, especially with the CCAAT box commonly found in gene promoters (Mantovani, 1999). In plants, NF-Y has been associated with development and responses to abiotic stress (Sorin et al., 2014). Ni et al. (2013) showed that miR169 directed the cleavage of NF-YA3 mRNA and that transgenic Arabidopsis that overexpressed NF-YA3 showed improved water retention and drought tolerance. According to Xu et al. (2014), miR169d-directed cleavage led to lowered NF-YA2 expression, which was reflected in the early-flowering phenotype. It has been found that in soya bean seven mRNAs encoding the NF-YA transcription factor were identified as miR169 targets in seed coats (Shamimuzzaman & Vodkin, 2012) and that miR169 targets the CCAAT-binding transcription factor (Zhang et al., 2008). The analysis of cold-responsive miRNAs in trifoliate orange indicated NF-Y was a putative target gene of miR169 (Zhang, Li, et al., 2014). Our results show a distinct negative correlation between the expression levels of miR169 and NF-YA in seedling roots of both Augusta and Fiskeby V: the overexpression of miR169 (by 39% and 85%) along with the simultaneous downregulation of NF-YA (by 82% and 54%) during chilling stress in Augusta and Fiskeby V, respectively (Figure 3).

miR319 has been associated with the regulation of numerous biological processes, such as gametogenesis, hormone biosynthesis and organ development (Jones-Rhoades & Bartel, 2004; Rhoades et al., 2002; Schwab et al., 2005; Sunkar & Zhu, 2004). In monocots, represented by sugarcane and rice, miR319 has been proven to regulate leaf morphogenesis under cold stress (Thiebaut et al., 2012; Yang et al., 2013). Herein, the low temperature treatments resulted in the upregulation of miR319 (by 61% and 42%) in the seedling roots of Augusta and Fiskeby V stressed at the VE stage, respectively, whereas in the leaflets of Fiskeby V, the expression of miR319 plummeted by 41% (Figure 2). According to the sequencing analysis performed by Zhang, Li, et al. (2014), miR319 was downregulated in the leaves of *Poncirus trifoliata* that was subjected to cold stress. Additionally, a

study conducted on cold-treated soya bean nodules also showed a decrease in miR319 synthesis (Zhang, Wang, et al., 2014).

miR319 has been found to cleave mRNA of TCP transcription factor in plants; however, it is possible that it also targets the GAMYB transcription factor, as predicted based on the sequence complementarity (Palatnik et al., 2007). In Arabidopsis, TCP transcription factor family has been demonstrated to take part in the control of leaf and flower development, branching, mitochondrial biogenesis and the regulation of the circadian clock (Aguilar-Martínez, Poza-Carrión, & Cubas, 2007; Giraud et al., 2010; Gonzalez, Welchen, Attallah, Comelli, & Mufarrege, 2007; Sarvepalli & Nath, 2011). GAMYB is an essential factor involved in gibberellin (GA) signalling in aleurone cells, where it binds to specific promoter sequences in GA-responsive genes (e.g. α-amylase) and it has been shown that GAMYB plays a role in flower development, as it interacts with the gene promoter regions in rice anthers (Tsuji et al., 2006). In sugarcane plants exposed to 48 hr of cold stress, GAMYB gene expression was reduced by 45% compared to that in the control. Moreover, the regulation of this transcription factor was confirmed to be directed by miR319 (Thiebaut et al., 2012). Furthermore, miR319, a cold-responsive miRNA, was predicted to target the MYB transcription factor in trifoliate orange (Zhang, Li, et al., 2014), and the prediction of Zhang, Wang, et al. (2014) identified the TCP and MYB transcription factors as putative targets of miR319a/b. However, in a study of the influence of chilling stress on vegetable soya bean, TCP or MYB transcripts could not be detected (Xu et al., 2016). Our predictions suggested GAMYB as one of the main targets of miR319, along with the TCP transcription factor (Appendix S4). In the roots of Fiskeby V seedlings stressed at the VE stage, the elevation in the expression of miR319 (by 62%) along with a simultaneous decrease in the synthesis of GAMYB (by 76%) has been observed (Figure 3). In the roots of Augusta seedlings, miR319 was at a similar level to that in Fiskeby V (increased by 64%); however, its target gene expression decreased by 30%.

Another member of one of the miRNA families conserved in the plant kingdom is miR397 (Jones-Rhoades & Bartel, 2004). The results of several studies have indicated that the expression levels of C-repeat/dehydration-responsive element binding factor (CBF/DREB) transcription factors as well as those of effector genes such as laccase or multicopper oxidase are influenced by the miR397 family (Dong & Pei, 2014; Jones-Rhoades & Bartel, 2004). According to our findings, cold stressed Augusta and Fiskeby V plants showed similar expression patterns for miR397 in seedlings and unifoliates, where it was downregulated by 80% and 37% in seedling roots and upregulated by 61% and 58% in unifoliates, respectively. Transgenic Arabidopsis plants overexpressing miR397a displayed an improvement in cold stress tolerance (Dong & Pei, 2014). Pan, Zhao, Yu, Bai, & Dong (2017) reported that the overexpression of miR397 corresponded with 8-fold and 3-fold decreased levels of laccase in *Beckmannia syzigachne* and *Oryza sativa*, respectively. Xu et al. (2016) identified miR397b in vegetable soya bean at the V1 stage; however, its expression was not profoundly changed in response to chilling stress. Laccases constitute the bulk of the multicopper oxidase family and have been found in higher plants and fungi. These glycoproteins are predominantly associated with the lignification process; however, their complexity has led to the proposal of additional functions such as flowering or root development (Cai et al., 2006; Dong & Pei, 2014; Hoegger, Kilaru, James, Thacker, & Kües, 2006). miR397 has been shown to target ice1 and laccases in *A. thaliana* and *Medicago truncatula* (Sun, 2012). Three laccase genes have been found to share sequence fragments with miR319 in *A. thaliana* (Dong & Pei, 2014). Another report states that miR397 in lotus, a legume plant, regulates the nodulation process via the modulation of rhizobial infection (Zhang, Wang, et al., 2014). Zhang, Li, et al. (2014) found that miR397 expression was elevated during cold stress in *P. trifoliata* and proposed that the laccase protein family may constitute potential target genes. Herein, the laccase gene was found to be differentially expressed in Fiskeby V plants treated at VE, V1 and R1 stage, with the exception of cotyledons of plants chilled at the VE stage. Nevertheless, a negative correlation of the expression levels of miR397 and its target gene was not observed in any of the analysed tissues.

It has been shown that the accumulation of ROS in plants results from a number of adverse environmental conditions, including temperature stress, drought, high salinity and heavy metals (Zhu, Ding, & Liu, 2011). To counteract the severe consequences of oxidative stress, plants employ ROS scavengers such as Cu/Zn-SODs (CSDs), the expression of which have been reported to be regulated by miR398 (Zhu et al., 2011). It was found that cold stress and wounding results in the downregulation of miR398 in seedlings of *Triticum aestivum* L. (Wang, Sun, Song, et al., 2014). Previously, Sunkar (2006) reported similar findings in *A. thaliana*. However, according to Zhang, Li, et al. (2014), the expression of miR398b in trifoliate orange was substantially increased during cold stress. In trifoliates of R1 stage and seedling roots of stressed Augusta plants, the expression of miR398 was decreased (by 73% and by 70%, respectively; Figure 2d). Interestingly, in Fiskeby V under chilling stress, miR398 was only slightly downregulated in seedling roots (by 14%) and was upregulated by 70% in the trifoliates of R1 stage plants (Figure 2d).

The accumulation of ROS is a common phenomenon involved in plant responses to various environmental stressors. Superoxide dismutases (SOD) are one of the types of enzymes engaged in the defence against ROS by processing superoxide radicals (Zhu et al., 2011). Copper is essential for the proper functioning of Cu/Zn-SOD; therefore, CCS, a copper chaperone for SOD, plays an indirect role in ROS scavenging (Chu et al., 2005). miR398 has been found to target both the SOD and CCS genes (Beauclair, Yu, & Bouché, 2010). In wheat, miR398 was found to be underexpressed during cold stress as well as after wounding (Wang, Sun, Song, et al., 2014). Accordingly, its putative target gene, SOD, was observed to be upregulated in both cases, suggesting its role in ROS management during these stresses (Wang, Sun, Song, et al., 2014). In Arabidopsis, miR398, in addition to SOD, regulated the expression of two Cu/Zn-SOD genes and one CCS gene (Zhu et al., 2011). Additionally, Sunkar (2006) proposed SOD as a potential target gene for miR398 in soya bean. Based on the target prediction results and a literature review, we chose CCS as a

potential associate of miR398 in soya bean during responses to chilling stress. Here, we found miR398 to be downregulated under chilling stress at every growth stage of Augusta. Conversely, upon chilling, miR398 was upregulated in Fiskeby V in trifoliates from the V1 and R1 groups. At the same time, the levels of CCS were elevated in the roots of both cultivars (Figure 3). These findings corroborate the role of miR398 in ROS scavenging during adverse conditions.

5 | CONCLUSIONS

In summary, this study showed that the growth and development of two cold-resistant soya bean varieties was affected in response to chilling stress. Augusta and Fiskeby V were most susceptible to chilling stress at the R1 stage, during which the majority of reproductive parameters were altered, including the chilling tolerance index in Fiskeby V. On the other hand, Augusta exhibited the lowest seed output when it was chilled at the VE stage. Nonetheless, Augusta proved to be better suited for Polish growing conditions due to its favourable performance during the field experiment. We described the expression profiles of several miRNAs involved in the chilling stress response. The differential expression of selected miRNAs in response to low temperature stress was detected primarily in roots and trifoliates of plants treated at the VE and R1 stages, respectively. Moreover, the genes that may have been targeted by the analysed miRNAs were identified and studied. The analysis of the miRNA and target gene expression profiles further elucidated the differences between the two varieties and confirmed that miRNAs may play a role in soya bean chilling stress responses. Additionally, the dissimilarities between the cultivars at the molecular level may explain the distinct phenotypes exhibited by Augusta and Fiskeby V.

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AUTHOR CONTRIBUTIONS

A.T., J.G.B., J.N. and T.T. provided ideas and designed the research; J.K., A.T., J.G.B. and J.N. performed the experiments and analysed the data; J.K. wrote the manuscript; A.T., J.N. and T.T. edited the manuscript; all authors read and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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ORIGINAL ARTICLE

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Cold stress-induced miRNA and degradome changes in four soybean varieties differing in chilling resistance

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Abstract

Chilling stress is one of the most important factors limiting soybean yield in the temperate climate. It significantly constraints the spatial distribution and agricultural productivity of plants, thereby affecting their growth and development. In this study, to determine the involvement of microRNAs (miRNAs) and their target genes in the chilling resistance of four soybean cultivars (Augusta, Fiskeby V, Toyomusume and *Glycine soja*) with varying stress susceptibility, 72 small RNA libraries and 24 degradome libraries for high-throughput sequencing were constructed. A total of 321 known miRNAs were identified, and 348 novel miRNAs were predicted in three analysed tissues. Moreover, under stress conditions, the differential expression of 162 known miRNAs, including well-conserved, legume- and soybean-specific miRNAs and 18 novel miRNAs, was found in the four tested cultivars. Degradome analysis allowed to assign the differentially expressed miRNAs to their potential target genes. They were found to be related to plant abiotic stress response mechanisms such as reactive oxygen species scavenging, flavonoid biosynthesis and regulation of osmotic potential based on GO and KEGG annotations. The findings of this study constitute a valuable insight into the function of miRNAs in the chilling resistance of soybean and may provide crucial knowledge in the development of new cultivars.

KEYWORDS
chilling, cold stress, degradome, plant stress responses, small RNAs, soybean

1 | INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] is one of the most important oilseed plants cultivated worldwide, with a production of 347 million metric tonnes in 2017 (https://www.soymeal.org/soy-meal-articles/world-soybean-production/). It is an exceptional source of oils and proteins used in human nutrition and in animal feed formulation. Additionally, soybean-derived oils are used in the production of biofuels (Wang & Komatsu, 2018). Symbiosis between soybean and *Bradyrhizobium japonicum* results in the formation of root nodules (Egamberdieva et al., 2018). The beneficial role of binding of atmospheric nitrogen is a distinctive aspect of soybean cultivation that considerably improves soil composition and helps to reduce the use of fertilizers.

An important issue of soybean cultivation in high latitudes is the lack of tolerance to low temperatures, which affects the nodulation

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process as well as the general growth and yield quality of the plants (Zhang et al., 2014). It has been predicted that in the future, due to climate change, the frosts will become more severe, especially upon seed set (Redden, 2021). Soybean requires relatively high temperatures for germination, growth, development and maturation, and the suitable temperature for soybean is 15–22°C at emergence, 20–25°C at flowering and 15–22°C at maturity (Liu et al., 2018). In temperate climatic conditions, soybeans may be exposed to chilling stress mainly during two periods. The first period is the emergence and early vegetative stages of plant development (V1–V3; Nleya et al., 2019), that is, from the last 10 days of April to mid of May. It has been suggested that the first hours of imbibition are crucial and that low-temperature–caused injuries are expressed as both reduced emergence of seedlings and reduced vigour and yield of surviving plants (Bedi & Basra, 1993; Skrudlik & Kościelniak, 1996). This issue is of major agricultural importance because germination in cold soil can markedly reduce productivity. The second most sensitive to chilling period is during the flowering phase because pod formation is a process critical for legume crop productivity. Temperatures of 15°C in day and between 15 and 9°C in night are biological minimums at this growth stage (Hume & Jackson, 1981a). The sensitivity of soybean to night temperatures below 15°C is reflected in changes occurring in metabolism, growth, development and yield (Alsajri et al., 2019; van Heerden et al., 2003; Kurosaki et al., 2003; Tyczewska et al., 2016). Exposure to low temperatures causes the accumulation of osmoprotectants such as proline and sucrose as well as reorganization of the membrane structure (Ahmad & Majeti, 2012; Michaelson et al., 2016). Commonly, under chilling conditions, the expression levels of stress-responsive genes, including many transcription factors and enzymes, are affected (Baillo et al., 2019). Moreover, a single night of cold, with minimum temperatures of 8°C, is sufficient to inhibit pod formation (Hume & Jackson, 1981b). Thus, under natural growth conditions, soybean yield is resilient to cold temperatures that fall to as low as 15°C. However, temperatures below this level pose a significant risk for reducing yield, especially when they fall to 10°C.

One of the mechanisms that regulate gene expression in plants relies on microRNAs (miRNAs). These are small noncoding RNAs that interact with genes involved in the regulation of plant growth, development and response to abiotic and biotic stresses (Hume & Jackson, 1981a). These interactions comprise mRNA cleavage based on the binding to complementary sequences within its target genes as well as translation inhibition by restriction of the ribosome binding process (Bartel, 2004). To date, a number of miRNAs in soybean has been reported to be associated with response to abiotic and biotic stresses such as drought, salinity (Liu et al., 2016), phosphate starvation (Xu et al., 2013), cold (Kuczyński et al., 2020) and cyst nematode infection (Li et al., 2012). However, the involvement of miRNAs in the chilling stress response in soybean is poorly explained and needs further investigation.

In this study, we compared the responses of four soybean cultivars differing in cold stress tolerance (Augusta, Fiskeby V, Toyomusume and *Glycine soja*) to chilling stress at the molecular level, with a focus on miRNAs and their target genes. The assessment of cold stress effects in three tissues [shoots (trifoliates), seedling roots and cotyledons] facilitated the comprehensive investigation of stress response in soybean early growth stages. The sequencing of small RNAs isolated from plants cultivated under control and stress conditions allowed to characterize miRNAs involved in the chilling stress responses. Additionally, the sequencing of degradome enabled to identify potential target genes of differentially expressed miRNAs in soybean. This study aimed to determine the responses of the above-mentioned four soybean cultivars to chilling stress at the molecular level and to elucidate the role of specific miRNAs in soybean tolerance system. Our results provide insights into cold stress response mechanisms and the regulatory roles of miRNAs in cultivated and wild soybean cultivars.

2 | MATERIALS AND METHODS

2.1 | Plant material

Four soybean varieties were chosen for the experiment: Augusta, Fiskeby V, Toyomusume and *G. soja*. Fiskeby V was bred by Dr. Sven A. Holmberg in Sweden, near the city of Norrkoping (58°30′N). Augusta was selected from two crosses: in the first step, a cross was made between Fiskeby V and line PI 194643, and line 104 was obtained; in the second step, line 104 was crossed with line 11 (*G. soja* wild species). Line 11 of *G. soja* grows in the natural environment of far Eastern Russia at latitudes similar to those of Poland and has a long-day–tolerant genotype. Therefore, Augusta has two sources of photoperiod insensitivity, and its chilling tolerance is derived from Fiskeby V. The seeds of the Augusta and Fiskeby V soybean cultivars were provided by Prof. J. Nawracała from the Poznan University of Life Sciences, Poland. The chilling tolerance of the Swedish cultivar Fiskeby V is presumed to be derived from the Sakhalin landrace Namikawa. *Glycine soja* is a wild soybean annual species that is native to China, Japan, Russia and parts of Korea and is a wild progenitor of the cultivated species *G. max*. *Glycine soja* accession PI 538411A was collected over Amur River (Far East of Russia) on latitude: 52°58′39″N and longitude: 127°21′44″E. Toyomusume was chosen as a chilling-sensitive genotype. It is a Japanese variety from Hokkaido Island, where it is cultivated mainly for tofu production.

TABLE 1 Scheme of the chilling stress treatment

Stage of soybean growth	Optimal growth temperature (°C)	Stress temperature (°C)	Duration of stress conditions (h)
VE – seedlings	20	4	48
V1 – vegetative	20	8	120

Prior to sowing, the soybean seeds were inoculated with *B. japonicum* (HiStick® Soy, BASF) to induce nodule formation. The soybean varieties Augusta, Fiskeby V, Toyomusume and *G. soja* were planted in pots filled with a mixture of all-purpose potting soil and sand in the ratio of 3:1. Plants were grown under controlled environmental conditions in a phytotron at 20°C with a relative humidity of 60% and a 16:8-h light: dark photoperiod prior to stressing treatment.

The plants were divided into two groups, with each group subjected to chilling treatment at a different developmental stage (Table 1). The first batch of plants was stressed at the VE stage (emerging seedlings) by keeping them at 4°C for 48 h in Percival chambers. The next batch of plants was exposed to 8°C for 120 h (5 days) at the V1 growth stage (first trifoliate). In the control and treated groups, 20 to 30 soybean plants were cultivated.

2.2 | sRNA library construction and sequencing

Total RNA was isolated from the trifoliates, seedling roots and shoots of the four soybean genotypes by using Direct-zol™ RNA Miniprep Plus (Zymo Research) according to the manufacturer's instructions. The quantity and purity of the total RNA were checked using NanoDrop ND-1000 (NanoDrop) and Agilent 2100 with a minimum RNA integrity number (RIN) threshold value of >8.0. Approximately 5–10 μg of total RNA was used for the sRNA library construction according to the protocol of TruSeq Small RNA Sample Prep Kits (Illumina). A total of 72 soybean sRNA libraries were constructed, from both chilled and control seedling roots and cotyledons as well as V1 stage shoots, all performed in triplicate. The libraries were sequenced on an Illumina Hiseq4000 at BGI Tech Solutions Co., Ltd.

2.3 | Bioinformatics analysis of sequencing data

The sequencing data were filtered to contain only fragments of at least 18 nt in size. The sequences were then further processed to contain at least five counts for each variety/tissue combination. The raw counts were subsequently analysed using edgeR Bioconductor package in R following the developer's instructions (Robinson et al., 2009).

The known miRNA sequences were identified using BLAST against all miRBase mature sequences downloaded in March 2019. Only sequences identical to the reference miRNAs were retained for further analyses. Novel miRNAs were searched using the miRDeepP2 program (Kuang et al., 2018) with default options and using *G. max* reference genome version 2.1 downloaded from ENSEMBL in May 2019. Only sequences that did not map to the reference miRBase dataset were retained for further analyses.

2.4 | ddPCR analysis

The profiles of four differentially expressed miRNAs were assayed by ddPCR. A set amount of extracted RNA (1 μg sRNA and 1.5 μg of total RNA) was reverse transcribed using SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific) as described in Varkonyi-Gasic et al. (2007). Stem-loop primers were designed for the miRNA reverse transcription reactions. The RT-PCR primers are listed in Table S1. To quantify the number of miRNA molecules in the plant samples, a ddPCR mixture containing 10 μl of ddPCR SuperMix Eva Green (BioRad), primers (the final concentration of each primer was 200 nM), template (reverse-transcribed, elongated miRNA) and RNase-free H_2O was used. A 20 μl reaction mixture was used to generate the droplets in an eight-well cartridge by using a QX100 droplet generator (Bio-Rad). The droplets were carefully transferred to a 96-well ddPCR plate and heat-sealed with a foil (Bio-Rad). The cDNA was then amplified in a T100 PCR thermal cycler (Bio-Rad) under the following cycling conditions: 5 min of denaturation at 95°C, followed by 40 cycles with a three-step thermal profile of 30 s of denaturation at 95°C, 30 s of annealing at 55°C and 45 s of extension at 72°C. Subsequently, the products were maintained at 72°C for 2 min for final extension. After amplification, the products were cooled to 4°C for 5 min, heated to 90°C for 5 min and finally cooled again to 12°C. The droplets were quantified in a QX100 droplet reader (Bio-Rad). Data acquisition and analysis were performed using QuantaSoft software (Bio-Rad). The positive droplets containing the amplification products were differentiated from the negative droplets by setting the fluorescence amplitude threshold to the lowest value of the positive droplet cluster. Yeast tRNA-Thr(TGT) molecules with a scrambled sequence were added to each RT reaction mixture as an internal control.

2.5 | Degradome sequencing

The quantity and purity of the total RNA were checked using NanoDrop ND-1000 (NanoDrop) and Agilent 2100 with a minimum RIN threshold value of >7.0. Approximately 20 μg of total RNA was used to prepare 24 degradome libraries, with three biological replicates for both stress and control conditions of the four cultivars. The mRNA was mixed with biotinylated random primers, and the RNA containing the biotinylated random primers was then captured by beads and ligated to adaptors, according to the BGI Tech Solutions Co., Ltd., procedure. Following purification, digestion, ligation and repurification, the cDNA library was sequenced (single end, 36 bp) with an Illumina Hiseq4000 at BGI Tech Solutions Co., Ltd. Clean reads of the degradome sequencing were obtained by removing low-quality reads and the adapter contaminants. The clean reads of the degradome data were then mapped to the soybean (*G. max*) genome. CleaveLand pipeline 4.3 (https://sites.psu.edu/axtell/software/cleaveland4/) was used to confirm the potential miRNA: target gene pairs with default parameters.

The putative targets for the selected sRNA reads were identified using CleaveLand4-4.5 (Addo-Quaye et al., 2009) program according to the developer's instructions. Prior to the analysis, the degradome data were adapter and quality trimmed using the cutadapt tool (Martin, 2011). mRNA sequences downloaded from the NCBI

RefSeq database in June 2019 were used as a reference for miRNA target detection.

2.6 | Gene expression analysis using real-time PCR

Total RNA was isolated from frozen powdered samples (100 mg) using NucleoSpin RNA Plant (Macherey-Nagel) following the manufacturer's protocol. Total RNA (2 μg) was used for cDNA synthesis with the Transcriptor First-Strand cDNA Synthesis Kit (Roche) using oligo (dT) primers according to the manufacturer's instructions. For real-time PCR analysis, the cDNA template was diluted two times. Real-time PCR analysis was performed to determine the expression levels of the *Glycine max* putative phytocyanin (Phyt), *Glycine max* transcriptional factor NAC-19 (NAC-19) and *Glycine max* malate dehydrogenase [NADP] (GmMDH), and each cDNA sample was analysed using Mono Color Hydrolysis UPL Probes (Roche) selected for each gene by using ProbeFinder Software (Roche). The PCR reaction mixtures were prepared according to the manufacturer's protocol. PCR conditions were as follows: initial incubation step at 94°C for 10 min, followed by 45 cycles of amplification (15 s at 94°C, 30 s at 60°C and 15 s at 72°C; single acquisition), with a final cooling step at 40°C for 2 min. The analysis was performed using a LightCycler 480 II instrument (Roche). Relative gene expression was calculated using the Roche Applied Science E-Method and normalized to the three reference genes (*Glycine max* actin-6 (ACT-6), SKP1/Ask-Interacting Protein 16 (SKIP16) and Eukaryotic elongation factor 1 β (ELF1B; Hu et al., 2009). All standard curves were generated by amplifying a series of twofold dilutions of cDNA. The primer sequences for the analysed genes and UPL probes are given in Table 2.

2.7 | GO analysis

GO functional classification was performed using Blast2GO (https://www.blast2go.com/) with the default settings. The sequences were blasted using NCBI BLAST service (QBlast) and a blastx-fast program. Blast expectation value (*E*-value) was set at 1.0E−3. Next, the GO ontology mapping and InterProScan were performed. GO mapping was performed against extensively curated gene ontology annotated proteins to obtain functional labels. The used data originate from the Gene Ontology Association and Uniprot ID-Mapping. The public EMBL-EBI InterPro web service was used to scan sequences against InterPro's signatures with the default settings. Go annotation was performed with the annotation cut-off set at 55 and *E*-value hit filter set at 1.0E−6. Enrichment analysis was performed using Fisher's exact test.

3 | RESULTS

3.1 | Analysis of small RNA sequencing libraries

To identify the miRNAs involved in the chilling stress response, 72 sRNA libraries were constructed for cold-stressed samples, and their corresponding controls that were sequenced by Illumina Technology. The number of raw sequencing reads per sample ranged from 20.2 to 43 million (Table 3).

After removal of the adapter sequences and low-quality reads, the total read count ranging from 16.6 to 35.9 million and the unique read count ranging from 240 to 525 thousands were obtained from these 72 libraries (Table 3). Quantification of miRNAs between the cultivars and treatments was preceded by the normalization of expression levels of the miRNA families to counts per million (cpm). The normalized miRNA families read frequencies ranged from 1 to 552,267 cpm for the trifoliates of V1 stage, from 1 to 134,679 cpm for the root samples and from 1 to 165,310 cpm for the cotyledon samples (Table 3). Size distributions based on the filtered reads were assessed and are shown in Figure 1. The majority of mapped sequences were found to be 21 nt long (18.1%), followed by sequences that were 20 and 22 nt long (12.7% and 12.3% respectively).

3.2 | Identification of known and novel miRNAs in soybean

The filtered reads from 72 sRNA libraries, from both chilled and control seedling roots and cotyledons as well as V1 stage shoots, all

TABLE 2 List of primers and UPL probes used for real-time PCR analysis

Gene shortcut	Full gene name	Forward primer (5′–3′)	Reverse primer (5′–3′)	UPL no.
ACT-6 [a]	***Glycine max* actin-6**	CAGGAATGGTTAAGGCTGGT	CGAGGACGACCTACAATGCT	67
SKIP16 [a]	**SKP1/Ask-Interacting Protein 16**	GGGATGGGATGGGATAGAAT	TCCCAAATAATGAAATTGAGACTTC	31
ELF1B [a]	**Eukaryotic elongation factor 1 β**	AGTTTTGTTTTTCTTTATTTGAATTGC	CAGCGCCACTGAATCTTACC	72
Phyt	*Glycine max* putative phytocyanin	GCACCACAGGCAATTCACTA	GGCAGTCTTGAGGGTGATTG	59
NAC20	*Glycine max* transcriptional factor NAC-19	CGAAATGGAAGACGTGAAGC	TCGGAAGCCTCGAAGTACAG	70
GmMDH	*Glycine max* malate dehydrogenase [NADP]	AAGATGCAGAATGGGCTTTG	GCCCATTTATGTCTAACAAGTCTG	85

[a]Reference genes are marked in bold.

TABLE 3 Statistics of soybean sRNA sequencing libraries

Sample	Total raw reads	Unique raw reads	Total filtered reads	Unique filtered reads
ARC	31748684	4672798	25733227	348281
ARS	27488230	4042803	22482467	340208,7
FRC	35274590	5755019	27375919	421643,3
FRS	33272581	4611872	27384545	402207,3
GRC	31371582	4520144	25182469	311193
GRS	32342809	3934417	27081801	294042,7
TRC	34799529	5073827	27851481	408338,3
TRS	32134038	4358886	26684040	394515,3
ACC	34831334	3881144	28492562	458136,7
ACS	32385665	3644302	26485865	438347,7
FCC	25825875	2208209	22059012	289776,7
FCS	26971906	2182955	23513888	277404,7
GCC	32915940	4407454	26261721	392516
GCS	31400723	3561122	26232399	362572,3
TCC	30884146	2790102	25515349	348449,3
TCS	34427857	3170062	29265725	369728,3
ASC	33943160	3816121	27873908	456142,3
ASS	31835213	3749304	26172147	453468,7
FSC	31749968	3875722	25430403	424815,3
FSS	32928969	2868753	28472060	382114
GSC	36552754	4686997	29270721	423320
GSS	30993728	4177839	24756494	413838,3
TSC	31087038	3448669	25297087	420317
TSS	36333521	3269836	29412734	406057,7

[a]First letter of the sample name designates cultivar: A – Augusta, F – Fiskeby V, G – *Glycine soja*, T – Toyomusume; second letter designates tissue: R – root, C – cotyledon, S – shoot; third letter designates conditions: C – control, S – stress.

performed in triplicate, were compared with the collection of mature (and precursor) soybean miRNAs from miRBase release 22.1. A total of 321 known soybean miRNA families were identified in all four cultivars (Table S2). In cotyledons of soybean at the VE stage, the most abundant miRNA family was miR159, except for Fiskeby V in which miR165 was the most highly expressed miRNA. In roots (radicles) of all plants collected at the seedling (VE) stage, the most extensively expressed miRNA family was miR319. In trifoliates of soybean collected at the V1 stage, the most abundant miRNA family was miR398. Additionally, among the highly expressed miRNA families in all the studied samples, there were mostly conserved miRNAs such as miR159, miR165, miR166, miR167, miR319, miR396, miR398, miR408 and miR482 and legume-specific miRNAs such as miR4414, miR1510 and miR3522. On the other hand, among the least expressed miRNA families, the ratio of conserved miRNAs to legume-specific miRNAs was substantially lower than that in highly expressed fraction. Most of the conserved miRNAs among the least abundant miRNA families were transcriptional variants of their highly expressed counterparts.

Additionally, 348 novel miRNAs were found in the four studied cultivars (213 miRNAs in Augusta, 220 in Fiskeby V, 224 in Toyomusume and 218 in *G. soja*) (Table S3), among which 129 miRNAs were common for all four cultivars.

3.3 | Differential expression of miRNAs in soybean

To identify the miRNAs that exhibit differential expression patterns under chilling stress, we used fold-change values of 1 and −1 and *p*-value of .05 as the threshold. A total of 162 differentially expressed miRNAs belonging to 137 miRNA families were identified between chilled and control samples of the four soybean cultivars. The distribution of differentially expressed miRNAs in shoots, cotyledons and roots of all studied varieties is given in Figure 2.

Among these differentially expressed miRNAs in all the tested cultivars, 93 were downregulated in chilled samples as compared to those in controls in at least one cultivar, 28 of which were downregulated by at least fivefold. MA plots of differentially expressed miRNAs in four studied cultivars are given in Figure 3.

On the other hand, 137 miRNAs were upregulated in samples from stressed plants compared to those in controls in at least one cultivar, 41 of which were upregulated by at least fivefold (Figure 3). The most prominent reduction in expression during chilling stress was noted for miR169, miR166, miR159 and miR5037 in cotyledons (VE stage); for miR159, miR160, and miR171 in seedling roots (VE stage) and for miR167, miR2111, miR5371, miR5037, miR398, miR4416 and miR160 in trifoliates from V1 stage. Conversely, the most remarkable increase in the expression levels was observed for miR6300, miR5368, miR6173 and miR1509 in cotyledons (VE stage); for miR319, miR9750, miR408 and miR2109 in seedling roots (VE stage) and for miR166, miR398, miR2119, miR399, miR4996, miR171, miR6300, miR5368, and miR169 in the trifoliates from V1 stage among the four cultivars. Several miRNA families, including miR159, miR319 and miR482, were differentially expressed universally in seedling roots, with miR159 and miR482 being downregulated in all the four cultivars, whereas miR319 was downregulated in Augusta and *G. soja* and upregulated in Fiskeby V and Toyomusume. Similarly, in the trifoliates of plants stressed at V1 stage, there were miRNAs differentially expressed in all the studied soybean cultivars, including miR10197, miR1507, miR1509, miR159, miR166, miR2111, miR3522, miR396, miR398, miR408 and miR4996, where miR10197 and miR2111 were downregulated, miR1509 and miR396 were upregulated in all cultivars, while miR1507, miR159, miR166, miR3522, miR398, miR408 and miR4996 were downregulated in Augusta and *G. soja* and upregulated in Fiskeby V and Toyomusume (Figure 4).

Regarding the differences in expressional patterns between the four cultivars, several notable miRNAs showed contrasting expression in the chilling-sensitive cultivar Toyomusume and the

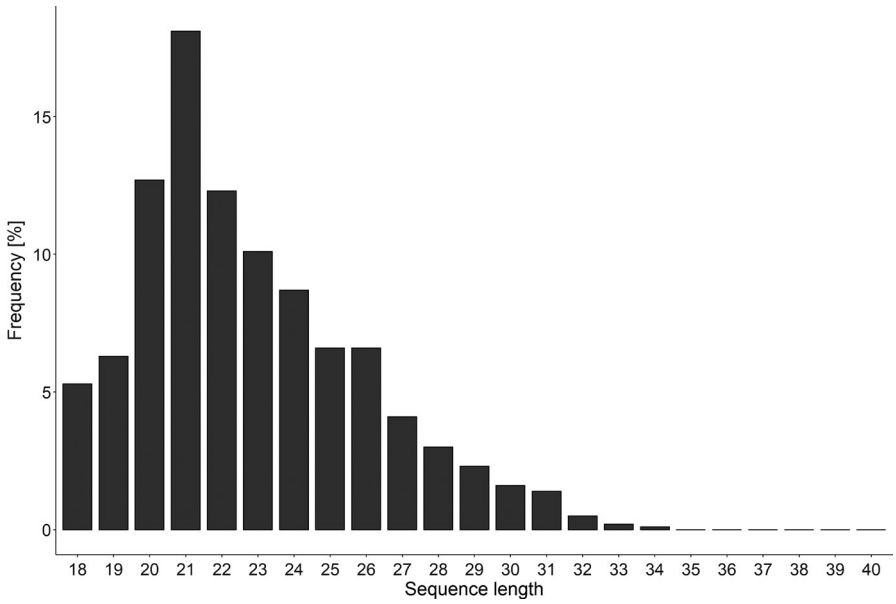

FIGURE 1 Sequence length distribution of miRNAs in sRNA libraries of soybean

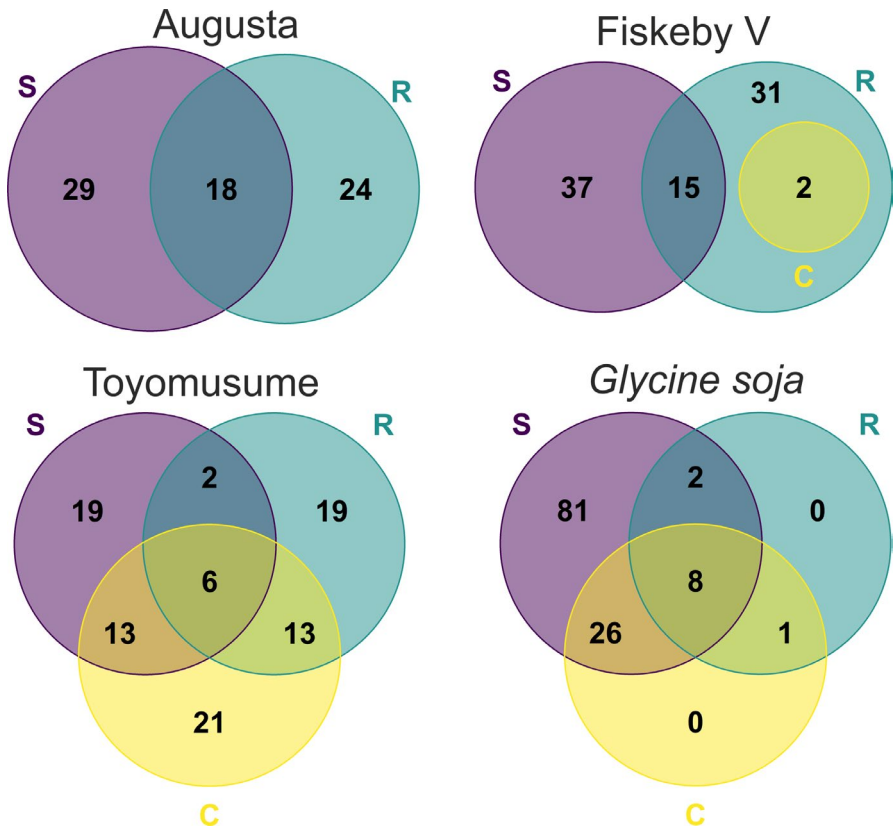

FIGURE 2 Quantitative distribution of differentially expressed miRNAs in shoots, cotyledons and roots of the studied cultivars; S – shoots, R – roots, C – cotyledons

chilling-tolerant cultivars Augusta, Fiskeby V and *G. soja*. In the seedling roots of Augusta, Fiskeby V and *G. soja*, miR169 and miR5770 were found to be downregulated by 2.3-, 3.3- and 2.4-fold and by 2.9-, 1.7- and 2.1-fold, respectively, while their expression was not significantly altered in Toyomusume, thus suggesting their involvement in the chilling stress response (Figure 5). Similar patterns were found in trifoliates (V1 stage) for miR156 and miR5770, where they were downregulated by 1.7-, 3.9- and 1.9-fold and by 2.1-, 3.6- and 2.3-fold in Augusta, Fiskeby V and *G. soja*, respectively, while their expression was again not altered significantly in Toyomusume. Interestingly, there were seven miRNAs (miR1507, miR159, miR166, miR 3522, miR398, miR408 and miR4996) with common expression trend of upregulation in Toyomusume and Fiskeby V, while being downregulated in Augusta and *G. soja*.

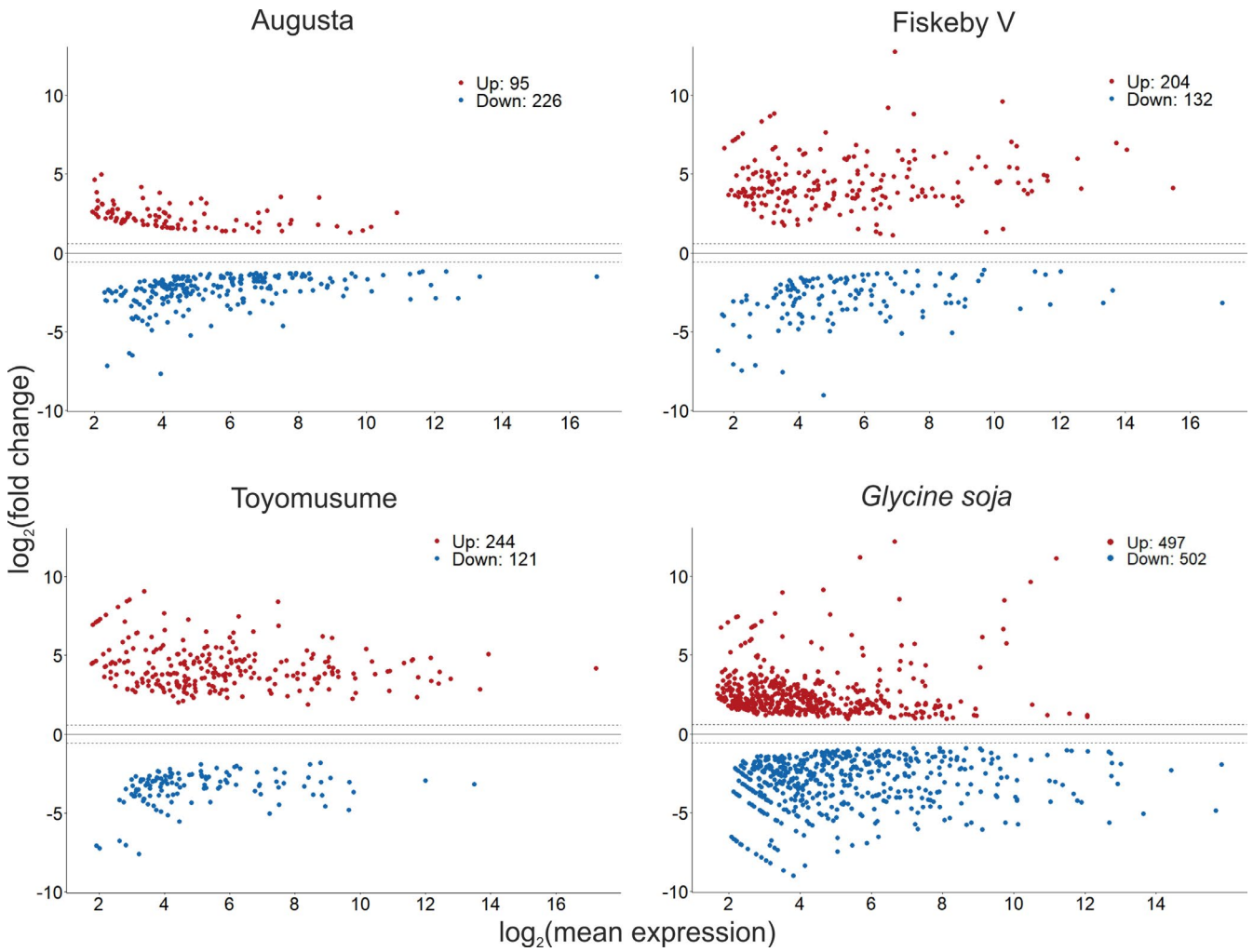

FIGURE 3 MA plots of differentially expressed miRNAs in four studied cultivars; red dots – upregulated, blue dots – downregulated

Additionally, 18 novel miRNAs were differentially expressed between stressed and control samples. Among them, four miRNAs were found in more than one sample. Two of them were common for cotyledons and trifoliates of *G. soja*, one was shared by trifoliates of *G. soja* and Augusta and one by trifoliates of *G. soja* and Fiskeby V.

3.4 | Validation of miRNA expression by ddPCR

To verify the reliability of sequencing results, the expression levels of four miRNAs (miR169, miR408, miR2109 and miR5770) responsive to chilling stress were evaluated by ddPCR (Figure 6). The ddPCR results showed that the expression patterns of four selected miRNAs were mostly in accordance with the assessment of sRNA sequencing (except for miR169 in Augusta and Fiskeby V, and miR408 in Toyomusume). The discrepancies in the fold change of particular samples can be attributed to the differences in the sensitivity and specificity of the two techniques. Moreover, in ddPCR, it is difficult to differentiate between the particular miRNAs belonging to one miRNA family, which may further explain some differences observed between sequencing and ddPCR results.

3.5 | miRNA target profiling by degradome sequencing

On the basis of the degradome sequencing and subsequent CleaveLand program analysis, we identified potential targets of soybean miRNAs that are involved in the chilling stress responses. In total, 2005 targets were identified in all 24 libraries, among which 1183 belonged to Category 0, 222 to Category 1, 293 to Category 2, 164 to Category 3 and 143 to Category 4, where Category 0 represented the best match between miRNA and target genes (Table S4). Further investigation revealed potential targets of differentially expressed miRNAs (Table 4). Examples of miRNAs alignments and their T-plots validated by degradome sequencing are given in Figure S1.

In seedling roots, miR159 targeted cytochrome P450 and pyruvate dehydrogenase (E1 component subunit alpha-3), while miR319

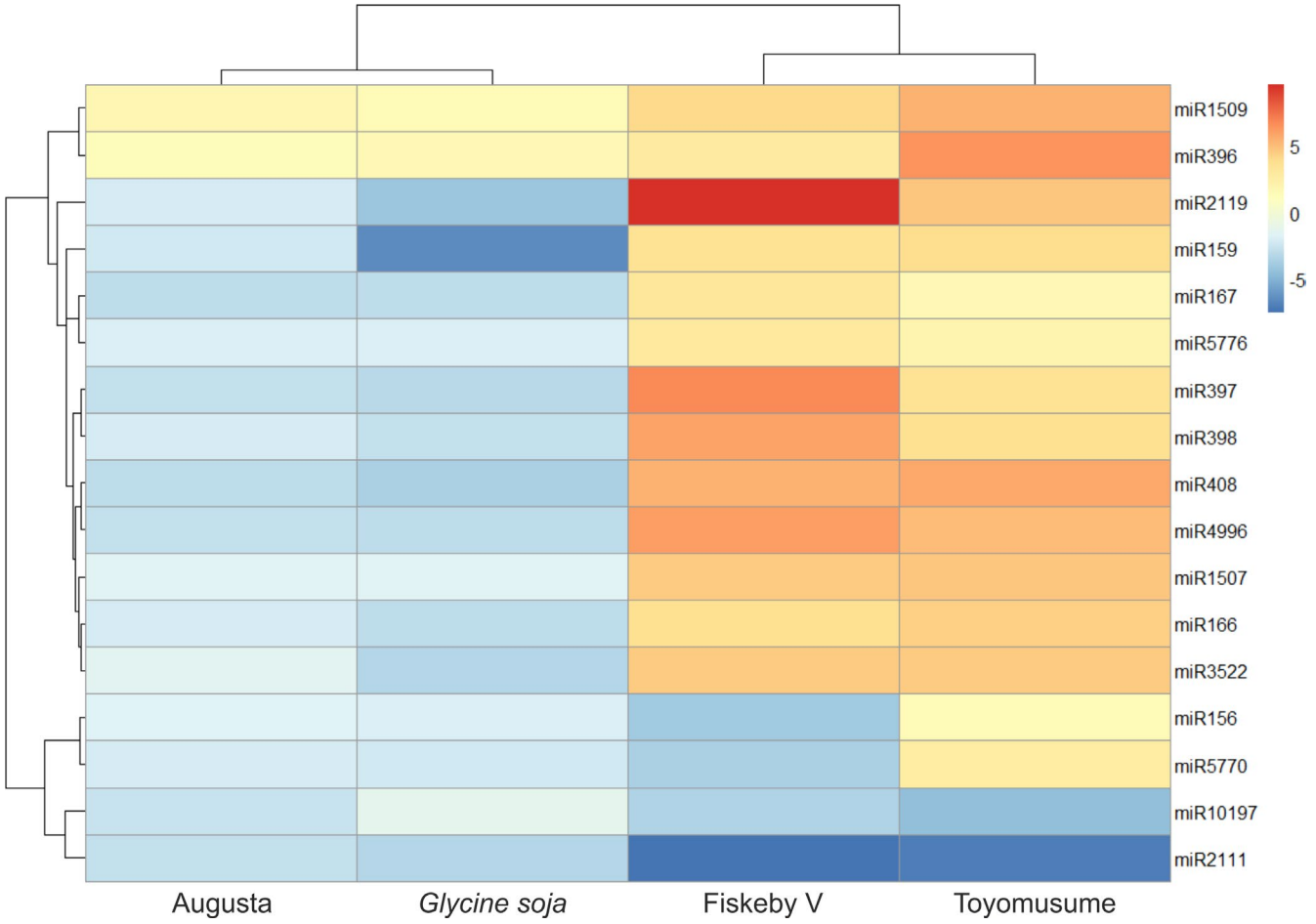

FIGURE 4 Heatmap of differentially expressed miRNAs in trifoliates of four studied soybean cultivars

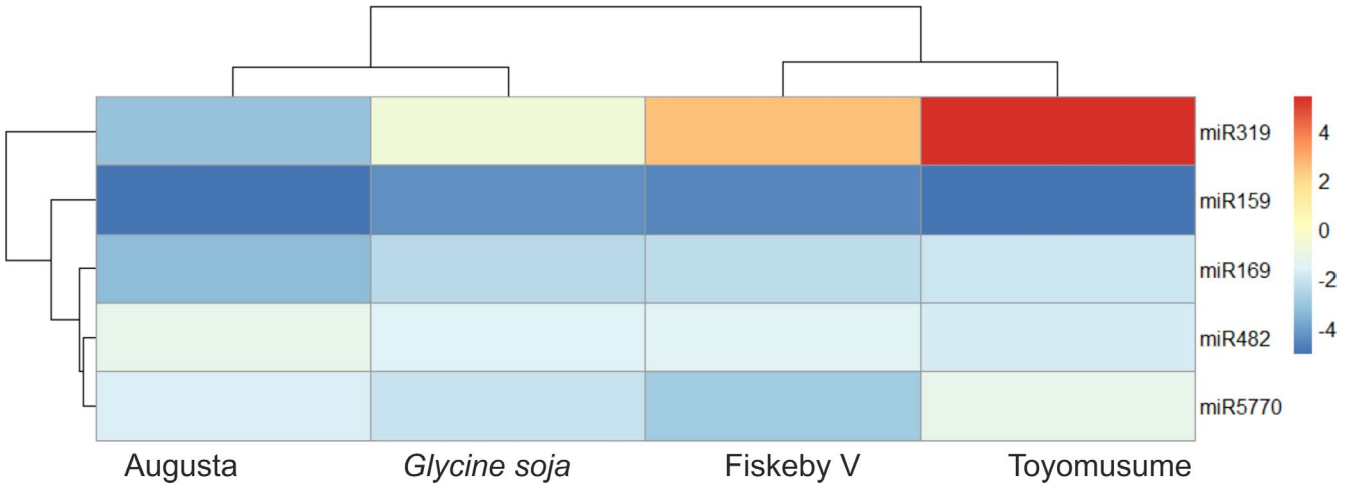

FIGURE 5 Heatmap of differentially expressed miRNAs in roots of four studied soybean cultivars

targeted TCP transcription, a protein from flavin-binding monooxygenase family, and cold-regulated protein (COR1; in *G. soja*). One of the most represented targets of miR482 was TIM21-like protein. Regarding the miRNAs and their targets in trifoliates of V1 stage, miR1507 was assigned to aspartic proteinase PCS1 and NBS-LRR disease resistance protein; miR156 was assigned to scarecrow-like protein 28, squamosa promoter binding-like protein (in Augusta) and FAD synthase (in *G. soja*); miR159 targeted CDPK-related kinase and pyruvate dehydrogenase (E1 component subunit alpha-3); miR166 targeted GAMYB transcription factor and homeobox-leucine zipper

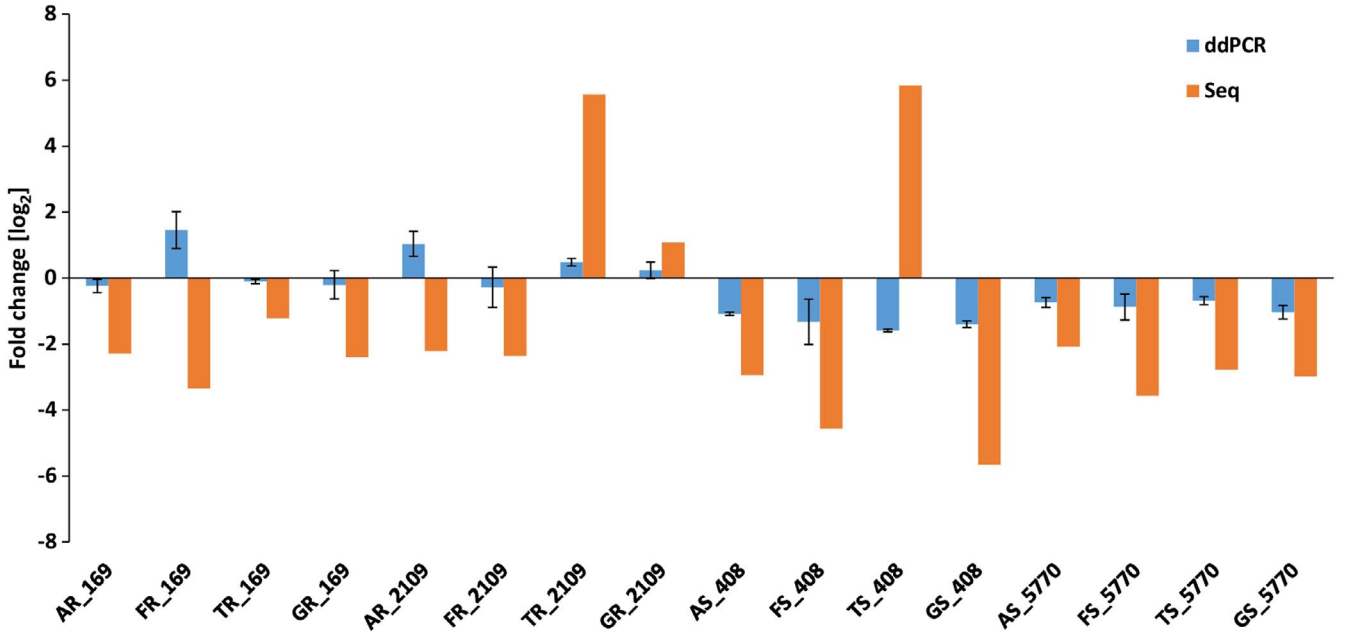

FIGURE 6 Validation of expression of four miRNAs at two developmental stages of four soybean cultivars. AS – Augusta roots, FR – Fiskeby V roots, TR – Toyomusume roots, GR – *G. soja* roots, AS – Augusta seedlings, FS – Fiskeby V seedlings, TS – Toyomusume seedlings, GS – *G. soja* seedlings

protein ATHB-14 like; miR2111 was found to cleave VSPA (vegetative storage protein A); miR3522 targeted COL2a (CONSTANS-like 2a); miR398 affected the expression of SOD (superoxide dismutase [Cu-Zn]) and CCS (Cu/Zn superoxide dismutase copper chaperone) (except for Toyomusume); miR408 was considered to alter the expression of HMA8 (chloroplast copper-translocating HMA8 P-ATPase) and NAC domain protein; and miR4996 targeted cysteine proteinase, polyphenol oxidase and omega-6 fatty acid desaturase (FAD).

Gene ontology terms were assigned to 378 target genes controlled by 16 differentially expressed miRNAs (Table S5). Target genes were described by 53 terms in *biological process* category, 31 terms in *molecular function* category and 25 terms in *cellular compartment* category. Highly represented terms included 'biosynthetic process', 'cellular nitrogen compound metabolic process' and 'cellular protein modification process' in *biological process* category; 'ion binding', 'DNA binding' and 'oxidoreductase activity' in *molecular function* category; and 'cell nucleus', 'protein containing complex' and 'membrane' in *cellular compartment* category (Figure 7). Moreover, according to the KEGG analysis, 378 target genes were significantly enriched in 67 pathways, of which the largest number of enzymes affected by the cold stress were those related to sugar metabolism: starch and sucrose metabolism (6), glycolysis/gluconeogenesis (5), galactose (5), amino sugar and nucleo sugar (5) and pyruvate (8) metabolism, pentose phosphate pathway (3) and pentose and glucuronate interconversion (3). Additionally, enzymes involved in carbon fixation in photosynthetic organisms (5), photosynthesis (1) and nicotinate and nicotinamide metabolism (1) were affected after cold treatment in soybean. Glutathione metabolism (3) was also altered due to chilling. The metabolism of various amino acids (including tyrosine, phenylalanine, glycine, serine, threonine, arginine and proline), purine and biotin, isoquinoline alkaloid biosynthesis as well as pyruvate and thiamine metabolism were also indicated as pathways influenced by cold stress (Table S6).

3.6 | Gene expression level analysis using real-time PCR

Three genes were selected for the analysis of changes in gene expression level under chilling in roots and cotyledons of four tested soybean varieties: *Glycine max* putative phytocyanin (Phyt, NM_001251440.2), *Glycine max* transcriptional factor (NAC-19, NM_001255827.1) and *Glycine max* malate dehydrogenase [NADP] (GmMDH, NM_001369219.1). In roots, following stress exposure, Phyt was upregulated in Augusta and Fiskeby V, contrary to Toyomusume and *G. soja*, where a significant downregulation of the gene's expression level was observed. NAC-19 transcription factor was highly upregulated in roots in all tested varieties. GmMDH in roots was upregulated in Augusta, Fiskeby V and Toyomusume, 50% downregulation was noted for *G. soja*. In cotyledons, the expression of Phyt was downregulated (by at least 50%) in all varieties except for *G. soja*, where it was upregulated (by 40%). NAC-19 transcription factor was upregulated in cotyledons of all tested varieties, and GmMDH was upregulated in Augusta and Toyomusume and downregulated in Fiskeby V and *G. soja*. The graphs presenting changes in the expression levels of chosen genes are presented in Figure 8.

TABLE 4 Target genes of some differentially expressed miRNAs involved in soybean chilling stress response

miRNA ID	Transcript	Transcript annotation	Cleavage site	Degradome category	Degradome *p*-value
gma-MIR1507a+|gso-MIR1507a+|vun-MIR1507a+|vun-MIR1507b+	XM_003538342.4	PREDICTED: *Glycine max* aspartic proteinase PCS1 (LOC100803089)	277	2	.038714958
gma-MIR166r+	NM_001250783.3	*Glycine max* GAMYB-binding protein (LOC732608)	1743	3	.091742104
crt-MIR166b+|csi-MIR166f+|gma-MIR166r+	XM_003524945.4	PREDICTED: *Glycine max* homeobox-leucine zipper protein ATHB-14-like (LOC100806322)	1055	0	.0012395
gma-MIR3522+|gso-MIR3522a+|gso-MIR3522b+	NM_001248914.2	Glycine max zinc finger protein CONSTANS-LIKE 2 (COL2B)	1017	0	.03432688
gso-MIR3522a+|gso-MIR3522b+	NM_001248512.2	Glycine max Cu/Zn superoxide dismutase copper chaperone (CCS)	741	0	.02167003
gma-MIR397a+	NM_001354136.1	Glycine max NAC transcription factor (LOC100814504)	617	3	.026758707
fve-MIR397+|gma-MIR397b+|lja-MIR397+	XM_003529084.3	PREDICTED: Glycine max laccase-7 (LOC100814926)	741	0	.002099926
MIR408-|ath-MIR408+|gma-MIR408a+|gma-MIR408b+|gma-MIR408c+|gma- MIR408+|zma-	NM_001255827.1	Glycine max NAC domain protein (NAC19)	726	0	.004468
gma-MIR5770a+	NM_001358538.1	Glycine max putative copper amine oxidase (LOC100819571)	228	2	.019549
gma-MIR482b+|gma-MIR482d+|gma-MIR482e+|gso-MIR482a+|gso-MIR482b+	NM_001254425.2	Glycine max TIM21-like protein, mitochondrial-like (LOC100779212)	244	0	.028829
MIR398b+|gma-MIR398c+|gma-	NM_001248369.1	Glycine max superoxide dismutase [Cu-Zn] (SOD1)	22	0	.002981
MIR398b+|gma-MIR398c+|gma-	NM_001248512.2	Glycine max Cu/Zn superoxide dismutase copper chaperone (CCS)	740	3	.069627

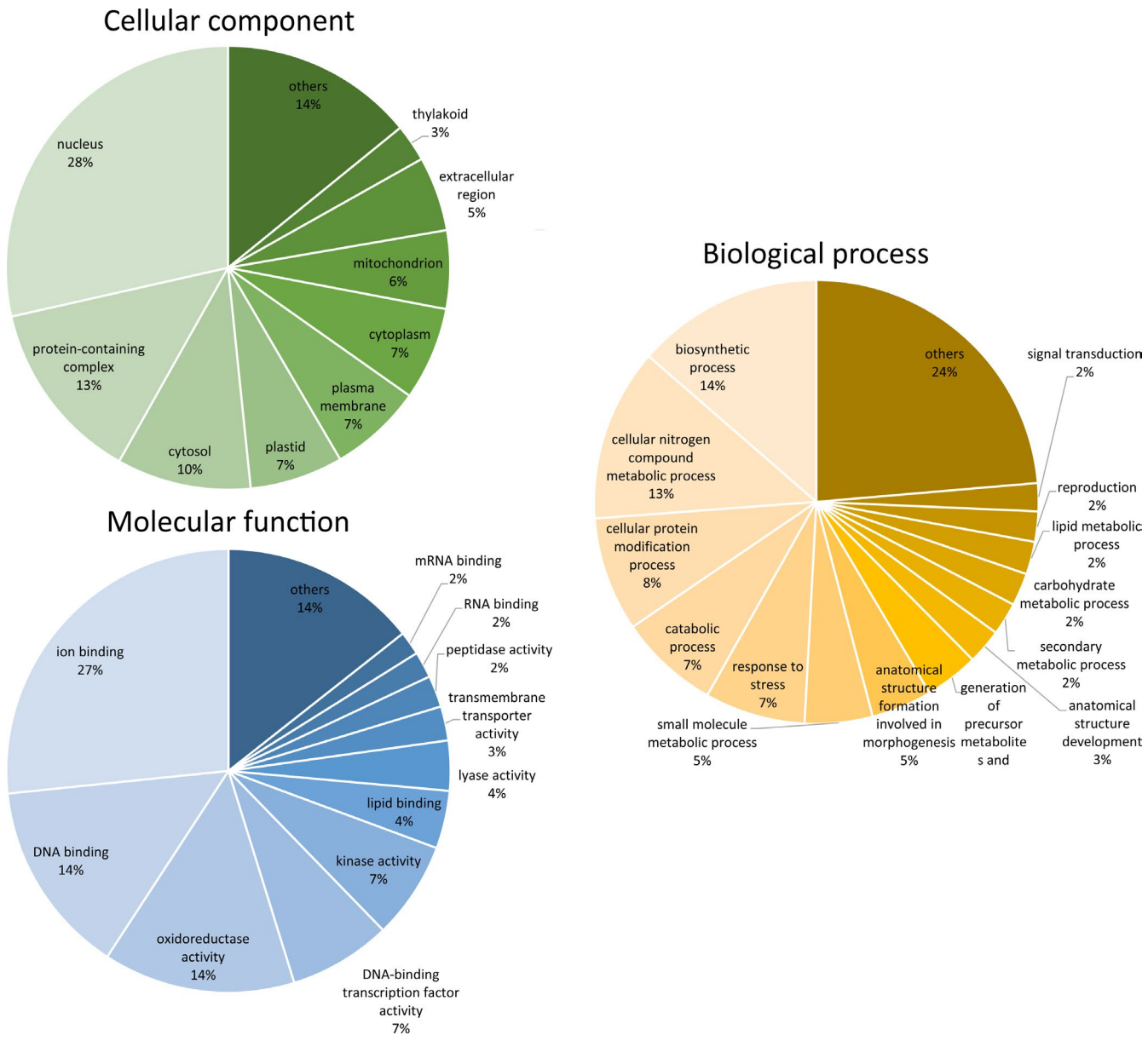

FIGURE 7 Gene ontology (GO) analysis of target genes of differentially expressed miRNAs

4 | DISCUSSION

Low-temperature conditions are one of the critical factors that influence plant growth, development and geographical distribution (Megha et al., 2018). Cold injury can affect plants in several ways, such as by causing disruption of energy generation by blocking photosynthesis, generating systemic oxidative stress caused by excessive production of reactive oxygen species (ROS) and disrupting membrane transport associated with reduced fluidity of the plasma membrane structure (Miura & Furumoto, 2013). miRNAs are crucial players in the regulation of various stress responses and constitute a major part of sequence-specific gene silencing machinery (Kumar, 2014). Nevertheless, the involvement of miRNAs in the chilling stress response in soybean needs further investigation. The present study aimed to identify miRNAs that participate in soybean's response to chilling stress. To achieve our objective, we used four cultivars (Augusta, Fiskeby V, Toyomusume and *G. soja*) that differ in their sensitivity to chilling stress. In addition to the traditional comparison of treated and control groups, Toyomusume, as the cultivar susceptible to low temperatures, served as the background in the analysis of the differential expression patterns of stress-responsive miRNAs. On the basis of the comparison of the aforementioned cultivars and their tissues sampled at two soybean developmental stages, we concluded that miRNAs play an important role in soybean's chilling stress tolerance mechanism. Additionally, we identified 321 known miRNAs along with 348 novel miRNAs in 72 libraries from the four tested soybean cultivars.

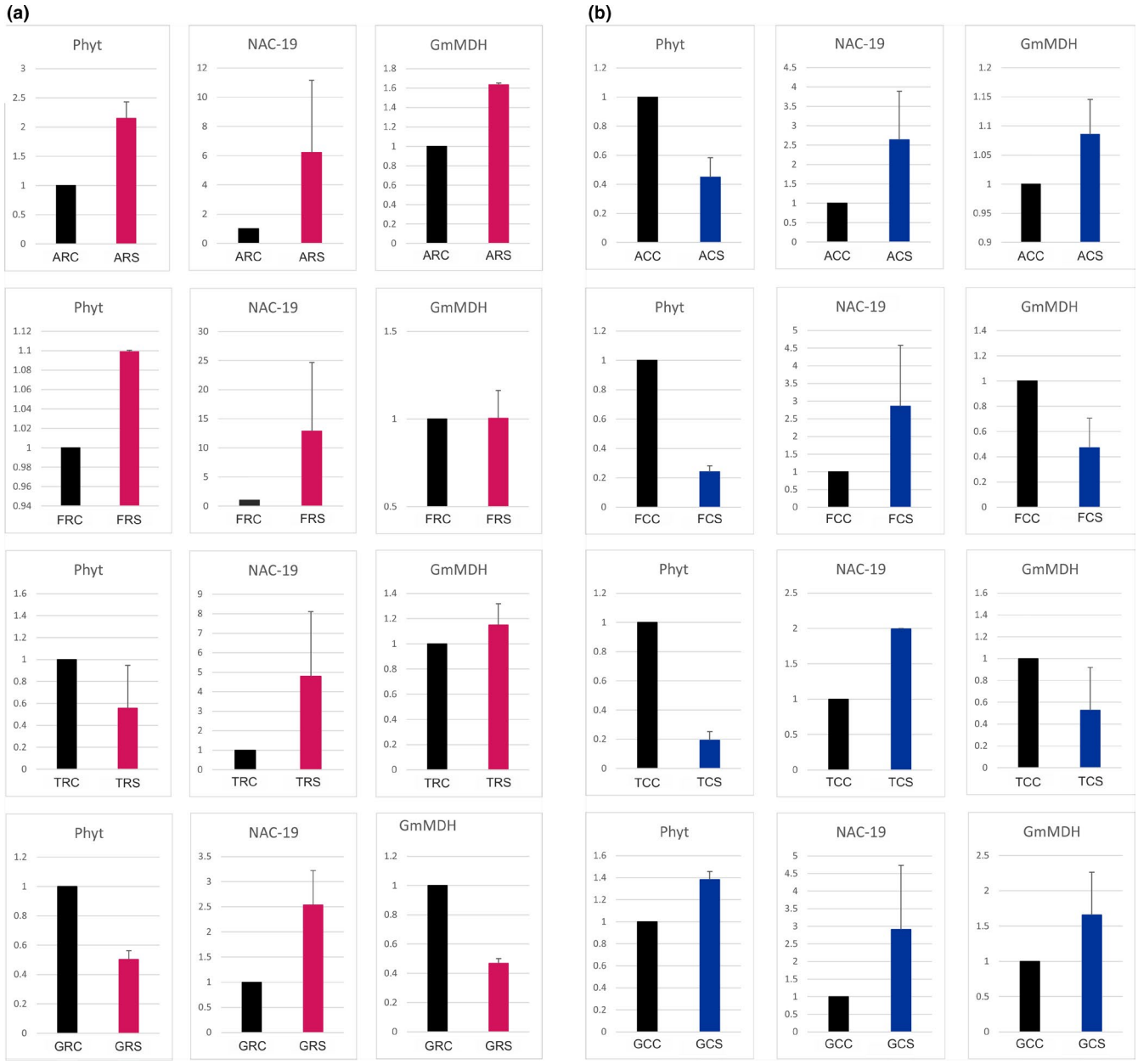

FIGURE 8 The relative gene expression levels (Y axis) of selected genes (Phyt, NAC-19 and GmMDH) in soybean varieties under chilling. (a) In roots, (b) in cotyledons. The results are presented as the mean ± SD, from two independent experimental and three technical repeats (treated vs. control). First letter of the sample name designates cultivar: A – Augusta, F – Fiskeby V, G – Glycine soja, T – Toyomusume; second letter designates tissue: R – root, C – cotyledon; third letter designates conditions: C – control, S – stress

4.1 | miRNA in chilling stress responses in soybean

Our approach based on the use of high-throughput methods broadens the knowledge of mode of action of miRNAs in soybean plants. A previous study showed that cold stress influences the growth of two tested varieties, namely Augusta and Fiskeby V, which was observed as phenotypic changes (Kuczyński et al., 2020). It was also shown that under chilling stress, the expression levels of several miRNAs (miR169, miR319, miR397 and miR398) and their target genes had changed. In the present study, amid the plethora of miRNAs that exhibited differential expression between the four studied cultivars, a group of miRNAs with a common expression pattern in all the analysed varieties caught our immediate attention. This group consisted of both conserved miRNAs (miR159, miR2111, miR396 and miR482) and legume-specific miRNAs (miR10197 and miR1509). Among these, miR1509 and miR396 were found to be upregulated in trifoliates of chilled soybean plants at V1 stage, whereas miR10197, miR159, miR2111 and miR482 were downregulated. In the analysis of the degradome libraries, genes that may be the targets of these miRNAs were identified. These genes included pyruvate dehydrogenase, auxin signalling F-box 2, aspartyl protease, TIM21-like protein, TMV resistance protein N and vegetative storage protein. Such

expression trends of these miRNAs suggest that their target genes play a universal role in controlling chilling stress response across different soybean cultivars. Most of the differentially expressed miRNAs reported in this study were found in the trifoliates. Expression profiles of some miRNAs showed specificity towards a particular tissue, in that they were found only in radicles, such as miR10190 and miR862, or in trifoliates, such as miR1511, miR168 and miR391. Interestingly, expressional patterns of other miRNAs such as miR1509, miR10440 or miR171 proved that chilling stress can cause one miRNA to be upregulated in one tissue but to be downregulated in another tissue. Differential expression of miRNA in various parts of soybean plants has been reported previously (Sun et al., 2016). Additionally, findings detailing the expression of miR157 in leaves and roots of *Prunus persica* (Eldem et al., 2012) support the existence of this phenomenon.

Thirteen miRNAs were found to have contrasting trends of expression in Toyomusume and at least two other cultivars. These trends were observed mainly in trifoliates (V1 stage); however, the differential expression of most of these miRNAs was detected in all tissues in at least one cultivar. miR156 was found to be downregulated in Augusta, Fiskeby V and *G. soja*, whereas no significant change in expression was observed in Toyomusume. Interestingly, in all other cases, Fiskeby V and Toyomusume shared the expressional pattern of upregulation, as opposed to Augusta and *G. soja*, in which the said miRNAs were downregulated during chilling stress. An exception to this tendency was miR319, whose levels increased in Augusta and *G. soja* and decreased in Fiskeby V and Toyomusume under low-temperature conditions. The legume-specific miR1507, which was downregulated in trifoliates of Augusta and *G. soja* and upregulated in Fiskeby V and Toyomusume, was predicted to control the expression of aspartic proteinase, which plays a role in protein turnover and biotic stress tolerance in plants (Mazorra-Manzano & Yada, 2008). Furthermore, two soybean-specific miRNAs, namely miR3522 and miR4996, exhibited analogical expression patterns to miR1507. miR3522 was assigned to the CONSTANS-LIKE 2B/A, which is involved in developmental processes, including flowering and root elongation (Steinbach, 2019). According to the degradome analysis, miR4996 potentially cleaves the transcripts of cysteine proteinase, which is responsible for the degradation of proteins from energetic reserves and proteins damaged due to stress conditions (Grudkowska & Zagdańska, 2004), and omega-6 FAD, which regulates the content of unsaturated fatty acids in the plasma membrane (Dar et al., 2017). Furthermore, many genes predicted to be targeted by differentially expressed miRNAs in this study remain uncharacterized, which leaves much room for future advancement in elucidating chilling stress response in soybean.

4.2 | Annotation of differentially expressed conserved miRNAs with their putative target genes

Conversely, the annotation of differentially expressed conserved miRNAs with their putative target genes proved to be more fruitful (compared to legume/soybean-specific miRNAs). miR156, which was downregulated in trifoliates of Augusta, Fiskeby V and *G. soja* but upregulated (not significantly) in Toyomusume, was predicted to control the expression of teosinte glume architecture 1 (TGA1), the transcriptional regulator belonging to the SBP (squamosa promoter binding-like protein) family, which was found to play a role in transition from juvenile to adult stage in maize, where it was also shown to be targeted by miR156 (Studer et al., 2017). miR159, which was downregulated in trifoliates of Augusta and *G. soja* but upregulated in Fiskeby V and Toyomusume, was predicted to target the CDPK-related kinase 6 (CRK6), which is reported to be involved in ROS metabolism and shown to have an extensive role in the abiotic stress tolerance response in *Oryza sativa*, *Zea mays*, *Populus trichocarpa* and *Brassica napus* (Bulgakov et al., 2011; Xiao et al., 2017). According to the study conducted on the vegetable soybean, the expression of miR159 was decreased due to chilling stress (Xu et al., 2016). miR166, having the same expressional pattern as miR159, was proposed to regulate ATHB-14, ATHB-15 and GAMYB. Another research group also reported ATHB-14 as a target gene of miR166 in soybean (Li et al., 2017). In that study, the authors found members of the miR166 family to be responsive to cold stress (Li et al., 2017). In *Camellia sinensis*, several members of the miR166 family were downregulated under drought stress, and a negative correlation was observed between the expression of miR166 and ATHB-14 like and ATHB- like (Guo et al., 2017). miR319 is known to target transcription factors from the family of TCP (TEOSINTE BRANCHED1/CYCLOIDEA/PCF) involved in leaf morphogenesis (Bresso et al., 2018). Our results showed that during chilling stress, miR319 was downregulated in roots of Fiskeby V and *G. soja*, with opposite tendencies in Toyomusume and Augusta. Furthermore, TCP2/3/4 were assigned as target genes of miR319 in the degradome analysis, which was corroborated by the work of another research group that described the relationship of miR319 and TCP3/4 in the context of flavonoid biosynthesis in soybean (Gupta et al., 2019). Another pattern of downregulation in Augusta and *G. soja* and upregulation in Fiskeby V and Toyomusume (not significantly) was observed for miR397 in trifoliates. Other authors reported that during water deficit in soybean, miR397ab showed downregulation in a resistant cultivar but upregulation in a sensitive cultivar (Kulcheski et al., 2011). Here, we predicted that NAC18/19 (Petunia No Apical Meristem (NAM), *Arabidopsis* transcription activation factors (ATAF1 and ATAF2), cup-shaped cotyledon 2 (CUC2)) and laccase-7 may be potential targets of miR397. NAC is a family of transcription factors involved in various developmental processes such as hormone signalling, fruit ripening and stress response (Hussain et al., 2017). According to Yang et al. (2019) NAC109 was upregulated in roots and shoots shortly after cold stress in soybean. Another research group reported that the expression of NAC19 was induced by ABA and JA, and it was engaged in the process of programmed cell death accompanied by ROS accumulation (Wang et al., 2015). Laccases in plants are mostly associated with the lignification process; however, they were also found to be involved in abiotic stress response in *Arabidopsis* (Wang et al., 2019). ROS are almost an inseparable factor

of abiotic stress in plants; therefore, the capacity of any given organism to neutralize these pernicious molecules is essential for endurance of adverse conditions (Dar et al., 2017). Nature's response to ROS is the development of the antioxidative system, including copper/zinc SOD (Feng et al., 2016). Our data showed downregulation of miR398 in trifoliates of Augusta and *G. soja*, and the opposite expression pattern was observed in Fiskeby V and Toyomusume. Further analysis suggests that Cu/Zn SOD and CSS are the target genes of miR398. Previous studies have shown that miR398 downregulates the transcription of its target genes, namely CCS and Cu/Zn SODs (CSD1 and CSD2), in *Arabidopsis*, thus corroborating its role in stress regulation (Beauclair et al., 2010; Guan et al., 2013).

4.3 | Novel miRNA with differential expression under chilling in soybean and their target genes

In the present study, 18 novel miRNAs were found that exhibited significant differential expression between control and chilled samples. The majority of these miRNAs were identified in cotyledons and trifoliates of *G. soja*. This may be explained by *G. soja* having the largest difference in its genome as compared to other tested cultivars that belong to the genus *Glycine*. Further analysis enabled to classify the target genes for some of the novel miRNAs found responsive to chilling stress. For example, novel miR151035 targeted the scarecrow-like (SCL6) protein in trifoliates of Fiskeby V. In *Arabidopsis*, SCL6 was found to coordinate the shoot branching process (Wang et al., 2010). Another study showed that SCL6 is involved in the nodulation process in soybean (Hossain et al., 2019). Novel miR119406 targeted ATP sulphurylase (ATPS) in trifoliates of *G. soja*. The expression of ATPS was observed to be induced due to cold treatment in soybean (Phartiyal et al., 2006). ATPS, as a crucial enzyme in the sulphur assimilation pathway, controls the rate of cysteine synthesis, which is one of the substrates of glutathione that plays a role in cold resistance of plants (Phartiyal et al., 2006). Interestingly, novel miR60377/64371/49212 targeted a protein containing F-box/kelch repeat in trifoliates of *G. soja* and Augusta as well as in cotyledons of *G. soja*. F-box proteins are a part of the E3 ubiquitin–protein ligase complex and were reported to be involved in the response to salinity, drought and heavy metal stress in *Medicago truncatula* (Song et al., 2015).

4.4 | Target gene expression analysis under chilling in soybean

The analysis of expression levels of selected genes showed changes caused by cold stress; one of the genes is transcription factor NAC-19 which belongs to one of the largest TF families that regulate plant growth, development and responses to environmental stresses (Diao et al., 2020; Zhang et al., 2018). In the present study, following chilling stress, NAC-19 was significantly upregulated in roots and cotyledons of all tested varieties. This result correlated well with observed downregulation of miR408 in cotyledons which was predicted to target NAC-19 (Table 4; Figure 6). To date, several reports indicated upregulation of many NAC TFs by cold stress in several different plant species like *A. thaliana*, *Brassica napus*, *Capsicum annum* L., *Glycine max*, *Oryza sativa*, *Triticum aestivum*, *Zea maize* and many more (reviewed in Diao et al., 2020).

NADP dehydrogenases are key components of NADPH production systems necessary to maintain redox balance in the cells, and preserving redox homeostasis is especially important during stress exposure (Begara-Morales et al., 2019; Sun et al., 2019; Wang et al., 2016). Malate dehydrogenase (GmMDH) belongs to a group of oxidoreductases that catalyse the conversion of malate and oxaloacetate, the reaction accompanied by reduction in the NAD(H) or NADP(H) pool. NADP is an important reducing agent for the synthesis of defensive substances and anabolic reactions (Sun et al., 2019; Wang et al., 2016). The role of MDH in stress responses has been proven, among others, in *A. thaliana* (Hebbelmann et al., 2012; Zhao et al., 2020), transgenic apple plants (Wang et al., 2016) and winter rye (Crecelius et al., 2003). Hence, it is not surprising that in roots of Augusta and Toyomusume varieties, GmMDH levels were upregulated, or maintained at the same level (in Fiskeby V). Only in one variety (*G. soja*), a decrease in the levels of GmMDH was observed. In cotyledons only, Augusta maintained increased expression level of GmMDH. An increase was also observed in *G. soja*. Phytocyanins (PCs) are ancient blue copper proteins which function as electron transporters. Previously it has been shown that PCs play important roles in cell differentiation and reorganization, organ development and also abiotic stress responses (Cao et al., 2015; Ma et al., 2011; Ruan et al., 2011). In the present study, an increase in the gene expression levels of *Glycine max* putative phytocyanin has been observed in roots of Augusta and Fiskeby V – the two cold-resistant varieties. On the contrary, in cotyledons, the expression levels were decreased in Augusta, Fiskeby V and Toyomusume. The differential expression profiles of enzymes involved redox homeostasis and electron transport, which may be responsible to some extent for increased/decreased susceptibility to abiotic stresses. However, more in-depth analyses are necessary to decipher the mechanism underlying the observed changes.

4.5 | Metabolic pathways affected in soybean under chilling

In the present study, we performed the GO analysis and KEGG pathway classification of genes predicted to be targeted by miRNAs that were differentially expressed under chilling stress. According to these analyses, terms that were highly represented included cellular nitrogen compound metabolism, cellular protein modification and stress response. These processes were strongly related to plant abiotic stress resistance. For instance, various protein modifications such as ubiquitination, sumoylation and phosphorylation activate numerous transcription factors crucial in abiotic stress response (Kosová et al., 2018). Furthermore, auxin-activated signalling,

regulation of transcription and secondary metabolism also constituted a substantial part of these classifications, suggesting a profound contribution in survival under low-temperature conditions. It has been established that auxins assist plants in coping with environmental stresses by regulating transcription factors and modulating growth and development (Bielach et al., 2017). Furthermore, secondary metabolites such as flavonoids, isoprenes and cinnamic acid derivatives that are known to be overproduced due to chilling stress can neutralize ROS (Isah, 2019; Yang et al., 2018).

It has been shown that reprogramming of the central carbohydrate metabolism plays a key role in cold acclimation in plants (Fürtauer et al., 2019; Hoermiller et al., 2017; Ritonga & Chen, 2020). These findings were corroborated in our study, as seen in the results of KEGG analysis, where it has been shown that sugar metabolism was among the pathways affected mostly by the 16 differentially expressed miRNAs. In Arabidopsis, starch metabolism is considered as a determinant of plant fitness under abiotic stress as it responds with great plasticity to various growth conditions. Additionally, various sugars stabilize biological membranes, liposomes, act as osmoprotectants or even stabilize photosynthesis during stress as the reduction in photosynthetic capacity is often accompanied by increased sugar accumulation (Fürtauer et al., 2019; Hajihashemi et al., 2018). Photosynthesis and CO_2 fixation were also affected and negatively regulated by cold stress (Banerjee & Roychoudhury, 2019; Calzadilla et al., 2019; Hajihashemi et al., 2018), which was further confirmed in our studies, as enzymes involved in carbon fixation, photosynthesis and nicotinate and nicotinamide metabolism were among the targets of miRNAs with changed expression levels under cold stress. Glutathione, organic sulphur repository, in its reduced form (GSH) is an essential metabolite in various biosynthetic pathways like detoxification and redox homeostasis (Rao & Reddy, 2008). To date several reports indicated the involvement of glutathione in responses to abiotic stresses (Hasanuzzaman et al., 2017; Kocsy, Szalai, et al., 2000, Kocsy, Von Ballmoos, et al., 2000; Spanò et al., 2017). At low non-freezing temperatures, high GSH content and glutathione reductase activity were detected in several plant species, indicating a possible contribution to chilling tolerance and cold acclimation (Kader et al., 2011). Glutathione metabolism was one of the pathways indicated in our KEGG analysis, where three enzymes: glutathione reductase (ec:1.8.4.2), 5-oxoprolinase (ATP-hydrolysing) (ec:3.5.2.9) and dehydrogenase (NADP+) (ec:1.1.1.49) were predicted as targets of miRNAs with changed expression under chilling. The classification of these metabolic changes may shed light on the role of target genes of differentially expressed miRNAs in stress responses of soybean.

5 | CONCLUSIONS

Cold stress is one of the major environmental factors that severely affects plant growth and development and negatively influences crop productivity. Some plants are able to cope with this stress and acquire chilling tolerance; in some species/varieties/single individuals, the exposure to this stress triggers developmental responses. As tender legumes, soybeans thrive in warm climates and are sensitive to cold. In the present study, to determine the involvement of miRNAs and their target genes in chilling resistance of four soybean cultivars varying in cold stress susceptibility, high-throughput sequencing was used to identify cold-responsive miRNAs and their target genes. A total of 321 known miRNAs were identified, and 348 novel miRNAs were predicted, of which 162 miRNAs, including well-conserved, legume- and soybean-specific miRNAs, and 18 novel miRNAs, respectively, had changed expression profiles. Interestingly, several miRNAs such as miR156, miR169 and miR5770 had similar expression patterns in Augusta, Fiskeby V and *G. soja*, which clearly contrasted from that in cold-sensitive Toyomusume variety. Altogether, the results suggest that these miRNAs may play a role in the chilling responses of soybean. Degradome analysis as well as GO and KEGG annotations allowed us to assign potential target genes to the differentially expressed miRNAs. Many of these genes were found to be related to plant abiotic stress response mechanisms such as ROS scavenging, flavonoid biosynthesis and regulation of osmotic potential. In summary, our findings provide valuable insights into the function of miRNAs in the soybean chilling resistance and may provide crucial knowledge in the development of new cultivars. Investigating the molecular mechanisms of soybean chilling stress responses will facilitate better understanding of the response of plant species to chilling and help to reduce the consequences of this major environmental stress on plants.

5.1 | Sequence data

These sequence data have been submitted to the Sequence Read Archive (SRA) BioProject (NCBI) databases under accession number PRJNA725380.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTIONS

A.T., J.G.-B. and T.T. – conceptualization; A.T., J.G.-B. and W.K. – methodology; A.T., J.G.-B. and W.K. – validation; T.T. – formal analysis; J.K., A.T., J.G.-B. and W.K. – investigation; T.T. – resources; A.T. and J.G.-B. – data curation; J.K. – writing–original draft preparation; J.K., A.T., J.G.-B., W.M.K. and T.T. – writing–editing; J.K. – visualization; A.T. – supervision; A.T. and J.G.-B. – project administration; T.T.

– funding acquisition. All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

Data openly available in a public repository that does not issue DOIs. Data available in article supplementary material.

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