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The role of miRNAs in legumes with a focus on abiotic stress response

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Abstract

Legumes are special group of nitrogen-fixing plants that are an essential component of cropping system and important source of food/feed for human/animal consumption. Like other crops, the productivity of legumes is threatened by environmental stresses caused due to global climate change. Abiotic stress tolerance is complex trait involving a suite of genes, the expression of which is controlled by transcription factors including gene/polypeptide sequences. Recently, microRNAs (miRNAs) have been increasingly recognised for their role in regulating the synthesis of polypeptides from different mRNAs including those that act as transcription factors. This review summarizes the current knowledge on the role of different miRNAs in response to main abiotic stresses in legumes. We found consistent as well as conflicting results within and between different legume species. This highlights that we have barely scratched the surface and very comprehensive and targeted experiments will be required in future to underpin the role of miRNAs in controlling the expression of important genes associated with abiotic stress tolerances.

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Introduction

Legumes belong to the family Fabaceae, previously Leguminosae, which includes some of the world's most important food and feed crops such as; *Glycine max* (Soybean), *Phaseolus vulgaris* (common bean), *Pisum sativum* (pea), *Cicer arietinum* (chickpea), *Medicago sativa* (alfalfa) and *Arachis hypogea* (peanut). Together, they account for one third of global primary crop production and are vital to meet the growing population demands. Legumes are rich in protein, oil, fibre and micronutrients, and are highly valued in the cropping cycle due to their ability to fix atmospheric nitrogen and act as a disease break between cereal or oilseed crops. Under conducive environmental conditions, legumes establish symbiotic relationships with arbuscular mycorrhizal (AM) fungi, leading to the formation of arbuscules, sites of phosphorous nutrient exchange (Parniske, 2008).

The three most intensively studied legume genomes, due to economic significance and/or suitable model features are: 1) *Medicago truncaluta*, a self-fertile plant with a small diploid genome (~500MB) with a short generation time; 2) *Lotus japonicus*, which has a diploid genome (about 470MB) and a short life cycle; and 3) *Glycine max*, with an amphidiploid genome (~1.1Gb) (Sato et al., 2010). These species genomes have been completely sequenced and a multitude of genomics tools are available for each in the public domain (http://www.plantgdb.org/MtGDB/ http://www.plantgdb.org/LjGDB/ and http://www.plantgdb.org/GmGDB/). Recently, the chickpea genome (wild and cultivated species) was sequenced by two different groups (Varshney et al., 2013; Jain et al., 2013).

Genomic tools developed for chickpea include BAC libraries, cDNA/EST databases, microarrays, high density linkage maps and mutant libraries.

In the last few years, small RNAs were determined to be important regulators of gene expression and plant growth (Chen, 2005; Jones-Rhoades et al., 2006; Mallory and Vaucheret, 2006). Consequently, interest has increased in studying their role in legumes, particularly in the symbiosis with the mycorrhizae and nitrogen fixation (Simon et al., 2009; Branscheid et al., 2010). There are two major classes of endogenous small RNAs in plants; microRNAs (miRNAs) and small-interfering RNAs (siRNAs). The miRNAs are ~20-24-nt non-coding single stranded RNAs, processed from imperfectly folded hairpin-like precursors by the Dicer-Like1complex (Jones-Rhoades et al., 2006; Ramachandran and Chen, 2008). Conversely, siRNAs (20-24 nt) are processed by other members of the Dicer protein family (DCL2, DCL3 and DCL4) from long, perfectly paired double-stranded RNAs (dsRNAs). These dsRNAs result from the transcription of inverted repeats, from the convergent transcription of sense–antisense gene pairs or due to activity of RNA-dependent RNA polymerases (RDRs) on aberrant transcripts (Allen et al., 2005; Vaucheret, 2006). Both miRNAs and siRNAs play important roles in plant growth and development (Jones-Rhoades et al., 2006; Khraiwesh et al., 2012).

The miRNAs regulate gene expression in plants by targeting mRNAs for cleavage or through translational repression (Bartel, 2004). They affect diverse processes such as leaf morphogenesis, floral organ identity, and root development (Mallory and Vaucheret, 2006; Sunkar et al., 2007). They also function in the feedback regulation of small RNA pathways and in the biogenesis of trans-acting siRNAs (Allen et al., 2005). They have been implicated in a

wide array of stress responses (Fujii et al., 2005; Sunkar et al., 2006; Zhang et al., 2006a; Yang et al., 2007) enabling plants to survive under adverse conditions such as drought, salinity, and high/low temperature. This involves triggering sophisticated mechanisms governed by complex gene networks. Although there have been significant in depth gene studies of the tolerance mechanisms, relatively little is known about the functional roles of miRNAs within them, particularly in non-model plants (Griffiths-Jones, 2006, Zhang et al., 2006b). Such studies are required to fully understand the mechanisms by which crop plants survive under adverse environmental conditions. Next Generation Sequencing (NGS) technology has greatly accelerated the discovery and characterisation of miRNAs in a range of diverse plant species (Lu et al., 2005; Rajagopalan et al., 2006; Fahlgren et al., 2007; Sunkar and Jagadeeswaran, 2008; Zhao et al., 2010; Chen et al., 2011; Li et al., 2011). In this review, we focus on the current understanding of miRNA involvement in combating abiotic stresses in legumes. However, up until recently, legumes have been orphaned from the developments in functional genomics (Varshney et al., 2012). Therefore, whilst discussing the role of miRNAs in abiotic stress tolerance in legumes, we also point out important research performed in model crops such *Arabidopsis* and rice. These miRNAs can be validated in legumes in future studies.

miRNA biogenesis in plants

Initially, in plants, miRNA is expressed as a long primary miRNA (pri-miRNA) by *RNA A Pol II* enzyme (Lee et al., 2004) (Figure 1). Subsequently, the primary miRNA is processed by *RNase III* enzyme, *Dicer-Like1* enzyme and associated proteins to a stem loop intermediate called miRNA precursor or pre-miRNA (Bartel, 2004; Kurihara and Watanabe, 2004). Next, it is processed into the miRNA/miRNA* duplex and is exported from the nucleus to the cytoplasm by

HASTY (Bollman et al., 2003; Han et al., 2004; Chen, 2005; Griffiths-Jones et al., 2006). The mature miRNA is then derived from one of the imperfect strands by the *HYL1* protein and the miRNA* is derived from the other strand (Han et al., 2004). Finally, the mature, methylated miRNA, is incorporated into the RNA-induced silencing complex (RISC) containing *ARGONAUTE1*, which directs the RISC to regulate gene expression by either mRNA cleavage or translational repression (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 (Ambros et al., 2003; Bartel, 2004; Bartel, 2009; Voinnet, 2009). Single mature miRNA can be present in several variant forms called isomiRNAs (isoforms of microRNAs) (Guo and Lu, 2010), which are caused by an imprecise or alternative cleavage of Dicer during pre-miRNA processing (Ebhardt 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.

Identification of miRNA in legumes

The first detected plant miRNAs were from *Arabidopsis thaliana* (Park et al., 2002; Reinhart and Bartel, 2002). Subsequently, they have been isolated from a wide range of species via genetic screening (Lee et al., 1993; Wightman et al., 1993), direct cloning after isolation of small RNAs (Fu et al., 2005; Lu et al., 2005) and computational prediction strategies (Bartel, 2004; Adai et al., 2005; Li et al., 2005; Sunkar et al., 2005; Wang et al., 2005; Jones-Rhoades et al., 2006). The genetic screening approach is very time consuming, laborious and costly, and has resulted in the identification of relatively few miRNAs (Lee et al., 1993; Wightman et al., 1993). On the contrary, the direct cloning and sequencing method identified many miRNAs became the method

of choice with the advent of NGS technology (Figure 2). Since 2005, this approach has been used successfully in many legumes such as *M. truncatula*, common bean, soybean, chickpea, peanut and lotus (Subramanian et al., 2008; Szittya et al., 2008). These species contain both conserved as well as novel miRNAs that may potentially regulate legume species-specific cell processes (Subramanian et al., 2008; Szittya et al., 2008). To date, a total of 1256 sequences belonging to 285 miRNA families have been identified from legumes in a publicly available miRNA database, miRBase (http://www.sanger.ac.uk/cgi-bin/Rfam/mirna/browse.pl accessed 19th March, 2013).

Knowledge of sequence and motif conservation together with NGS technology has enabled the development of computational algorithms for miRNAs identification from genome and EST databases (Adai et al., 2005; Li et al., 2005; Sunkar et al., 2005; Wang et al., 2005). Using this approach, conserved miRNAs have been identified from several species of the Fabaceae family (Zhang et al., 2006a; Sunkar and Jagadeeswaran, 2008). However, since many miRNAs are likely to be family or species specific, this approach is not sufficient, highlighting the continued need for large scale NGS sequencing.

NGS sequencing was first applied to identify novel non-conserved miRNAs in Arabidopsis (Rajagopalan 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 Jagadeeswaran, 2008; Szittya et al., 2008). To date, a limited number have been identified via this approach in legume species (Dezulian et al., 2006; Zhang et al., 2006b; Li et al., 2008; Zhou et al., 2008). These include: *Acacia auriculiformis* (7

precursors, 7 mature), *Arachis hypogaea* (23 precursors, 32 mature), *Acacia mangium* (3 precursors, 3 mature), *Glycine max* (506 precursors, 555 mature), *Glycine soja* (13 precursors, 13 mature), *Lotus japonicus* (3 precursors, 4 mature), *Medicago truncatula* (675 precursors, 719 mature), *Phaseolus vulgaris* (8 precursors, 10 mature), and *Vigna unguiculata* (18 precursors, 18 mature). These have been deposited in a publicly available miRNA database, miRBase (http://www.sanger.ac.uk/cgi-bin/Rfam/mirna/browse.pl accessed 19th March, 2013). Several sets of novel species specific (legume) miRNAs have been reported, including 87 novel and 42 conserved in soybean (Subramanian et al., 2008; Wang et al., 2009; Joshi et al., 2010). In excess of 100 novel miRNAs were identified in *M. truncatula* (Szittya et al., 2008; Jagadeeswaran et al., 2009; Lelandais-Briere et al., 2009). Further, six stress responsive and 16 evolutionarily conserved miRNA families were identified from *P. vulgaris* (Arenas-Huertero et al., 2009). 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). Further, NGS technology has also been successfully used to systematically identify stress-associated miRNAs (Li et al., 2010; Chen et al., 2011; Li et al., 2011; Wang et al., 2011; Zhou et al., 2012) (Table 1).

The role of miRNA in abiotic stress

Due to their sessile nature, plants have evolved various complicated mechanisms to overcome a variety of environmental stresses such as drought, salinity, extreme temperatures and availability of micronutrients in soil. The miRNAs play an important role in these mechanisms by regulating the expression of thousands of genes. To date, many miRNAs involved in a variety of abiotic stress responses have been predicted. However, few have been functionally confirmed.

In this section we summarize miRNAs that are predicted to be involved in various stress responses in legumes.

miRNA expression in response to drought, cold and salinity

Plants suffer from variety of abiotic stresses, however drought, heat, cold and salt stress are more frequently encountered. Drought is one of the most ubiquitous environmental stresses affecting plant growth and development as majority of crops are grown under rainfed conditions. Recent studies in various plants species suggest that miRNAs play important role in drought tolerance. These include conserved miRNAs such as miR164, miR169, miR171, miR396, miR398, miR399, miR408 and miR2118 (Liu et al., 2008). Their expression patterns vary with species. For example, miR169 was down-regulated in *Arabidopsis* and *M. truncatula* (Li et al., 2010; Trindade et al., 2010) but up-regulated in common bean (in response to abscisic acid treatment) and rice (Arenas-Huertero et al., 2009; Zhao et al., 2009). In *M. truncatula*, miR398a,b and miR408 were strongly up-regulated in shoots and roots under drought stress (Trindade et al., 2010). The miR398 and miR408 repress the *COX5b, CSD1* and plantacyanin genes (Trindade et al., 2010).

Recently, Wang et al., (2011) identified 22 members of 4 miRNA families were up-regulated and 10 members of 6 miRNA families were down-regulated in response to drought stress in *M. truncatula*. Among the 29 new miRNAs/new members of known miRNA families, 8 miRNAs were responsive to drought stress with 4 miRNAs each being up- and down-regulated. The known and predicted targets of the drought-responsive miRNAs were found to be involved in diverse cellular processes including development, transcription, protein degradation,

detoxification, nutrient status and cross adaptation. Further, a number of novel legume miRNA were also identified in *Phaseolus vulgaris*. For instance, pvu-miRS1, pvu-miR1514a, miR159.2, pvu-miR2118 and pvu-miR2119 accumulated upon drought and ABA treatments (Arenas-Huertero et al., 2009). Novel miRNAs may target regulatory elements for cellular processes that may be unique to legumes (Arenas-Huertero et al., 2009).

Using a different approach, small RNAs were sequenced from two cowpea genotypes (CB46, drought-sensitive, and IT93K503-1, drought-tolerant) that grew under well-watered and drought stress conditions. About 157 miRNA genes that belong to 89 families were identified by mapping small RNA reads to cowpea genomic sequences (Barrera-Figueroa et al., 2011). Among the 44 drought-associated miRNAs, 30 were up-regulated in drought condition and 14 were down-regulated. Although miRNA expression was in general consistent in two genotypes, 9 miRNAs were predominantly or exclusively expressed in one of the two genotypes and 11 miRNAs were drought-regulated in only one genotype, but not the other (Barrera-Figueroa et al., 2011). In a similar study in soybean, drought tolerant and sensitive genotypes were subjected to drought stress and miRNAs that were differentially expressed characterised. By sequencing drought tolerant and sensitive genotypes as well as rust tolerant and sensitive seedlings, they identified a total of 24 families of novel miRNAs that had not been reported before, six families of conserved miRNAs that exist in other plants species, and 22 families previously reported in soybean (Kulcheski et al., 2011). They observed the presence of several isomiRNAs during the analyses. A striking feature however was that majority of the miRNAs (miR166-5p, miR169f-3p, miR1513c, miR397ab and miR-Seq13), were up-regulated during water deficit stress in the sensitive plants whilst, for the tolerant genotypes, these miRNAs were down-regulated

(Kulcheski et al., 2011). The miRNAs that were differentially expressed in the tolerant/sensitive genotypes under drought stress may potentially be regulating genes associated with drought tolerance/sensitivity and should be further investigated.

Salt stress is also responsible for decline in crop productivity and approximately 6% of the global arable land is affected by excess salt (Munns and Tester, 2008). Several studies have demonstrated that plants express a variety of miRNAs in response to salt stress (Sunkar and Zhu, 2004; Lu et al., 2008; Arenas-Huertero et al., 2009). Soybean miRNAs searches have also identified some potential candidates (Zhang et al., 2008; Chen et al., 2009). In one study, soybean miRNAs associated with abiotic stresses (drought, salinity, and alkalinity) were identified and analyzed with deep sequencing. One hundred and thirty three conserved miRNAs representing 95 miRNA families were expressed in soybeans under these treatments (Li et al., 2011). Out of these, 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. In addition, other genome-wide studies in *Arabidopsis*, rice, soybean, maize and *Populus* have identified salt responsive miRNAs such as miR393, miR394, miR396 and miR156 (Sunkar and Zhu, 2004; Liu et al., 2008; Ding et al., 2009; Gao et al., 2011; Li et al., 2011).

Recently, the expression profiles of nine different miRNAs were analysed in *Phaseolus vulgaris* seedlings in response to 0.4 M NaCl and drought stress. The miR395 was most sensitive to both stresses and was up-regulated by 616 and 2810-folds by 1.00% PEG and 0.4 M NaCl, respectively (Nageshbabu and Jyothi, 2013). Further, miR396 and miR172 were up-regulated after exposure to both the stresses. The miR396 has been shown to function in leaf development

(Liu et al., 2009) and expression of miR396 has been shown to be induced under high salt, cold, and drought stresses (Liu et al., 2008). Interestingly, over-expression of miR396 leads to an increased tolerance to drought stress (Feng-Xi and Di-Qiu, 2009). The authors found that individual miRNA expression profiles varied between the two different stresses, indicating that salt and drought stresses induce differential miRNA expression through different mechanisms, such as oxidative stress or inhibition of plant growth. They also reported that salt and drought conditions induced the expression of *APX* and *ADH*, two stress-related plant genes, in *Phaseolus vulgaris*.

To understand the dynamic regulation of miRNAs in functioning nodules during salt stress response, deep sequencing of miRNAs was performed in normal and salt stressed-soybean mature nodules (Dong et al., 2013). The authors identified 110 known miRNAs belonging to 61 miRNA families and 128 novel miRNAs belonging to 64 miRNA families. Among them, 104 miRNAs were dramatically differentially expressed (>2-fold or detected only in one library) during salt stress. The miR159b,c, miR169c and miR319a,b, were highly down-regulated and gly_1, gly_3, miR171p and miR4416d were highly up-regulated by salt (Dong et al., 2013). Further, when the 128 novel miRNAs representing 64 families were compared with other known plant miRNAs in the miRBase database, they found that 66 miRNAs representing 27 known miRNA families had identifiable locus in these plant species, 12 miRNAs were conserved in legumes, and strikingly 10 miRNAs (miR1513d, miR1520s, miR4357b,c, miR4416b, miR4416c, miR5037e, miR862c, miR1507d, miR4405b, miR862d) were only found in soybean (Dong et al., 2013).

In cowpea, the expression of 18 conserved miRNAs belonging to 16 distinct miRNA families was evaluated under salt stress. Using the miRNA sequences, 15 potential target genes were predicted and all of them were identified as transcription factors. Seven of these predicted miRNAs (vun-miR156a, vun-miR159b, vun-miR160a, vun-miR162a, vun-miR168a, vun-miR169b and vun-miR408) were experimentally validated in the root tissues and found to be up-regulated during salt stress as revealed by qRT-PCR (Paul et al., 2011). In *Arabidopsis,* miR498 was expressed during drought and cold stress treatment, and expressed in cowpea during salt stress (Liu et al., 2008). Whilst in common bean, increased accumulation of miRS1 and miR159.2 was observed in response to NaCl addition (Arenas-Huertero et al., 2009). They also proposed that miR398 targets a superoxide dismutase in common bean and in other plants such enzymes play important roles in the oxidative stress response (Arenas-Huertero et al., 2009). These findings suggest that whilst there is some similarity, the response to salt stress may be species or even genotype-specific, potentially involving different miRNA-mediated regulatory strategies.

Interestingly, four miRNAs associated with cold tolerance in Arabidopsis (miR319, miR393, miR397, miR402) were analysed for similar role in sweet pea (*Pisum sativum*). Primers to these miRNAs were designed and their role in pea was investigated using RT-PCR. They showed that miR319, miR393, miR397, and miR402 probably exist in pea, and the level of their expressions increased after the cold treatment (Wang and Long, 2010).

miRNA involved in symbiosis

A striking feature of legumes is their ability to fix atmospheric nitrogen *via* establishment of a symbiotic relationship with soil rhizobacteria in specialized organs known as root nodules (Schultze and Kondorosi, 1998; Gresshoff, 2003). Nitrogen fixation is a complex process that involves a tight and synergistic regulatory network of genes from both the plant legume and the rhizobacteria. Nodule organogenesis begins with rhizobial root infection and the formation of root nodule primordial. This process is induced by compounds known as Nod factors which are secreted by the bacteria. Several legume receptor, receptor kinase, kinase and transcription factor (TF) genes are essential for this and subsequent steps of signal transduction cascades (reviewed by Oldroyd and Downie, 2004). Various studies were conducted to understand the signaling mechanisms regulating these processes (Gresshoff, 2003; Searle et al., 2003). Recently, miRNAs have been implicated in the legume–rhizobia symbiosis signaling regulation (reviewed by Simon et al., 2009). Initial studies indicated the involvement of only two miRNAs, miR166 and miR169, in the *Bradyrhizobium japonicum* infection and nodule development in *M. truncatula* and soybean (Combier et al., 2006; Boualem et al., 2008; Subramanian et al., 2008). The miR169 post-transcriptionally regulates the CCAAT-binding complex, *HAP2*-type TF (*HAP2.1*), which is a key regulator of nodule development (Combier et al., 2006). Meanwhile, miR166 down-regulates expression of the class-III homeodomain-leucine zipper (*HD-ZIP III*) TF involved in symbiotic nodule and lateral root development (Boualem et al., 2008; Subramanian et al., 2008).

Subramanian et al. (2008) identified 35 miRNAs involved in the early stages of soybean-*Rhizobium* nodule development and that were responsive to *Bradyrhizobium japonicum* inoculation. Subsequently, soybean specific miRNAs (22) and novel miRNAs from mature nodules (4) were identified (Wang et al., 2009). Further, using NGS and bioinformatic

approaches, 87 more novel miRNAs were identified in soybean and their target genes were predicted (Zhang et al., 2008; Wang et al., 2009; Joshi et al., 2010). In particular, up-regulation of miR168 and miR172 and down-regulation of miR169 was observed during the early soybean-*Rhizobium* nodulation process. These were involved in altering the concentration of Auxin Response Factors (*ARF*s), necessary for phytohormone homeostasis (Subramanian et al., 2008). Similarly, 11 miRNA families were identified to be involved in the later stages of the soybean-*Rhizobium* nodulation process (Wang et al., 2009). Transgenic expression of miR482, miR1512 and miR1515 resulted in increased soybean nodulation, with differential expression among non-nodulating and super nodulating genotypes (Li et al., 2010). These studies reveal that soybean-*Rhizobium* nodulation is a complicated gene regulatory cascade leading to symbiosis establishment and operation involving many miRNAs.

Similar studies for identifying miRNA involved in symbiosis were conducted in *M. truncatula*. Eight novel miRNAs, four of which were *M. truncatula*-specific, were identified in shoot:root libraries. A total 100 novel candidate miRNAs were mapped in the *M. truncatula* genome, which were mined from deep sequencing of nodules and root tip sRNA libraries (Jagadeeswaran et al., 2009; Lelandais-Briere et al., 2009). *In situ* studies revealed that novel miRNAs, miR2586 and miR107, accumulated in the nodule meristem, while miR396 accumulated in the root tips. This led to speculation that these miRNAs function in stem cell renewal. Similarly, miR172 and miR398 were enriched in the nodule invasion zone, suggesting a function in cell differentiation or bacterial release into the plant cytoplasm. The predicted targets for *Bradyrhizobium*-responsive miRNAs were TFs, proteins involved in hormonal signaling pathways and cell cycling (El Yahyaoui et al., 2004). The miR169 was proposed to be involved

in nodule development via regulating expression of TF *MtHAP2-1*. Also although the function remains unknown, both miR2568 and miR107 showed differential expression among nodulating and non-nodulating genotypes (Combier et al., 2006; Lelandais-Briere et al., 2009; Wang et al., 2009; Turner et al., 2012). The nodular expression of many miRNAs is influenced by nutrient (phosphorus, iron, nitrogen and manganese) stress conditions in common bean. For example, under manganese toxicity, 11 miRNAs were strongly induced and another 11 were strongly inhibited in leaf or root tissues (Valdes-Lopez et al., 2010).

miRNA involvement in nutrient homeostasis

Phosphorus (P) is a major component of many macromolecules involved in many essential plant metabolic processes. Taken up by roots as inorganic phosphorus (Pi) from soil, it is one of the most limiting micronutrient for plant growth within agricultural systems. Therefore in order to meet the phosphorus requirement, many plant species develop mutualistic associations with Arbuscular Mycorrhizal (AM) fungi. The establishment of such associations and their subsequent functioning are reliant on complex signalling and interaction. Accordingly, microRNA are vital signalling and regulatory factors in P starvation stress (Schachtman and Shin, 2007; Chen et al., 2008; Valdes-Lopez and Hernandez, 2008; Yuan and Liu, 2008). Recently, miR399 was reported to play a key role in maintaining Pi homeostasis in Arabidopsis. The miRNA is induced during phosphorus starvation, causing repression of the ubiquitin conjugating enzyme, *UBC24*, a repressor of phosphate transporters (Chiou et al., 2006). This miRNA is also induced during P deficiency signalling in common bean and *M. trucatula* (Valdes-Lopez and Hernandez, 2008). Under P deficiency, mycorrhizal roots of *M. truncatula* have increased levels of miR399, which limits *Mt*PHO2 expression, compared to levels in non-

mycorrhizal roots. Thus this miRNA is proposed to be involved in the regulation of AM symbiosis. In common bean, the miRNA PvmiR399 regulates the MYB transcription factor PvPHR1, which plays a key role in regulating the expression of target genes involved in phosphorous transport, remobilization and homeostasis (Valdes-Lopez and Hernandez, 2008). Further, deep sequencing studies revealed that 10 miRNAs (miR157, miR160, miR165, miR166, miR169, miR393, pvumiR2118, gma-miR1524, gma-miR1526 and gma-miR1532) were differentially regulated under P deficiency in several common bean organs (Valdes-Lopez et al., 2010).

Deep sequencing of *Arabidopsis* has revealed additional P starvation-responsive miRNAs such as miR156, miR778, miR827 and miR2111 (up-regulated during P starvation) and miR169, miR395 and miR398 (down-regulated during P starvation) (Hsieh et al., 2009; Pant et al., 2009). In Soybean, 57 miRNAs were differentially expressed under P deficiency (Zeng et al., 2010). Subsequently, deep sequencing of soybean root and shoot libraries constructed under P stress identified 60 known and conserved responsive miRNAs, belonging to 35 families. Also, 16 novel predicted miRNAs were identified (Sha et al., 2012). In a larger study, 167 miRNAs, belonging to 35 families, were identified via differential expression in response to P deficiency in white lupin of which, 17, 9 and 10 were found to be up-regulated, while 7, 6 and 12 were down-regulated in roots, stems and leaves, respectively (Zhu et al., 2010). Further, four small RNA libraries from leaves and roots of soybean plants grown under phosphate-sufficient and P-depleted conditions were sequenced recently. Collectively, 25 miRNAs were induced and 11 miRNAs were repressed by P starvation in soybean (Xu et al., 2013). They identified organ-

specific expression of some miRNAs highlighting different role of the same miRNAs in different organs.

Apart from P, other nutrients such as copper (Cu), sulphur (S), Aluminum (Al) and Nitrogen (N) are essential for the essential plant metabolic processes (Grotz and Guerinot, 2006). Several miRNAs have been associated with maintaining these processes. For example, in *Arabidopsis*, miR397, miR398, miR408, and miR857 were proposed to maintain Cu homeostasis during Cu deficiency through the regulation of Cu:zinc superoxide dismutase (*CSD1* and *CSD2*), plantacyanin and various laccases (Abdel-Ghany and Pilon, 2008; Yamasaki et al., 2008). Similarly, miR395 regulates ATP sulphurylase (*APS4*) and a sulphate transporter (*AST68*) when maintaining S homeostasis during S deficiency. This has been reported in legume species (Szittya et al., 2008; Kawashima et al., 2009). Further, in *Arabidopsis*, under N limitation, miR167 is associated with lateral root outgrowth (Gifford et al., 2008), and the repression of miR169 and miR398a during N limitation is also reported (Pant et al., 2009).

A deep sequencing study in soybean from libraries of Al^{3+} treated and non-treated roots identified an additional 30 Al^{3+} stress responsive miRNAs. Of these, 10 were conserved miRNAs that belonged to seven families, 13 were unconserved and seven were novel (Zeng et al., 2012). More recently, several *M. truncatula* miRNA (miR160, miR319, miR396, miR1507 miR1510a and miR390) were identified as down-regulated and a further two (miR166 and miR171) not responsive to Al^{3+} treatment (Chen et al., 2012). In Soybean, miR396, miR390 and mir1510a-p5 were up-regulated, miR156, miR164 and miR169 were down-regulated and miR1510a was non responsive to Al^{3+} (Zeng et al., 2012). Using a computational approach, (Zhou et al., 2008),

identified 26 new miRNA candidates including miR160, miR166, miR319, miR393, and miR398 that were responsive to mercury, cadmium and Al^{3+} stresses. Their differential expressions were subsequently assessed in various *M. truncatula* organs and tissues.

Several studies have reported that alteration of the availability of one nutrient affects availability of other nutrients, resulting in unexpected interactions among miRNAs (Grotz and Guerinot, 2006; Haydon and Cobbett, 2007). For example, in common bean, Cu content increased under nitrogen and iron deficiency and miR398 and miR408 were down-regulated, Conversely, under acidic and manganese toxicity conditions, Cu was decreased and miR398 was up-regulated. Similar results were reported for *Arabidopsis*, wherein the *SQUAMOSA* promoter binding protein-like 7 (*SPL7*) activated the transcription of miR397, miR398, and miR408 under low Cu conditions (Yamasaki et al., 2009; Valdes-Lopez et al., 2010). Valdes-Lopez et al., (2010) showed that some miRNAs were expressed similarly under the same stress conditions in different organs, suggesting their possible interaction in response to the same nutrient stress. For example, pvu-miR1511, gma-miR1513, gma-miR1515, and gma-miRNA1516 were strongly expressed, specifically, in iron deficient leaves, indicating their participation in the iron signal transduction pathway in this organ.

Conclusions: similar and conflicting results that need further investigation

miRNA discovery has opened a new avenue to better detect and understand complex regulatory systems in plants and in particular those involved in abiotic stress tolerances. This review summarizes recent developments in legume miRNAs and their versatile roles in various stress responses, in particular focusing on abiotic stresses.

To date, hundreds of plant miRNAs have been identified using several traditional methods from a variety of species. In addition, large numbers of miRNA targets have been computationally predicted, some of which have been validated. Furthermore, the most recent applications of NGS together with the gamete of available genomics tools, has provided sufficient data to discover and characterise even low copy miRNAs that are expressed during a particular stress. These studies reveal the potential for enormous adaptive functional diversity during stress tolerance responses and provide a rich foundation for future functional research. This information will provide the ability to select for adaptive diversity leading to development of stress tolerant crops. Specifically, a better understanding of the role of miRNA in the symbiotic relationships of legumes in overcoming several important agriculturally limiting environmental stresses is of high priority.

As observed from the above review, although we have gained a lot of insight into role of miRNAs in response to abiotic stresses, there are both, similar and contradicting gene expression results in response to various abiotic stresses in legumes. For instance, under drought stress, miR171, miR156 and miR395 are up-regulated in *Vigna unguiculata* and *Glycine max*, and miR159 is up-regulated in *Phaseolus vulgaris* and *Vigna unguiculata*. On the contrary under drought stress, miR1510 is up-regulated in *Glycine max* whilst being down-regulated in *Medicago truncatula*. Similarly, miR396 was down-regulated in *Medicago truncatula* and *Vigna unguiculata*, whilst being up-regulated in *Glycine max*. More similarities and differences of miRNA expression in different legume species can be found in Table 1.

While the similarities in miRNA expression under an abiotic stress condition are encouraging and these miRNAs are promising candidates in improving abiotic stress tolerance in legumes, we cannot choose to ignore the differences. The differences in miRNA expression in different legumes could possibly be due to differences in the cultivation of plants, the level of treatment applied, differences in time points at which tissues were sampled, or even differences in tissues analysed (roots, shoot, leaves, *etc*.). A good example highlighting differences in cultivation and tissue sampling is that in soybean, whilst miR482 was found to be up-regulated under drought stress in one study (Li et al., 2011), it was down-regulated in both, tolerant and sensitive genotypes in another study (Kulcheski et al., 2011). Moreover, some studies have used the same genotype to look at miRNA expression in response to several abiotic stresses (example: drought, salt and alkalinity by Li et al., 2011). The genotype used in these studies could be tolerant to one abiotic stress but sensitive to the other, therefore generating conflicting results of miRNA expression when compared to other genotypes of same species or to a different species.

The bottom line is abiotic stress response is very complex and as researchers, we have to come up with and agree on a universal stress treatment, tissue sampling, tissue processing and data analysis pipeline so that results between different species become comparable. However, different species react to drought, salt, and other treatments differently. For example, one species could be more drought tolerant than other enabling it to tolerate low to moderate drought without change in gene expression. Therefore, the stress treatment and tissue collection points should be based on the physiological state of the plant (example, leaf oxidation status, osmotic potential, chlorophyll content, *etc*.) rather than days after sowing or treatment. Also, it is very important to conduct physiological experiments and field trials to know if a particular genotype is tolerant or

sensitive to a particular environmental stress before drawing conclusions about its involvement in abiotic stress tolerance.

Further, once candidate miRNAs and their target genes are identified, they should be validated using transgenics. Some of this work has already been started in legumes. Recently, Ni et al. (2013) identified and characterized a gene, *GmNFYA3*, which is target gene for miR169 in soybeans (*Glycine max*). Real time RT-PCR analysis indicated that *GmNFYA3* was induced by abscisic acid (ABA) and abiotic stresses, such as polyethylene glycol, NaCl and cold. Subcellular localization analysis suggested that *GmNFYA3* may activate its specific targets in the nucleus. Co-expression in *Nicotiana benthamiana* and 5′ RACE assays indicated that miR169 directs *GmNFYA3* mRNA cleavage *in vivo*. Overexpression of *GmNFYA3* resulted in Arabidopsis with reduced leaf water loss and enhanced drought tolerance. In addition, the transgenic Arabidopsis exhibited increased sensitivity to high salinity and exogenous ABA. Moreover, the transcript levels of ABA biosynthesis (*ABA1*, *ABA2*), ABA signaling (ABI1, ABI2) and stress-responsive genes, including *RD29A* and *CBF3*, were generally higher in *GmNFYA3* plants than in wild-type controls under normal conditions. These results suggest that the *GmNFYA3* gene and miR169 function in positive modulation of drought stress tolerance in soybean and other crops.

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Figure 1: The schematic diagram of the miRNA biogenesis in plants

Figure 2: Flow chart for miRNA isolation and characterisation using Next Generation Sequencing (NGS) technology

Table1: miRNAs expression pattern under important abiotic stress conditions in legume species.

miRNA	Plant species	Response	Reference
Drought stress			
miR398a,b miR398b,c	*Medicago truncatula*	Up-regulated Down-regulated	Trindade et al 2010 Wang *et al.*,, 2011
miR408	*Medicago truncatula*	Up-regulated	Trindade et al 2010
miR399k miR2089 miR2111a-f,h-s miR2111g miR2111u,v miR5274b miR5558	*Medicago truncatula*	Up-regulated	Wang *et al.*,, 2011
miR4414a miR5554a-c	*Medicago truncatula*	Down-regulated	Wang *et al.*,, 2011
miR1510a-3p, 5p miR1510a	*Glycine max* *Medicago truncatula*	Up-regulated Down-regulated	Li *et al.*,, 2011 Wang *et al.*,, 2011
miR164a-c miR164	*Medicago truncatula* *Vigna unguiculata*	Down-regulated	Wang *et al.*,, 2011 Barrera-Figueroa *et al.*,, 2011
miR169 miR169g,j miR169 miR169d	*Medicago truncatula* *Medicago truncatula* *Vigna unguiculata* *Glycine max*	Down-regulated Down-regulated Down-regulated Up-regulated	Trindade et al 2010 Wang *et al.*,, 2011 Barrera-Figueroa *et al.*,, 2011 Li *et al.*,, 2011
miR396a miR396 miR396e	*Medicago truncatula* *Vigna unguiculata* *Glycine max*	Down-regulated Down-regulated Up-regulated	Wang *et al.*,, 2011 Barrera-Figueroa *et al.*,, 2011 Li *et al.*,, 2011
miR2118 pvu-miR2118	*Medicago truncatula* *Phaseolus vulgaris*	Up-regulated	Wang *et al.*,, 2011 Arenas-Huertero *et al.*, 2009
pvu-miR393 pvu-miR1514a pvu-miR2119 pvu-miRS1	*Phaseolus vulgaris*	Up-regulated	Arenas-Huertero *et al.*, 2009
pvu-miR159.2 miR159	*Phaseolus vulgaris* *Vigna unguiculata*	Up-regulated	Arenas-Huertero *et al.*, 2009 Barrera-Figueroa *et al.*,,

			2011
miR171c miR171 miR171b-5p	*Medicago truncatula* *Vigna unguiculata* *Glycine max*	Down-regulated Up-regulated Up-regulated	Wang *et al.*,, 2011 Barrera-Figueroa *et al.*,, 2011 Li *et al.*,, 2011
miR156 miR156f	*Vigna unguiculata* *Glycine max*	Up-regulated	Barrera-Figueroa *et al.*,, 2011 Li *et al.*,, 2011
miR395 miR395a	*Vigna unguiculata* *Glycine max*	Up-regulated	Barrera-Figueroa *et al.*,, 2011 Li *et al.*,, 2011
miR162 miR166 miR167 miR319 miR403 miR828 vun_cand001 vun_cand010 vun_cand041 vun_cand057	*Vigna unguiculata*	Up-regulated Up-regulated Up-regulated Up-regulated Up-regulated Up-regulated Up-regulated Up-regulated Down-regulated Down-regulated	Barrera-Figueroa *et al.*,, 2011
miR166-5p miR169f-3p miR397a,b miR1513c miR-Seq13	*Glycine max*	Up-regulated in sensitive but Down-regulated in tolerant genotype	Kulcheski *et al.*,, 2011
miR-Seq11 miR-Seq15	*Glycine max*	Up-regulated in sensitive but unchanged in tolerant genotype	Kulcheski *et al.*,, 2011
miR-482bd-3p	*Glycine max*	Down-regulated in both tolerant & sensitive genotypes	Kulcheski *et al.*,, 2011
miR482b miR482	*Glycine max* *Vigna unguiculata*	Up-regulated Down-regulated	Li *et al.*,, 2011 Barrera-Figueroa *et al.*,, 2011
miR4415b miR-Seq07	*Glycine max*	Up-regulated in both tolerant & sensitive genotypes	Kulcheski *et al.*,, 2011
miR394a miR829.1 miR1507a	*Glycine max*	Up-regulated	Li *et al.*,, 2011

miR1508a
miR1509a
miR1515
miR1520d,e,f,k,l,n,q
miR4341
miR4342
miR4345
miR4349
miR4351
miR4352a,b
miR4358
miR4359b
miR4360
miR4361
miR4362
miR4364a
miR4365
miR4366
miR4367
miR4369
miR4371a-c
miR4374b
miR4375
miR4378a
miR4379
miR4380a
miR4385
miR4387a,b
miR4390
miR4391
miR4393b
miR4394
miR4396
miR4397
miR4398
miR4399
miR4400
miR4401
miR4404
miR4405
miR4406
miR4407
miR4408
miR4409
miR4410
miR4411

Salt stress			
pvu-miR159.2 gma-miR159b,c	*Phaseolus vulgaris* *Glycine max*	Up-regulated Down-regulated in root nodules	Arenas-Huertero *et al.*, 2009 Dong *et al.*,, 2013
miR169d gma-miR169b,c	*Glycine max*	Up-regulated Down-regulated in root nodules	Li *et al.*,, 2011 Dong *et al.*,, 2013
miR395a miR395b,c	*Glycine max*	Up-regulated Up-regulated in root nodules	Li *et al.*,, 2011 Dong *et al.*,, 2013
miR482*,b gma-miR482	*Glycine max*	Up-regulated Up-regulated in root nodules	Li *et al.*,, 2011 Dong *et al.*,, 2013
miR1510a-5p gma-miR1510a	*Glycine max*	Up-regulated Up-regulated in root nodules	Li *et al.*,, 2011 Dong *et al.*,, 2013
miR1520d,e,l,n,q gma-miR1520b gma-miR1520c	*Glycine max*	Up-regulated Up-regulated in root nodules Down-regulated in root nodules	Li *et al.*,, 2011 Dong *et al.*,, 2013 Dong *et al.*,, 2013
miR2118 pvu-miR2118	*Glycine max* *Phaseolus vulgaris*	Up-regulated	Li *et al.*,, 2011 Arenas-Huertero *et al.*, 2009
pvu-miR393 pvu-miR2119 pvu-miRS1	*Phaseolus vulgaris*	Up-regulated	Arenas-Huertero *et al.*, 2009
miR4342 miR4344 miR4349 miR4351 miR4359b miR4363 miR4366 miR4369 miR4371c miR4374b miR4378a miR4380a miR4385 miR4387b miR4394 miR4397 miR4401 miR4404	*Glycine max*	Up-regulated	Li *et al.*,, 2011

miR4405 miR4406 miR4407 miR4408 miR4409 miR4411			
gly_1 gly_2 gly_3 gly_4 gly_5a gly_5b gly_6 gma-miR166a,b gma-miR390a-3p miR171g,j,u miR171o miR171p miR399i,j,k miR408a,c miR4416c miR4416d	*Glycine max*	Up-regulated in root nodules	Dong *et al.*,, 2013
gly_15 gly_16 gly_17 gly_18 gly_19 gma-miR160 gma-miR319a,b gma-miR1517 gma-miR1523 miR4416b miR5037e miR5559	*Glycine max*	Down-regulated in root nodules	Dong *et al.*,, 2013
Cold stress			
pvu-miR2118	*Phaseolus vulgaris*	Up-regulated	Arenas-Huertero *et al.*, 2009
Abscisic acid			
miR169 pvu-miR159.2 pvu-miR393 pvu-miR2118 pvu-miR2119	*Phaseolus vulgaris*	Up-regulated	Arenas-Huertero *et al.*, 2009

pvu-miR1514a
pvu-miRS1

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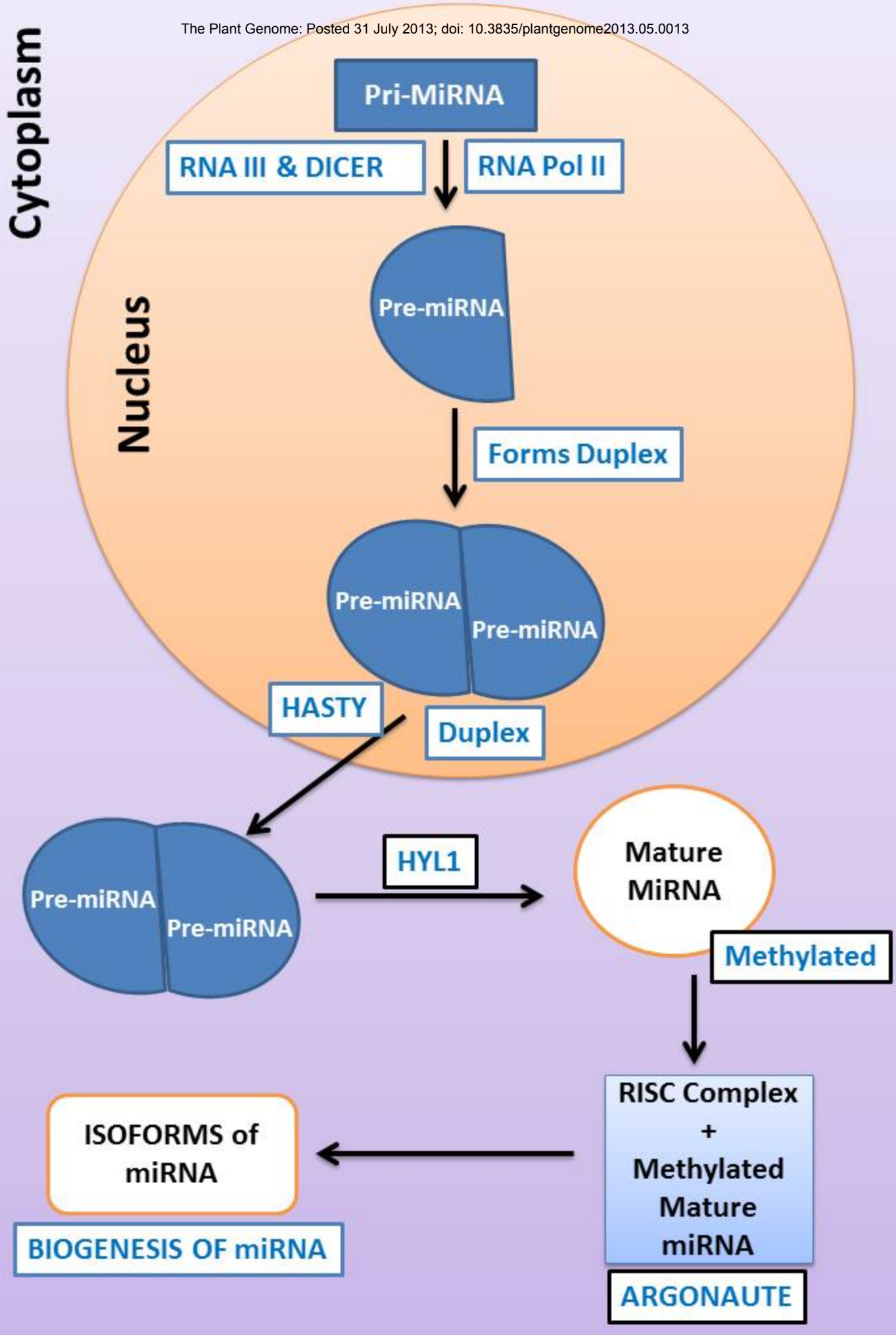

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