Abiotic Stress Tolerance in Soybean: Regulated by ncRNA

YASIN JESHIMA KHAN¹, HUSNARA TYAGI¹, ANIL KUMAR SINGH² AND SANTOSHKUMAR MAGADUM

Division of Genomic Resources, ICAR-NBPGR, PUSA campus, New Delhi, India

INTRODUCTION

Soybean (Glycine max) is an economically important legume crops for being one among the global sources of protein and oil, as a food and livestock feed. Crops are under continued environmental stresses limiting growth and development resulting poor crops yield lower than their genetic potential (Singh and Bhatt BP 2012). Transcriptomics, proteomics and metabolomics have been employed to improve understanding of the biological processes and molecular/cellular mechanisms involved in plant stress responses. Soybean is having a published annotated high-quality genome sequence. Over the past decade, small RNAs were determined to be important regulators of gene expression and plant growth (Chen, 2005; Rhoades et al., 2006; Mallory and Vaucheret, 2006). Small RNAs (19-24 bp) are key regulators of gene expression that guide both transcriptional and post-transcriptional silencing mechanisms in eukaryotes. Small RNAs comprise two major classes: microRNAs (miRNAs) and short-interfering RNAs (siRNAs). Current studies have demonstrated that microRNAs (miRNAs) act in several plant pathways associated with tissue proliferation, differentiation and development along with the response to abiotic stresses (Kulcheski et al., 2011). Kulcheski et al. (2011) identified new miRNAs in soybean by constructing eight libraries of small RNAs from water deficit and rust affected plants. In plants, miRNA genes are transcribed by RNA polymerase-II enzymes (Pol II) generating primary miRNA (pri-miRNA) (Lee et al., 2004). Pri-miRNA forms an imperfect foldback structure, which is processed into a stem-loop precursor (pre-miRNA) by nuclear RNaseIII-like enzymes called DICER-LIKE proteins (Bartel, 2004; Kurihara and Watanabe, 2004). The resulting pre-miRNA contains a miRNA: miRNA* intermediate duplex, formed by a self-complementary fold-back structure. A mature miRNA sequence can range from 19 to 24 nucleotides (nt) in length and act as a regulatory molecule in post-transcriptional gene silencing by base pairing with target mRNAs. The same mature miRNA can also present several iso-miRNAs which are isoforms of conserved microRNAs (Guo and Lu, 2010). They are caused by an imprecise or alternative cleavage of Dicer during pre-miRNA processing. Iso-miRNAs have been recently identified in both plants and animals.

In soybean (Glycine max (L.) Merrill), the major legume crop worldwide, 35 novel miRNA families were identified for the first time by Subramanian et al. (2008) and studied the role of

ABSTRACT

Plants respond through a cascade of reactions resulting in varied cellular environment leading to alterations in the patterns of protein expression resulting in phenotypic changes. Single cell genomics and global proteomics came out to be powerful tools and efficient techniques in studying stress tolerant plants. Non-coding RNAs are a distinct class of regulatory RNAs in plants and animals that control a variety of biological processes. Small ncRNAs play a vital role in post transcriptional gene regulation by either translational repression or by inducing mRNA cleavage. The major classes of small RNAs include microRNAs (miRNAs) and small interfering RNAs (siRNAs), which differ in their biogenesis. miRNAs control the expression of cognate target genes by binding to complementary sequences, resulting in cleavage or translational inhibition of the target RNAs. siRNAs too have a similar structure, function, and biogenesis like miRNAs but are derived from long double-stranded RNAs and can often direct DNA methylation at target sequences. In this review, we focus on the involvement of ncRNAs in combating abiotic stresses of soybean. This review emphasis on previously known miRNAs as they play important role in several abiotic stresses like drought, salinity, chilling and heat stress by their diverse roles in mediating biological processes like gene expression, chromatin formation, defense of genome against invading viruses. This review attempts to elucidate the various kinds of non-coding RNAs explored, their discovery, biogenesis, functions, and response for different type of abiotic stresses and future aspects for crop improvement in the context of soybean, a representative grain legume.

Keywords: Abiotic Stress, Drought, ncRNA, Salt, Soybean

* Corresponding author email: Yasin.Jeshima@icar.gov.in

¹IARI Regional Station, Kalimpong, Shillong, Meghalaya, India
²ICAR Research complex, Patna, Bihar, India
miRNAs in soybean-rhizobial symbiosis. Interestingly, Zhang et al. (2008) used a comparative genome-based in silico screening of soybean EST databases and quantitative PCR to provide evidence for 69 miRNAs belonging to 33 families. Wang et al. (2009) identified 32 miRNAs belonging to 11 miRNA families in soybean root nodules. The identification of nine novel miRNAs in wild soybean (Glycine soja) was also reported by Chen (2005). Another study looked at four different soybean tissues (root, seed, flower and nodule) and identified 87 novel soybean miRNAs (Joshi et al., 2010). Song et al. (2011) identified 26 new miRNAs and their related target genes from developing soybean seeds.

Although these studies resulted in a large number of miRNAs identified in soybean, none of them validated these microRNAs under abiotic stresses. Currently, there are 203 miRNAs identified in Glycine max (miRBase, release 16, http://www.mirbase.org/). A large number of candidate genes, proteins and pathways have been identified using omics approaches for detection of genes (genomics), mRNA (transcriptomics), protein (proteomics), and metabolites (metabolomics). miRNAs miR395, miR397b, and miR402 participate in stress responses leads to plant adaptation for environmental challenges Phillips et al. (2007).

**Biogenesis of miRNA**

Biogenesis of miRNA has been described in several reviews. The biogenesis of miRNAs starts with RNA pol II or III (Lee et al., 2004) dependent transcription of a miRNA gene locus generating a long primary RNA (pri-miRNA). In the miRNA biogenesis pathway, primary miRNAs (pri-miRNAs) are transcribed from nuclear-encoded MIR genes by RNA polymerase II (Pol II) miRNAs are small regulatory RNAs of 20–22 nt that are encoded by endogenous MIR genes. Mature miRNAs are produced from a pathway starting with primary miRNA transcripts (pri-miRNAs) transcribed from miRNA genes by RNA polymerase II leading to precursor transcripts with a characteristic hairpin structure (Mallory et al., 2008). In plants, the processing of these pri-miRNAs into pre-miRNAs is catalyzed by DCL1 and assisted by HYPOASTIC LEAVES 1 (HYL1) and SERRATE (SE) proteins (Han et al., 2004). DCL plays a significant role in recruiting DCL1 to pri-miRNA (Yu et al., 2008). Pre-miRNA hairpin precursor is finally converted into 20- to 22-nt miRNA/miRNA* duplexes by DCL1, HYL1, and SE (Bollman et al., 2003; Han et al., 2004; Chen, 2005; Griffiths, 2006). The duplex is then methylated at the 3' terminus by HUA ENHANCER 1 (HEN1) and exported into the cytoplasm by HASTY (HST1), an exportin protein. In the cytoplasm, one strand of the duplex (the miRNA) is incorporated into an AGO protein, the catalytic component of RISC, and guides RISC to bind to cognate target transcripts by sequence complementarity (Bartel, 2004). The miRNAs thus produced direct cleavage of a specific messenger RNA (mRNA) based on sequence homology between the miRNA and a target mRNA (Bartel, 2004). Single mature miRNAs can be present in several variant forms called iso-miRNAs (isofoms of microRNAs) (Guo and Lu, 2010), which are caused by an imprecise or alternative cleavage of Dicer during pre-miRNA processing (Ebbhardt et al., 2010; Guo and Lu, 2010; Naya et al., 2010). Therefore, isolation of miRNAs and their targets is essential for understanding their role in gene repression and plant growth and development. In addition to the control of targets at the posttranscriptional level, miRNAs regulate gene expression by causing epigenetic changes such as DNA and histone methylation.

**miRNA Discovery**

The first detected plant miRNAs were from Arabidopsis thaliana (Park et al., 2002; Reinhart et al., 2002). Plant miRNA discovery was initially dominated by the identification of conserved miRNAs expressed across the diverse plant species (Axtell and Bowman, 2005). Next Generation Sequencing (NGS) is a powerful tool for miRNA discovery. NGS was first applied to identify novel non-conserved miRNAs in Arabidopsis (Rajagopal et al., 2006; Fahlgren et al., 2007) and later in California poppy, rice, wheat, soybean, Medicago truncatula and Nicotiana attenuata (Barakat et al., 2007; Yao et al., 2007; Pandey et al., 2008; Sunkar and Jagadeseewaran, 2008; Sztitya et al., 2008). In soybean (Glycine max) the major legume crop worldwide, Subramanian et al. (2008) identified 35 novel miRNA families for the first time. Li et al. (2011) identified 50 novel miRNAs, with the 10 most highly expressed candidates. Expression of reported miRNAs and discovery of novel small RNAs are in trends. Expression analysis are nucleic acid hybridization-based technologies (Northern hybridization, RNase protection, primer extension, real-time RT/PCR, microarray hybridization or even more sophisticated technologies), while the discovery is based on direct sequencing of small RNAs. The starting material for detection of small RNAs can either be total RNAs or size fractionated total RNA, which is best isolated using standard Trizol (guanidineumisothiocyanate/acetic phenol) method. Size fractionation is needed for application of microarrays or for small RNA cloning, and is not required for Northern hybridization, RNase protection, primer extension or real-time RT/PCR. Size fractionation of nucleic acids is generally carried out using denaturing polyacrylamide gel electrophoresis, although some specialized methods have been developed to enrich for the fraction of small RNAs. Large RNAs, including the abundant rRNAs, can be removed by precipitation in the presence of polyethylene glycol (PEG-8000) and NaCl. Alternative methods depend on the use of silica-based column separation (miRNA Isolation Kits from Ambion and Invitrogen, respectively).

Total RNA may have different types of small RNAs that differ in size; two approaches of miRNAs identification and characterization are viz., computational approach and experimental validation. Computational programs like MiRScan (Lim et al., 2003) and MiRALign among others yielded immense number of conserved miRNAs. Alignment based tools nineteen have been utilized in identification of miRNA families in legumes viz., Medicago truncatula, Lotus japonicas and Glycine max. Experimental approach involving conventional cloning and deep sequencing of small RNA libraries has disclosed variety of low abundant, non-conserved, tissue and environment specific miRNAs (Lu et al., 2005; Sunkar et al., 2007; Yao et al., 2007; Subramanian et al., 2008). An Expressed Sequence Tag (EST) and a Genome Survey Sequence (GSS) approaches were developed to identify...
miRNAs (Zhang et al., 2005). The EST- GSS approach identified 69 miRNAs belonging to 33 families in soybean and five miRNAs in Glycine soja and Glycine clandestine (Zhang et al., 2008). In legumes besides conserved miRNAs, species specific novel miRNAs have also been discovered. Investigations on soybean by various workers have yielded 87 novel and 42 conserved miRNAs (Subramanian et al., 2008; Wang et al., 2009; Joshi et al., 2010). Based on computational predictions and sequencing approach, a large number of miRNA gene families (482), miRNA precursors (1039) and mature miRNA (1114) sequences have been identified from soybean and related legume species (Ramesh et al., 2013). Thus the miRNA discovery and functional validation were based generally on the investigations of hardly any conserved miRNA families (Bartel, 2004).

Recent reports have demonstrated that hundreds of miRNAs have been discovered or identified in soybean. Chen (2005) reported 15 conserved miRNA belonging to eight different families and nine novel miRNA comprising eight families in wild soybean seedlings. Joshi et al. (2010) identified 129 miRNAs based on sequencing and bioinformatic analyses of which, 42 miRNAs were conserved in soybean and other species, while 87 were novel miRNAs. Song et al. (2011) identified 26 novel miRNAs in developing soybean seeds by deep sequencing. According to Li et al. (2011), small RNA sequences compared with miRBase 16.0 based on sequence similarity identified 133 known miRNAs corresponding to 95 miRNA families in the soybean. 10 miRNAs (miR1513d, miR1520s, miR4357b, miR4357c, miR4416b, miR4416c, miR5037e, miR862c, miR1507d, miR4405b, miR862d) were only found in soybean. These observations suggest that conserved miRNAs may be essential for controlling basic cellular and developmental pathways (e.g. cell cycle) in plants.

**Stress responsive miRNAs in soybean**

Plants combat and exposed to abiotic stress under or over expression of certain miRNA and lead to the synthesis of new miRNAs to withstand stress. During various abiotic stresses such as drought, salinity, temperature fluctuation and oxidative environment, several stress regulated miRNAs have been identified in different plants. Genome wide analysis of micro RNAs were carried out in drought, cold, salinity, water lodging, temperature stressed soybean plants belonging to different developmental stages by using molecular techniques. Salinity is also a major problem in agriculture. Several salt regulated micro RNAs have been identified that plays either a direct or indirect role in salt stress alleviation. In a study of Arabidopsis and legumes plant crops, reveal that miRNAs under specific condition can control or regulate the expression of specific genes associated with abiotic stress tolerances (Mantri et al., 2013).

Experiment of Li et al. (2011) showed that 133 conserved miRNAs representing 95 miRNA families were expressed in soybean under three treatments. In addition, 71, 50, and 45 miRNAs are either uniquely or differently expressed under drought, salinity, and alkalinity, respectively, suggesting that many miRNAs are inducible and are differentially expressed in response to certain stress. Thus the miRNAs act as a master regulator modulating various gene expressions in plants in response to abiotic stress (Gupta et al., 2014) and plant growth (Chen, 2005; Rhoades et al., 2006; Mallory and Vaucheret, 2006). The miRNAs act by cleavage or through translational repression (Bartel, 2004). They play in feedback regulation of small RNA pathways and in the biogenesis of trans-acting siRNAs (Allen et al., 2005). They play major role in stress responses (Fujii et al., 2005; Sunkar et al., 2007; Zhang et al., 2006; Yang et al., 2007) enabling plants to survive under adverse conditions such as drought, salinity, and high/low temperature.

**Drought stress**

To understand the regulatory networks of stress tolerance, quantitative and qualitative analyses of gene expression are necessary. In order to restore the cellular function and make plants more tolerant to stress, transferring a single gene may not be sufficient to reach the required tolerance levels (Bohnert et al., 1995). To overcome such constraints, enhancing expression of a stress inducible transcription factor that regulates a number of other genes is a promising approach (Yamaguchi-Shinozaki et al., 1994; Chinnusamy et al., 2005). Eight libraries of small RNAs were constructed to identify new miRNA in soybean to verify those that are possibly drought regulated and submitted to Solexa sequencing. Sequencing and subsequent analyses of library 269 miRNAs detected. The libraries were developed from drought-sensitive and tolerant seedlings of soybean with or without stressors (Kulcheski et al., 2011). They examined miRNA expression profiles during abiotic stresses to soybean. miRNAs were up-regulated during drought stress in the sensitive plants. miRNAs (miR434a, miR157*, and miR171a) were identified as functional regulation factors in the resistance of stress. Kulcheski et al. (2011) showed that drought is the major abiotic stress factor to negatively affect soybean productivity around the world. The impact of limited water during the flower formation can cause shorter flowering periods, and water stress during the later phases of soybean reproductive development has been reported to accelerate senescence, which decreases the duration of the seed-filling period.

**Salt stress**

Salt stress (abiotic stresses) is also a serious problem for crop plants worldwide. In salt stress condition where excessive salts in soil solution cause inhibition of plant growth or plant death. High salt stress leads to disruption of homeostasis in water potential and ion distribution which occurs at both the cellular and the whole plant levels. Drastic changes in ion and water homeostasis lead to molecular damage, growth arrest and even death of plants. Salt stress furthermore induces the ABA synthesis which closes stomata when transported to guard cells that ultimately decreases photosynthesis activity and leads to oxidative stress. According to Gupta et al. (2014), salinity is major problem in agricultural agronomy. A huge number of salt regulated micro RNAs have been identified in soybean in the salt stressed that plays either a direct or indirect role and regulate up and down in salt stress alleviation.

The miR159b c, miR169c and miR319a, b, miRNA identified in soybean which were highly down-regulated and gly1, gly3, iR171p and iR4416d were also identified in soybean which
highly up-regulated by salt. So many gene transcripts gets up or down regulated during salt stress suggesting the tight regulation of transcription during stressed condition in plants. Therefore, post-transcriptional gene regulation plays a crucial role in the plant salt response. In previous studies it has been reported that members of miR167, miR319, and miR393 were similarly regulated in stress tolerance (Gao et al., 2010; Sunkar et al., 2007). In this study, members of miR1520n miR4374b, and miR4396 were up-regulated simultaneously under salt, and drought stresses. Mangrauthia et al. (2013) identified 133 conserved miRNAs representing 95 miRNA families were differentially expressed in soybeans under different stress treatments along with 50 miRNAs differently expressed under salt stress (Li et al., 2011). The targets sulfurylase and ASPI genes are regulated by miR395 in salt induced soybean line under sulfate starvation conditions with non-specific salt stress responding pathways, such as the maintenance of energy supply (Ding et al., 2009 and Bartel, 2004). Salinity is a significant problem affecting physiological, biochemical and molecular processes of plants and is predicted to become a larger problem in the coming decades. The detrimental effects of high salinity on plants can be observed at the whole-plant level or in the cellular level in terms of plant death and/or decrease in productivity (Mangrauthia et al., 2013). Above the study of miRNA in salt stress, we can say that several miRNA have been identified for salt stress that play a major role in salt stress and express in up and down regulation under salt stress. According to the study of Mangrauthia et al. (2013) by modifying their gene expression through the post transcriptional gene regulation, plants respond to salt stress. Hence, in addition to their roles in growth and development and maintenance of genome integrity, miRNAs are also important components in plant stress responses.

**Rust stress**

Eight libraries of small RNAs were constructed to identify new miRNA in soybean to verify those that are possibly rust-stress regulated and submitted to Solexa sequencing. Sequencing and subsequent analyses of library 269 miRNAs detected. The libraries were developed from rust-susceptible and resistant seedlings of soybean with or without stressors (Kulcheski et al., 2011). Most miRNAs did not vary during the fungus infection in the susceptible genotype and for the tolerant genotype; most of the miRNAs remained unchanged. miRNAs (miR434a, miR157b*, and miR171a) genes were identified as functional regulation factors in the resistance of stress. Mishra et al. (2013) identified and isolated some compatible bacterial and fungal isolate and their effectiveness against plant disease. The mode of action may be regulated by miRNA. Kulcheski et al. (2011) studied rust-stress assay and constructed the four libraries of small RNAs from leaves which were compounded by: 1) leaves of rust-susceptible seedlings with mock inoculation (Rust- Susceptible Leaf Control (RSLC)); leaves of rust-susceptible seedlings with rust-spore inoculation (Rust-Susceptible Leaf Treated (RSLT)); leaves of rust-resistant seedlings with mock inoculation (Rust-Resistant Leaf Control (RRLC)); and leaves of rust-resistant seedlings with rust-spore inoculation (Rust-Resistant Leaf Treated (RRLT)).

**miRNA response for root nodulation in soybean**

Li et al. (2010) studied that three miRNA’s viz., miR482, miR1512, miR1515 were established for their function in root nodulation as identified in soybean. In other study, Li et al. (2010) examined that the gene expression levels of six families of novel miRNAs and investigated their functions in nodule development in soybean. miRNAs also play critical and diverse roles in symbiotic nitrogen fixation. To identify potential regulators early in nodule development, Subramanian et al. (2008) inoculated soybean (Glycine max) roots with Brady rhizobium japonicum and identified differentially expressed miRNAs. For the example, miR168 and miR172 were upregulated at 1 or 3 hpi but returned to basal levels by 12 hpi; miR159 and miR393 were upregulated by 3 hpi and continued to maintain these levels to 12 hpi; miR160 and miR169 were downregulated in response to rhizobia. Also, rhizobial infection changed the levels of miR166, miR932, miR164, and miR168, which target ARFs (ARF10, ARF16, and ARF17), TIR1, NAC1, and AGO1, respectively (Li et al., 2010). The results suggested that miR482, miR1512, and miR1515 might have specific and important functions during soybean nodulation. Cloning and sequencing of miRNAs from functional nodules of soybean revealed conserved miRNAs (miR167, miR172, miR396, and miR399), whereas four other families had sequences homologous to Gm-miR1507, Gm-miR1508, Gm-miR1509, and Gm-miR1510, which play a role in nitrogen fixation.

Table 1: Differential expression of miRNAs

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<th>miRNA</th>
<th>Plant species</th>
<th>Response</th>
<th>References</th>
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<tr>
<td>miR1510a-3p, 5p</td>
<td>Glycine max</td>
<td>Up-regulated</td>
<td>Li et al., 2011</td>
</tr>
<tr>
<td>miR169d</td>
<td>Glycine max</td>
<td>Up-regulated</td>
<td>Li et al., 2011</td>
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<tr>
<td>miR396e</td>
<td>Glycine max</td>
<td>Up-regulated</td>
<td>Li et al., 2011</td>
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<tr>
<td>miR171b-5p</td>
<td>Glycine max</td>
<td>Up-regulated</td>
<td>Li et al., 2011</td>
</tr>
<tr>
<td>miR156f</td>
<td>Glycine max</td>
<td>Up-regulated</td>
<td>Li et al., 2011</td>
</tr>
<tr>
<td>miR395a</td>
<td>Glycine max</td>
<td>Up-regulated</td>
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### Future aspect

In this context, our goal was to identify new miRNAs and to discover those that may be regulated by moisture stress. Using high-throughput sequencing, we sequenced small RNAs from the roots of drought-sensitive and tolerant seedlings in response to control or water deficit conditions. A total of 256 miRNAs were detected by sequencing and the expression profile of several miRNAs varied during abiotic and biotic stresses. We consider that the identification of these miRNAs is important to understanding small RNA-mediated gene regulation in legumes under water deficit stress.

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