Review Article

Fine tuning of auxin signaling by miRNAs

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ABSTRACT

microRNAs (miRNAs) constitute a major class of endogenous non-coding regulatory small RNAs. They are present in a variety of organisms from algae to plants and play an important role in gene regulation. The miRNAs are involved in various biological processes, including differentiation, organ development, phase change, signaling, disease resistance and response to environmental stresses. This review provides a general background on the discovery, history, biogenesis and function of miRNAs. However, the focus is on the role for miRNA in controlling auxin signaling to regulate plant growth and development. **[Physiol. Mol. Biol. Plants 2008; 14(1&2) : 81-90]** *E-mail : neeti@icgeb.res.in*

Key words : *microRNAs; Biogenesis; Functions; Auxin; Plant development*

MicroRNAs (miRNAs) have recently been identified as the most important class of endogenous non-coding small RNAs. They comprise RNA molecules of 19-24 nucleotide (nt) length (Carrington and Ambros, 2003; Bartel, 2004) that are evolutionarily conserved across distantly related species, from worms and algae to higher eukaryotes (Pasquinelli *et al*., 2000; Axtell and Bartel, 2005; Zhang *et al*., 2006a; Molnar *et al*., 2007). A growing body of evidence has unveiled the significance of miRNA in regulating essential biological processes, including normal growth of cells and development of organisms as well as in maintaining the integrity of genomes.

History

The first endogenous ~22-nt RNA were identified as key regulatory molecules in the pathway controlling the timing of larval development in the nematode, *Caenorhabditis elegans*. Two research groups independently identified lin-4 as a post-transcriptional regulator of the heterochronic gene *lin-14*, which plays an important role in controlling the temporal pattern formation in *C. elegans* (Lee *et al*., 1993; Wightman *et al*., 1993). Seven years later, another small regulatory RNA, let-7 was found to control developmental timing and cellular differentiation in *C. elegans* (Reinhart *et al*., 2000). The sequence and developmental expression pattern of let-7 was highly conserved in a wide range of animal species, suggesting its important role in regulating biological processes (Pasquinelli *et al*., 2000). These findings initiated the discovery of miRNA and other small RNAs in different organisms. The identification of miRNAs in plants was reported in the year 2002 simultaneously by different groups (Llave *et al*., 2002; Reinhart *et al*., 2002; Park *et al*., 2002). Since then there has been an exponential increase in the identification of miRNAs as well as in understanding the significance of their roles in multiple biological processes.

Currently, around 5,071 miRNA sequences from primates, rodents, birds, fish, worms, flies, plants and viruses have been identified and deposited in publicly available, miRBase Sequence database-release 10.0 (Griffiths-Jones *et al*., 2006). Computational approaches have estimated that miRNA comprise 1-5 % of the total protein-coding genes (Lai *et al*., 2003; Lewis *et al*., 2005). It is further estimated that in humans about 30 % of protein-coding genes may be regulated by miRNAs (Lewis *et al*., 2005) and this value may be extended to the miRNA-regulated gene networks operative in plants. Recently algorithms based on intragenomic matching of potential miRNAs and their targets coupled with support vector machine classification of miRNA precursors, predicted the existence of around 1200, 2500 and 2100 miRNA candidate genes in *Arabidopsis*, *Populus* and *rice*, respectively (Lindow *et al*., 2007).

Identification

Identifying new miRNAs or confirming the computational

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predictions involves the use of any of the three primary strategies. A first approach has been the identification of miRNAs through genetic screens. This technique led to the identification of lin-4 and let-7miRNAs in nematode. The second method involves classification after direct cloning of small RNAs. This has enabled identification of several tissue-, development- or physiology- specific miRNAs in plants (Llave *et al*., 2002; Reinhart *et al*., 2002; Sunkar and Zhu, 2004, Sanan-Mishra *et al*., unpublished). The third requires computational predictions to postulate the putative miRNAs, which can be subsequently validated. Several computational programs have been developed and successfully applied to predict miRNAs in a diverse animal and plant species. Some of the available softwares include, miRScan (Lim *et al*., 2003), miRseeker (Lai *et al*., 2003), findMiRNA (Adai *et al*., 2005), miRAlign (Wang *et al*., 2005b), ProMiR (Nam *et al*., 2005) and microHARVESTER (Dezulian *et al*., 2006). The application of computational methods are limited by the requirement of genome sequences, yet the EST and GSS databases have been employed to identify several miRNAs in 71 different plant species, (Zhang *et al*., 2005, 2006a). To date, more than 1200 miRNAs have been identified with cloning methods or computational approaches from a variety of plants.

Biogenesis

The biogenesis of miRNA is a multiple-step process that requires the participation of several enzymes. It has been very well documented in animals (Denli *et al*., 2004; Han *et al*., 2004). miRNA genes are distinct from the protein coding genes and can exist at any location of a genome, including introns, exons or regions between two protein coding genes. They are transcribed as independent units to primary miRNAs (pri-miRNAs) by RNA polymerase II (Lee *et al*., 2004). The pri-miRNAs may contain 7-methyl guanosine cap at its 5' end and a poly-adenylated tail at its 3' end (Cai *et al*., 2004). They acquire specific hairpin-shaped stem-loop secondary structures that are processed by dsRNA-specific ribonuclease III enzyme, DROSHA, to release 60-70 nt precursor miRNAs (pre-miRNAs). The pre-miRNA, having a 5' phosphate and a 3' overhang of 2 nt, are then transported to the cytoplasm by EXPORTIN-5 (EXP5) (a member of the Ran transport receptor family). Once in the cytoplasm, pre-miRNAs are further processed from the end of the hairpin structure stem by DICER, a second RNase III endonuclease, in to 20-22 nt long miRNA:miRNA* duplex (Zhang *et al*., 2004). The mature miRNA is incorporated into RNA-induced silencing complex (RISC) which induces gene silencing by preventing protein synthesis (Schwarz *et al*., 2002; Hammond, 2005). In animals it is achieved by inhibiting the translation machinery while in plants it is achieved by degradation of the target transcript.

There are several other differences in the biogenesis pathway of plant miRNAs. The processing of pri-miRNAs to pre-miRNAs in plants is mediated by the Dicer-like protein 1 (DCL1) instead of DROSHA (Park *et al*., 2002; Reinhart *et al*., 2002; Papp *et al*., 2003; Kurihara and Watanabe, 2004). This step also involves cooperation of other proteins like HYPONASTIC LEAVES1 (HYL1) (Han *et al*., 2004, Vazquez *et al*., 2004), HUA ENHANCER1 (HEN1) (Boutet *et al*., 2003; Park *et al*., 2005) and SERRATE (SE) (Yang *et al*., 2006a). The further cleavage of pre-miRNA to a miRNA:miRNA* duplex is mediated by DCL1 in nucleus (Papp *et al*., 2003; Kurihara and Watanabe, 2004) and the duplex is transported to cytoplasm by HASTY (HST), the plant ortholog of EXP5 (Park *et al*., 2005) where it gets incorporated into RISC complex.

Targets of micro RNA

In plants miRNA-mediated gene regulation is achieved by degradation of the target transcripts by the slicer component of RISC, ARGONAUTE (Ago1). The target is specified by the perfect or near-perfect sequence complementarity between the miRNA and its respective binding site on the targeted mRNA (Aukerman and Sakai, 2003; Wang *et al*., 2005a; Williams *et al*., 2005b). Experimental evidence indicates that there is usually a single miRNA binding site anywhere along the target mRNA. Exact base pairing is required at nucleotides 3-10 however a few mismatches are permitted at the very ends of the sequences, especially at the 3' ends (Mallory *et al*., 2004a, b).

A majority of the conserved plant miRNA targets constitute the transcripts of crucial transcription factors or other regulatory proteins that play an important role in basic organogenesis or signal transduction, (Allen *et al*., 2004; Bartel, 2004; Jones-Rhoades *et al*., 2004; Mallory *et al*., 2004b; Kidner and Martienssen, 2005). In addition, non-conserved miRNAs may function in the specialized processes like biosynthesis of cell wall metabolites (Lu *et al*., 2005) or cotton fiber development (Zhang *et al*., 2005). Recently we have identified some novel miRNA from rice that are predicted to target a wide range of non-DNA binding transcripts from receptor kinases to essential enzymes (Sanan-Mishra *et al*., unpublished).

Identifying miRNA targets is thus a very important step in studying the miRNA functions in plant development. Several approaches have been employed

towards this end. The genetic approach is one of the methods to identify miRNA targets. It is based on the abnormal expression of target mRNAs in the miRNA loss-of-function mutants. Another strategy is to predict miRNA targets by computational methods. MIRcheck (Jones-Rhoades and Bartel, 2004), findMiRNA (Adai *et al*., 2005) and miRU (Zhang, 2005) are the available software's for identifying miRNA targets in plants. The predicted targets can be subsequently verified by adopting PCR based strategies.

Functions of micro RNA

The diversity of the miRNA genes and their expression patterns in different tissues or development stages implies that, as a class, miRNAs play versatile roles in plant growth and development. This is evident from the study of loss of function mutants of enzymes involved in miRNA biogenesis. *dcl1* mutant shows reduced expression level of mature miRNAs and exhibits many developmental abnormalities including immature embryos, altered leaf shape and morphology, delayed floral transition, and female sterility (Park *et al*., 2002; Reinhart *et al*., 2002; Liu *et al*., 2005; Kurihara *et al*., 2006). Similarly, *hst* mutant also exhibits pleiotropic abnormalities, such as abnormal leaf and flower morphology, accelerated phase transition and reducing fertility (Bollman *et al*., 2003).

miRNAs influence all aspects of plant development by regulating essential transcription factors and/or crucial signal transduction pathways. They are involved in regulating the various aspects of shoot morphogenesis from meristem establishment and maintenance (Juarez *et al*., 2004, McHale and Koning, 2004, Mallory *et al*., 2004a; Juarez *et al*., 2004; Zhong and Ye, 2004; Bowman, 2004; Bao *et al*., 2004; Kim *et al*., 2005) to leaf development including abaxial-adaxial polarity (Palatnik *et al*., 2003), organ boundaries (Laufs *et al*., 2004), shape and complexity (Ori *et al*., 2007).

The apical meristem establishment is controlled by targeting several members of the NAM/ATAF/CUC(NAC)-domain transcription factors that play an important role in both embryogenic development and meristem formation (Aida *et al*., 1997; Takada *et al*., 2001; Hibara *et al*., 2003, Laufs *et al*., 2004; Guo *et al*., 2005). Leaf development is regulated by modulating the expression of class-III homeodomain leucine zipper (HD-ZIP) transcription factor genes, which control leaf patterning along adaxial/abaxial axis (Juarez *et al*., 2004). PHABULOSA (PHB), PHAVOLUTA (PHV), and REVOLUTA (REV) are three closely related Arabidopsis HD-ZIP transcription factors that are the targets of miR165 and miR166 (Emery *et al*., 2003; Bao *et al*., 2004; Bowman, 2004; Juarez *et al*., 2004; Mallory *et al*., 2004b; Zhong and Ye, 2004; Kim *et al*., 2005; Williams *et al*., 2005b; Ko *et al*., 2006). Altered expression of miR165 and miR166 resulted in leaf developmental abnormalities in Arabidopsis and corn (Juarez *et al*., 2004). It was recently observed that overexpression of miR165 affects apical meristem formation, organ polarity establishment and vascular development in Arabidopsis (Zhou *et al*., 2007). In addition miR159/Jaw is found to regulate a subset of TCP transcription factor genes that control leaf development (Palatnik *et al*., 2003).

miRNAs have been found to regulate the transition from vegetative to reproductive phase in plants. APETALA 2 (AP2) is one of the class A genes that control plant flowering time and floral morphology (Lohmann and Weigel, 2002). Over-expression of miR172 inhibited translation of AP2 and AP2-like mRNAs, resulting in early flowering and disrupting floral organ identity in Arabidopsis and maize (Aukerman and Sakai, 2003; Chen, 2004; Later *et al*., 2005). miR159, miR156, and miR171 are also involved in phase transition and floral development (Chard *et al*., 2004; Schwab *et al*., 2005). This is evident from the fact that miR171 is predominantly expressed in inflorescence instead of leaf (Llave *et al*., 2002). Similarly, over-expression of miR159 results in perturbed anther development and delayed flowering in a short-day photoperiod (Chard *et al*., 2004). miR156 also affected plant phase transition, including early formation of rosette leaves, by negatively regulating the expression of a Squamosa promoter binding protein like (SPL) plant-specific transcription factor (Schwab *et al*., 2005).

Recent studies have demonstrated that role of miRNA in regulating plant adaptive responses to biotic and abiotic stresses (Zhang *et al*., 2006b). Zhang *et al* (2005) showed that 25.8 % of sets containing miRNAs were identified in plant tissues stressed with pathogen, salt and drought. miR319 was over-expressed by cold stress (Sunkar and Zhu, 2004) while miR402 was strongly induced by drought, cold, and/or salinity (Sunkar and Zhu, 2004). Recently, Lu *et al*. (2005) identified 48 miRNA sequences from the *Populus* genome. Some of them were induced by mechanical stress and may function in a critical defense system for structural and mechanical fitness. miRNAs are also induced in response to nutrient stress as evidenced by the induction of miR395 under sulfate starvation conditions (Jones-Rhoades and Bartel, 2004) and miR399 under phosphate starvation (Allen *et al*., 2005). miR395 participates in sulphate assimilation and allocation by modifying the expression of ATP sulphurylase (APS) and a sulphate transporter

(AtSULTR2;1). Up-regulation of miR399 results in the down-regulation of UBC24 encoding an ubiquitin-conjugating E2 enzyme, which is critical in Pi homeostasis.

The role of miRNAs in virus-induced gene silencing is well documented. Recently, we have identified several miRNAs in rice that are up-regulated in response to Tungro virus infection (N. Sanan-Mishra unpublished results). Viral suppressors of gene silencing like HC-Pro, p19, p21, and p69 are important pathogenicity factors that help in disease symptom appearance and various developmental abnormalities in infected plants (Chapman *et al*., 2004). Several studies have shown that these suppressors are involved in the regulation of miRNA activities (Kasschau *et al*., 2003; Chen *et al*., 2004; Chapman *et al*., 2004). HC-Pro inhibited the expression level and activity of miR171, and caused developmental deficiency (Kasschau *et al*., 2003). P69 enhanced the expression and activity of miRNAs, and consequently enhanced plant resistance to pathogens (Chen *et al*., 2004). A recent study demonstrated that bacterial flagellin-derived peptide induced an over-expression of miR393 in Arabidopsis resulting in increased plant resistance to pathogens (Navarro *et al*., 2006). miR393 targets the F-box auxin receptors (TIR1, AFB1, AFB2, and AFB3), to inhibit growth of Pseudomonas syringae (Navarro *et al*., 2006).

Recently, Zhang and colleagues proposed that designed artificial miRNAs (amiRNAs) may be used to suppress gene expression of targeted genes to study gene function (Zhang *et al*., 2006b). This hypothesis was confirmed in a few plant species by two recent studies (Alvarez *et al*., 2006; Schwab *et al*., 2006) which demonstrated that amiRNAs specifically turn off their predicted targets. Both groups also found that amiRNAs were expressed in a specific manner by using inducible or tissue-specific promoters (Alvarez *et al*., 2006; Schwab *et al*., 2006). This suggests that amiRNAs can be designed to knock down several related, but not identical, targeted genes simultaneously. This provides a powerful tool to study gene function with multiple genes in a gene family.

Auxin and micro RNA in plant development

The term auxin is derived from the Greek word "auxein" which means "to grow". Auxins constitute the oldest and one of the most well studied class of phytohormones. An expanse of literature is available on the various aspects of auxin action (for recent reviews see Fleming 2006; Hartig and Beck 2006; Quint and Gray, 2006; Teale *et al*., 2006; Tanaka *et al*., 2006; Able, 2007; Spaepen *et al*., 2007; Vieten *et al*., 2007). Recent years have witnessed a tremendous leap in our understanding of the role of auxin and mechanism of its regulation (Dharmasiri *et al*., 2005; Woodward *et al*., 2005).

In plants each developmental process integrates a network of events that are regulated by different phytohormones and interactions among hormonal pathways modulate their effects. Auxin is recognized as the key hormone that alone or in combination with other hormones is responsible for modulating many aspects of plant growth including root and shoot architecture, organ patterning, vascular development and tropic response to light and gravity (Millner, 1995). It is also involved in delaying leaf senescence, promoting fruit ripening, and controlling abscission of fruits and dormancy in seeds. The auxin supply from the apical bud helps in maintaining apical dominance by stimulating the production of ethylene, which suppresses growth of lateral buds. Synthetic auxins are effective herbicides.

The interaction of auxin with its receptor initiates a complex signaling cascade that culminates in the induction of several transcription factors. These in turn regulate various downstream genes to affect a specific response. A genetic screen for auxin-resistant mutant identified TIR1 (transport inhibitor response 1) as a receptor for auxin. TIR1 encodes an F-box protein (Ruegger *et al*., 1998) and is a component of SCF-type ubiquitin-ligases, which are responsible for targeting specific proteins for degradation after tagging them with multiple ubiquitin residues (Vierstra, 2003). In fact the name SCF itself is derived from three foremost identified members: Skp1, Cullin, and F-box protein.

Auxin Response Factors (ARFs) are a major class of transcription activators and repressors that facilitate the auxin signal by binding to specific cis-elements in the upstream regions of auxin-inducible genes (Fig. 1). One such promoter sequence, is the TGTCTC auxin response elements (AuxRE) (see Guilfogle and Hagen, 2001) that is present in the primary/early induced gene families including GH3s, SAURs (small auxin up-regulated Ranks) and the AUX/IAA (Auxin/Indole-3-acetic acid inducible) (Hagen *et al*., 2002). GH3 encodes auxin conjugated enzymes, which act to reduce free auxin levels (Staswick *et al*., 2005) and AUX/IAA encode rapidly turned over transcriptional repressors of auxin-inducible gene (Tiwari *et al*., 2004). The Arabidopsis genome contains 23 ARF genes a few of which have been characterized (Wang *et al*., 2005a). ARFs are characterized by an amino terminal DNA-binding domain, a mid domain and two carboxy-terminal dimerisation domains (Tiwari *et al*., 2003). The ARF family can be classified into sub-groups depending

on the composition of the middle region between the DNA binding and dimerisation domains. ARFs with glutamine-rich (Q-rich) middle regions act as transcriptional activators. These are represented by 5 members including ARF 5, 6, 7, 8 and 19. The remaining 17 ARFs have serine-rich (S-rich), serine-glycine rich (SG-rich), serine-proline rich (SP-rich) or serine-proline-leucine rich (SPL-rich) middle regions and representatives of each type have been shown to act as repressors of transcription. ARF1 has been functionally characterized as a transcriptional repressor (Ulmansov *et al*., 1999a).

The auxin signaling is itself tightly regulated and found to be conserved among rice, Arabidopsis, poplar, Medicago and Lotus. A number of genes in auxin signaling cascade are confirmed as targets of miRNAs. miR393 targets four closely related F-box genes, including the auxin receptor TIR1 (Ruegger *et al*. 1998; Gray *et al*. 2001). miR393 also targets At3g23690, a basic helix-loop helix transcription factor that is homologous to GBOF-1 from tulip, which Genbank annotates as an auxin-inducible gene. Some of the ARFs have also been shown to contain potential miRNA binding sites. ARF10, ARF16 and ARF17 are regulated by miR160 (Rhodes *et al*., 2002) while miR167 negatively regulates the expression of ARF 2, ARF 3, ARF 4, ARF6 and ARF8 (Rhodes *et al*., 2002; Bartel and Bartel, 2003; Mallory *et al*., 2005; Sorin *et al*., 2005; Williams *et al*., 2005a; Yang *et al*., 2006b). miR160 and the target ARFs are conserved between dicots and monocots (Mallory *et al*., 2005).

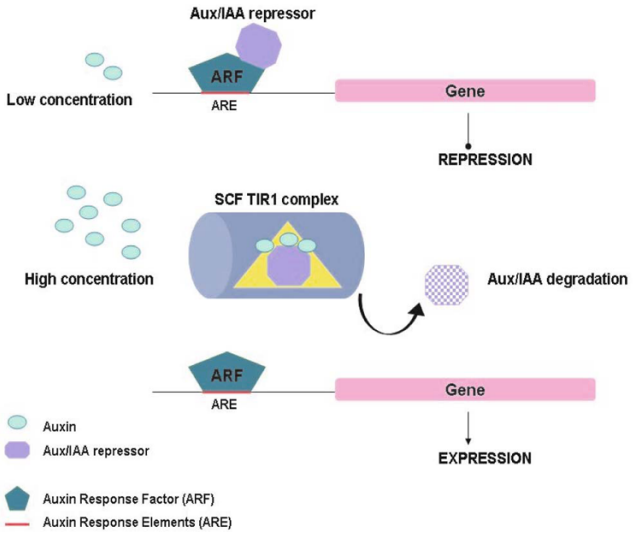

Fig. 1. Auxin regulated gene expression in plant cells. A schematic representation to show concentration-dependent role of auxin as repressor or activator of gene expression.

Auxin and miRNAs independently regulate the ARF levels to direct normal growth and development of aerial organs as well as lateral root production (Fig. 1). ARF10 and ARF16 dictate the root cap cell formation to mediate the direction of root tip growth. In absence of miR160 mediated regulation of ARF10 and ARF16 severe developmental abnormalities occur (Wang *et al*., 2005a). These include abnormal roots, leaf curling, serrated laminas, early flowering with altered floral morphology, defective embryos and reduced fertility (Liu *et al*., 2007). The ARFs alter expression of GH3-like early auxin responsive genes that are essential suppressors of active auxin levels. In Arabidopsis there are 20 GH3 homologs, which fall into three classes. The group II GH3 proteins which include YDK1/GH3.2, GH3.3, GH3.5 and DLF1/GH3.6 have been shown to conjugate IAA to amino acid in vitro (Staswick *et al*., 2005). miR160-regulated ARF17 acts directly to repress GH3.5 and DFL1/GH3.6 transcripts leading to increased free IAA levels. Similarly miR167 guides the cleavage of ARF8 transcript, which apparently negatively regulates free IAA levels by controlling GH3-like gene expression (Tian *et al*., 2004). Although, the exact mechanism of GH3-like gene regulation by ARF is not very clear, nonetheless it is indicative of an intricate interaction between auxin levels and miRNAs during plant development (Fig 2).

miR164 attenuates auxin signals by targeting five members of the NAM/ATAF/CUC (NAC) domain transcription factor family (Rhodes *et al*., 2002; Guo *et al*., 2005). Of these, NAC1 is involved in modulating lateral root development while Cup Shaped Cotyledons 1 (CUC1) is implicated in meristem development and separation of aerial organs (Mallory *et al*., 2004b; Hibara

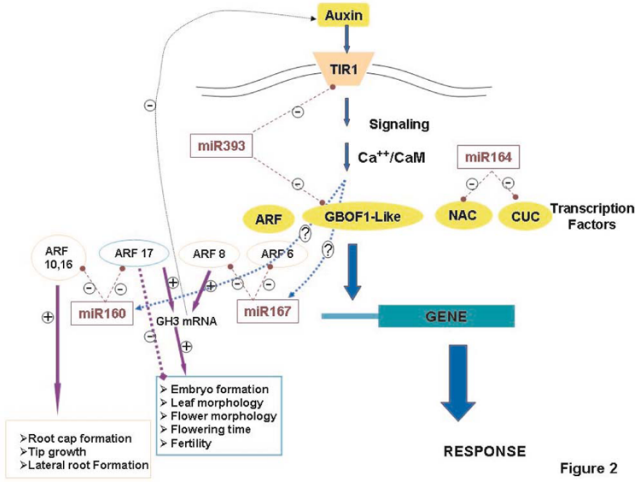

Fig. 2. A schematic representation to show miRNA modulated auxin regulatory networks operative in plant cells.

et al., 2006). NAC1 acts down stream of TIR1 to transmit auxin signals promoting lateral root emergence as discussed above. Prolonged auxin treatment results in miR164 mediated cleavage of NAC1 transcripts (Guo *et al*., 2005). The specificity of the regulatory pathway is highlighted by the fact that out of the three miR164 loci, viz. MIR164a, MIR164b, MIR164c, cleavage of NAC1 is mediated by MIR164b alone (Reinhart *et al*., 2002). An additional regulation is operational at the protein level as the highly unstable NAC1 protein is regulated by the RING motif E3 ligase, SINAT5 (Arabidopsis homologue of Drosophila protein SINA) (Xie *et al*., 2002; Guo *et al*., 2005).

The complexity in the present scenario is supplemented by evidences for the regulation of miRNA biogenesis by auxin. *In vitro* studies have indicated that phytohormones required for proliferation of cells may act as "silencing suppressors" in callus and tumors. ARF8 and TIR1 proteins are positive regulators for the cell response to the proliferation promoting hormone, auxin, in culture conditions (Navarro *et al*., 2006). Expression of OsGH3-2, an rice IAA-conjugating enzyme, is positively regulated by ARF8 (Yang *et al*. 2006b). Several lines of evidences suggest that lower concentration of exogenously supplied auxin is capable of suppressing miRNA biogenesis. Yang *et al* (2006b) have proposed a signal transduction pathway, auxin-miR167-ARF8-OsGH3-2, which is important for determining the cellular free auxin level, that guides appropriate auxin responses. Further, miR167 levels decreased in response to 1mM or lower concentration of 2,4-D.

To demonstrate the suppressor activities of auxins we used the GFP silenced lines of *Nicotiana benthamiana*. We observed that exogenous application of different types of auxins can reverse the GFP silencing in tobacco (Fig. 3) (Singh-Teotia *et al*., unpublished). Although the mechanism of hormone action is not completely known yet, it is possible that the totipotency in plants is due to their ability to shut-off or silence the miRNAs to revert the cells normal developmental pathway. This may be used as an effective tool for survival and adaptation of plants in response to the environmental pressures.

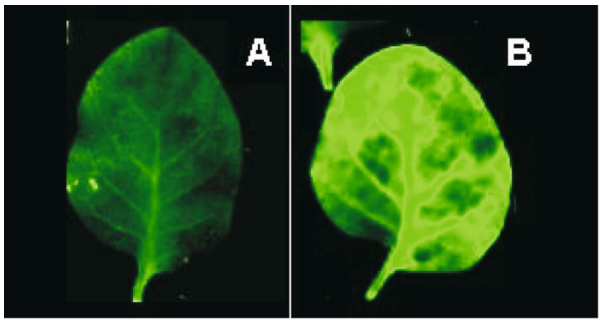

Fig. 3. Reversal of GFP fluorescence by exogenous auxin application. Leaves obtained from the GFP silenced tobacco line when exposed to low concentrations of auxin show GFP fluorescence in UV light. Representative picture of untreated leaf (A) and leaf exposed to auxin, IAA (B).

CONCLUSIONS

miRNAs have versatile functions in development, signaling, stress and disease resistance and are emerging as crucial regulators of gene expression. Although numerous miRNAs have been identified from different organisms, there is still a lot of ambiguity relating to miRNA-regulated pathways and their applications. Lot of study is also required to decipher the regulation of miRNA expression and the components involved in their origins. The recent identification of "mirtrons" in nematodes and insects (Ruby *et al*., 2007) has opened the possibility for the existence of specialized pathways of miRNA biogenesis in plants. Further investigation of the regulatory pathways upstream of these miRNAs in sensing the hormonal or environmental signals and regulatory pathways downstream of the miRNA target genes will offer a new insight for understanding the development and regulation of adaptation in plants. The prospects of miRNA-related studies will become apparent with the identification and understanding of miRNA targets and their functions.

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