Advance On Detect Methods of Plant Microrna Expression

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Abstract—**microRNAs (miRNAs) are an extensive class of endogenous, non-conding, short (21~25 nt) RNA molecules directly involved in signal transduction and play an important role in gene expression regulation. The detection of miRNA expression is a very important first step in miRNA exploration. With the discovery of new plant microRNAs and the functional research of miRNA thoroughly, new demand has been put forward to the plant miRNA detecting methods. Recently, plant miRNA expression detecting methods is mostly based on the hybridization and PCR techniques. In order to provide some suggestions for the plant miRNA detection research, this review summarizes the progress of several kinds of plant miRNA expression detecting methods, discusses the advantages and the disadvantages of several kinds of methods and prospects the plant miRNA detecting methods.**

Key words-plant microRNA, expression detection, hybridization, PCR

I. INTRODUCTION

MiRNAs are a class of non-coding small RNAs of roughly 21-25 nucleotides in length, which play important roles in regulating gene expression and signal transduction by directing gene splicing or depressing gene translation in polycells biology, It is reported that several thousand of miRNA have been identified in animals and plants in the past years(Lee R C et al., 1993; Garcia D., 2008; Bonnet E et al., 2004). To date, 15172 miRNAs have been identified in plants, including 213 from Arabidopsis, 377 from rice, 234 from Populus, 170 from maize, 148 from sorghum, 230 from Physcomitrella, 375 from Medicago truncatula, 148 from soybean, 37 from Pinus taeda, 64 from Selaginella moellendorffii, 46from Brassica napus and 36 from sugar-cane (miRBase release 16.0,October,2010,http://microrna.sanger.ac.uk/sequences/)(Suh et al., 2004; Griffiths J.S., 2006). These plant miRNAs have been identified and showed that miRNA play essencial roles in growth and development in model plants such as Arabidopsis thaliana and rice. (Mallory A.C and Vaucheret H., 2006)

However,It is known that miRNAs regulate gene expression in different tissues and cells temporally and spatially , and express of miRNA genes rapidly and exactly. With the identity and research of plant miRNAs, many new techniques have been explored for testing plant miRNA combined with the traditional methods such as Northern, which provided more and more help for deep research of plant miRNAs. This paper discussed traditional and present plant miRNA detection methods to gain insight into plant miRNA detection research.

II. PLANT MiRNA DETECTION TECHNIQUES

A. Plant miRNA hybridization detection

1) PAGE/Northern hybridization

PAGE and subsequent Northern hybridization are simple and traditional methods to detect miRNA expression(Sempere L.F et al., 2004). PAGE can effectively segregate non-coding RNA (<200bp) in total plant tissue. While Northern hybridization can effectively detect low abundance RNA by selecting LAN-modified oligonucleotide probe to hybridizate and quantify detect the molecular weight of miRNA with the help of RNA marker (Calin G.A., 2004; Valoczi A., 2004). Several of the newly identified miRNAs exhibited tissue or developmental stage-specific expression patterns under the four stresses such as dehydration、hyperhaline、low temperature、ABA stress in Arabidopsis thaliana shoot, It is reported that the expression of miR393 increased strikingly; miR389a decreased;miR397b and miR402 increased slightly; while miR319c is only association with low temperature (Sunka et.al 2004) and Li et.al (2010) detected the transgenic plants of miRNA which induced bradyrhizobium ddjaponicum bradyrhizobium japonicum functionally, high expression of miR483、miR1512、miR1515 ficilitated the sharp increase of rootnodule of soybean, however, the expression level was little related to the lateral length and density. Different miRNAs exhibited tissue or developmental stage-specific expression patterns also have found in rice (Wang et.al 2004) miR1、miR6、miR10 expressed more than the adult; and miR11 expressed most in the root, while miR16 most in the leaf. Wang et al. improved the traditional Northern blot hybridization for the liquid Northern which used fluorescently labeled oligonucleotide probes and characterized by simple and specific miRNA determination and quantitation.By applying the liquid Northern ,the entire detection process was completed within a few hours, and multiple miRNAs can be simultaneously detected in a single experiment.(Xiaosu Wang et al., 2010)

Although Northern hybridization can measure specific gene expression in different tissues or organs, it is incapable of the temporal and spatial expression of genes in microscopic and submicroscopic level, in addition, PAGE/Northern hybridization requires the samples to reach microgram to avoid false negative, and the sensitivity is low to detect rare miRNAs. With the development of molecular hybridization and mature methods of immune histochemistry, in situ hybridization(ISH) is providing another effective approach for us.

2) In situ hybridization

In situ hybridization can detect temporal and spatial expression of miRNAs conveniently and directly,MiRNA oligonucleotide probe were labeled and locked to hybridizite with nucleotide sequences under test in the tissue slices in situ hybridization,and observed the existence and location of nucleotide under electron microscope by autoradiography(Zeng Y and Cullen B.R., 2003). Oligonucleotide probe were modified with nice two-fold chain stability and sensitivity by Banding with complementary RNAs, therefore,increases cross compatibility and avoids the drop of small RNAs in hybridization and subsequent elution prosesses(Kloosterman W.P et al., 2006).The expression behavior of miR169 and its target gene were found in Medicago truncatula (Combier J.P et.al. 2006), the results showed that miR169 played a key role in the development process of root nodule and negatively regulated transcription factor MtHAP2-1, thereby ruled the growth of root nodule meristem. Chen et.al. have detected miR172 and AP2 which is the target gene regulating floral organ characteristic, miR172 accumulated abundantly in the early entire flower primordium and later inner two wheel flower primordium, while AP2 could only function in the out two wheels despite its expression in the four wheels of flower primordium organs(Chen X., 2003). Lelandais B.C et al. applied in situ hybridization to detect the expression behavior of miRNAs in *Medicao truntula* root nodule，the results showed that miRNA160、miRNA169、miRNA172 、miRNA393 and miRNA171 expressed abundantly in Nodule meristem, miRNA167 expressed abundantly insurrounds in the vascular bundle, miRNA396 had no expression(Lelandais B.C et al., 2009). Nogueira F.T et al. used in situ hybridization to detect the expression behavior of miRNA390 which controlled biosynthesis of the ta-siRNAs in the Corn Seedling，the results showed that miRNA390 expressed abundantly in the vascular bundle of seedling leaves.(Nogueira F.T et al., 2009) Palatnik et al. detected the expression behavior of TCP4 transcription factor which was regulated by miR319 during the wild-type embryogenesis, leaf development and the jaw-D seedlings, the results showed that miR319-regulated TCP4 had an instructive role in regulating the growth of cotyledons and leaves, both of which are affected in jaw-D mutants.(Palatnik et al., 2003)

ISH was convenient and intuitive to be used to detect the expression behavior of miRNAs.however, there were less about quantitative information of miRNAs. The results showed that the hybridization efficiency and sensitivity need to be further improved.

3) MicroRNA chip technology

Chip technology is a faster and more widely and more useful method for the research of miRNA expression (Yan N et al., 2007). Compared with the traditional techniques of miRNA,DNA chip technology has many obvious advantages, First, gene diagnosis can be generally finished in 30 min with remarkable acceleration. Secondly, hundreds of gene sequences can simultaneously be detected every time owing to high efficiency of detection and the process of detection parallel.Third, the false-positive and false- negative rate of gene diagnose significantly reduced by controlling rigorous degrees of the molecular hybridization.enclosed by crossing infection. Antisense cDNA probes were pointed on the nylon membrane, and hybridized with small molecular weight RNA sample with 5 'end of radioactive marker, then obtained signal after radiation from enhancement. Therefore, it is a new, idea approach with a higher sensitivity and a high throughput for miRNA detection by oligonucleotide microarray probe (Castoldi M et al., 2006). Most miRNA chip employs fluorescent detecting and connects with luminous groups such as biotin by covalent binding (Direct labeling method) (Krichevsky A.M et al., 2003),or employs markers in the stage of cDNA synthesis or PCR amplification (indirect labeling method) (Thomson J.M et al., 2004). Michael J er al. detected the expression of miRNA in different tissues at seedling stage of Arabidopsis, the results showed that the expression levels of miRNA have tissue-specific through different ways. The expression of miR156 family and miR157 family were down-regulated sharply, they exhibited lower expression level in stem, cauline leaf and inflorescence, the same as the expression level of miR398,miR396 and miR163 while miR172 expression level was in contrast(Axtell M.J.,and Bartel D.P., 2005). Zhou et al. screened miRNA in Arabidopsis thaliana related to UV - B radiation , the verification experiment proved that miRNA genes were regulated positively by UV-B radiation (Zhou et al 2007). Combining with northern blot analysis Zhao et al. successfully found four rice miRNA induced by drought and identified the two of them, miR-169g and miR-393. MiR-169g expressed especially striking in the roots induced by drought in miRNA 169 family alone (Zhao et al 2007) .The expression of the known 117 miRNA is researched by Han H.L et al. under high salt, drought and cold in Arabidopsis.14 miRNA expression levels changed , of which,10 miRNA responsed to high salt signal and cold signal, four response drought signal.The expression of miRl68, miRl71 and miR396 were induced in three kinds of stress condition, whereas miRl65, miR319 and miR393 were up-regulated under high salt and cold treatment, miRl67 was induced by high salt and drought stress(Han H.L et al., 2008). The reproducibility is verified in miRNA expression profiling of the rice seedlings roots, leaves and consistent with Northern hybrid results . (Liang et al 2005)

Now, Capital Biochip Corporation (http://www.capital bio.com/) invented plants miRNA chip, probe number re ached 426, which mainly comes from cloning or bioinfo

rmatics prediction from 9 types of plants such as Rice, Arabidopsis and so on. LC SCIENCES Corporation (htt p://www.lcsciences.com) have developed miRNA chips s pecifically for Rice, Corn and Arabidopsis, whose probe number were 142, 43 and 153, respectively. miRNA c hip is undoubtedly the best choice of high-throughput te chnique to detect plant microRNA expression, it can ide ntify all known plants miRNA expression profiling in th e short time. However, It cannot be effectively detect t he unknown miRNA, particularly the non-conservative miRNA.And the production and detection of gene chip not only require expensive equipment, but the result is semi-quantitative and poorer repeatability; So it cannot o ptimize the hybrid environment of miRNA and distingui sh highly similar miRNA.

4) High-throughput sequencing of small molecules RNA

The second generation techniques have many advantages such as high-throughput, high efficiency and accurary, therefore, it is very suitable for sequencing of plants microRNA which has low abundance, tissue specificity and spatial temporal specificity owing to deep coverage ,huge output and short sequence.So it is very effective method to find those important microRNA in specific growth phase or particular stress of plant material and analyse comprehensively the expression of small molecules RNA participated in the above process (Fahlgren N et al., 2007). In recent years, researchers began using high-throughput sequencing technologies such as MPSS (Brennecke J et al., 2007), 454-FLX（454 Life Sciences）(Margulies M et al., 2005) and Solexa（Illumina）(Cokus S.J et al., 2008) to excavate plant microRNA large-scalely, and reflect the miRNA expression levels in different tissue of plants through deep analysis. For example, Moxon et al. sequenced deeply and analyzed the microRNA expression of tomato leaves and fruits using 454-FLX, It is showed that the expression of the most of microRNA have tissue-specific. Among them, the expressive quantity of miR1917, miR1918 and miR1919 in fruit are higher than leaves; and miR1917 participated in fruit development process through adjusting its target genes LeCTR1 and LeCTR2 (CTR family members, negative regulated the tomato fruit ripening process which response to ethylene reaction)(Moxon S et al., 2008). Nobuta K et al. applied MPSS and 454-FLX to analyze the microRNA expression of Wild-type and *rdr2* Mutant of *Arabidopsis*, It is showed that ta-siRNAs and part of long-21nt miRNA were enrichment in *rdr2* Mutant, however, those long-24nt siRNAst which derived from the genome heterochromatin decreased significantly 。MicroRNA of soybean root were sequenced and analyzed deeply using 454 - FLX which infected with Bradyrhizobium , miR159 and miR393 were up-regulated significantly while miR160 and miR169 were decreasing tendency, It was speculated that these microRNA may be involved in response to the infection process of rhizobia (Subramanian etc 2008).

Although high-throughput sequencing can detect the expression of organizational microRNA by abundance analysis, However, it should be noted that northern blotting is the standard technique for quantifying miRNAs in current methods. Mix sample of wheat organizational microRNA analysed by 454 - FLX deep sequencing, 58 miRNA genes were reached , including 35 conservative miRNA and 23 special miRNA of wheat, and then the expression of part microRNA were verified by PAGE/Northern hybridization and RT- PCR, It is showed that miR156 was high in root and leaf, while lower in other organizations , miR171 was in various organizations of wheat, but highest in root .(Yao etc 2007)

B. Detection of plants miRNA by PCR

1) Semi-quantitative RT-PCR

There are basically two kinds of methods to validate the authenticity of plants miRNA in present , one is PAGE/Northern hybrid which detects the existence of miRNA candidates, and the other is RT - PCR which detects whether miRNA is expressed or not .Semi-quantitative RT-PCR is a simple, rapid, specific and common technology for RNA relative quantification and it was used to test the expression level of plant miRNA precursor.However, the expression level of plants miRNA precursor does not necessarily consistent with the mature miRNA(Wei Li., and Kangcheng Ruan., 2009). Our lab have identified the authenticity of predicted miRNA through adding tail and primer extensions RT-PCR method in this laboratory(Fig. 1), 7 screened miRNAs (zea-miR173 、 zea-miR397 、 zea-miR528、zea-miR529、zea-miR833、zea-miR834 and zea-miR863) could be detected in the different position (leaves、roots and leaf sheath)of maize inbred line R15 and Y478, this result proved that these seven miRNA is real and those expression were expressed in different materials/organization. Therefore, it was very effective, high sensitivity, and simple operation to adopt RNA tailed with poly(A) and primer extensions PCR (Shi R and Chang V.L., 2005) , and low-cost to verify the authenticity of the expression of plant miRNA (Zhang Q et al., 2007).
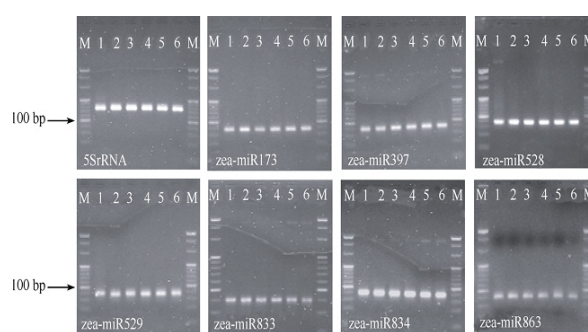

Figure 1. PCR assay for miRNAs tailed with poly(A)1:Y478 leaves; 2:Y478 roots; 3:Y478 sheath; 4:R15 leaves; 5:R15 roots; 6:R15 sheath

Various improvement techniques have been developed such as primer extensions RT-PCR, stem-loop primer RT-PCR and tailed RT - PCR of miRNA and so on. Varkonyi-Gasic et al. applied the stem-loop pulsed RT-PCR to detect the expression level of miRNAs of *Arabidopsis thaliana*, the result demonstrated that miRNAs are

differentially expressed in the phloem sap and the surrounding vascular tissue, and the stem-loop pulsed RT-PCR which enabled fast, sensitive and specific miRNA expression profiling was suitable for facilitation of high-throughput detection and quantification of miRNA expression.(Varkonyi-Gasic E et al., 2007). Six new miRNA was verified in rice roots and leaves under cadmium stress,and six miRNA were all different.(Huang S.Q etc 2009) Among them, miR601, miR602 and miR603 were up-regulated by cadmium stress in rice root however, miR604 was down-regulated in the root but miR602 and miR606 were down-regulated in rice leaves, while miR601 in rice leaves and miR606 and miR605 in roots were no changes. In our lab, 22 miRNAs and the precursor of miR168 of R15 (disease resistant) and Y478(sensitive disease) were detected under *Rhizoctonia solani* stress by semi-quatitative RT-PCR, 4 miRNAs precursor sequence between resistant and sensitive materials, miR168, miR165,miR173 and miR845, explained that these miRNAs could be relevant with the regulatory of corn sheath blight. 10 kinds of miRNAs were expressed in leaves of apple seedling both in vivo and in vitro, and miRNA156 and miRNA157 in young leaves of seedling were higher than in vivo than in vitro.

2) Real-time RT-PCR

Real - time PCR can be very accurate to analyse quantitatively the expression of miRNA (Gramantieri L et al., 2007 and Moschos S.A et al., 2007). Stem-loop real-time fluorescence quantitative RT-PCR (Chen C.F et al., 2005) and real‐time RT-PCR of extended primer (Shi R et al., 2005) emerged from the change of miRNA template and prime owing to the shorter length of mature miRNA. Stem-loop real-time fluorescence quantitative RT-PCR is high specificity and sensitivity to test miRNA expression, including design reverse transcription primer with stem-loop structure and conduct real-time PCR using specific molecular probe with fluorescently labeled miRNA (Zhang H.H et al., 2007). The basic principle of real-time fluorescence quantitative RT - PCR with tailed RNA and extended primer is to use tailed RNA enzyme (Poly A polymerase) to extend the miRNA by adding multiple adenylic acid to the 3 'end region of detected RNA, then to use reverse transcription primer similar with Olig (dT) 5 ' end region to go on the reverse transcription process to extend products, and obtain the reverse transcription products about 80nt in the end. And then, the products act as a template for the PCR amplification, the full-length of miRNA act as 3 'end primers, and then based on the 5' end of template to design upstream primer for the PCR amplification, and combined with the real-time PCR to detect the expression level of miRNA. Compared to the stem-loop real-time fluorescence quantitative PCR, real-time fluorescence quantitative RT-PCR with tailed RNA and extented primer is high sensitivity, simple operation, low-cost, it is very effective to test the expressio level of miRNA from cloning   tissue (Wei Li and Kangcheng Ruan., 2009). The expression of miRNA was successfully detected in plants (Shi et al., 2005) and miRNA398 was successfully

detected in arabidopsis thaliana and poplar under the ABA and salt stress (Jia X 2009) It is showed that miRNA398 was increasing firstly and then began to decline at 48h,then reached the lowest in poplar; miR398 was unchanged under salt stress in arabidopsis thaliana, while opposite under ABA stress in poplar.
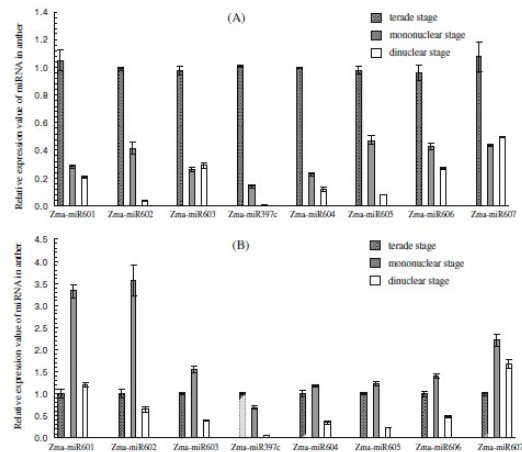

Figure 2.   Difference of miRNA expression patterns between CMS line and its maintainer with the development of pollen. A.miRNA expression patterns in the anther of maintainer line N48-2. **B**. miRNA expression patterns in the anther of CMS line C48-2 (our lab , Shen Y.O et al., 2010)

In our lab, 8 miRNAs were deteced at different stages of anthor in C cytoplasmic male sterile line C48-2 and maintenanceline N48-2(Fig.2), 8 miRNAs occurred at tetrad stage of these two materials, it was showed there were distinct expression patterns for the eight miRNAs between the two lines in the process of microspore developmen, each of the miRNAs tended to be down-regulated after the tetrad stage in a fertile line, however, most of the miRNAs in the cytoplasmic male sterile line were shown to be up-regulated from the tetrad to mononuclear stage, displaying special expression patterns differing from the ones in fertile line (Shen Y.O et al., 2010).Combated with in situ hybridization MiR165a and miR166b were detected in the root tip tissue of arabidopsis thaliana at the seedling stage,(Annelie Carlsbecker et al., 2010) and miR165a and miR166b were lower in exodermis of the root tip, but increasing sharply in endodermis of the root tip.However,there are some disadvantages such as easy to combine with nonspecific double-stranded DNA, production of false-positive, the high demand of specificity primers and so on.

III.   CONCLUSIONS

Detection technology of plant microRNA is constantly improving, semi-quantitative, high specificity, high sensitivity, with the development of microRNA chip andhigh-throughput sequencing, exploration of the functional microRNA and in-depth study on its function have become a hot spot of research, Combing with application bioinformatics and plant microRNA database. Therefore, there has also put forward new requirements for

detection technology of plant microRNA. At present, the detection of plant microRNA expression rely mainly on the combination the traditional techniques with emerging high-throughput sequencing , for example, We predicted miRNA through high-throughput screening of miRNA chip, then tested candidate miRNA through a real–time PCR and Northern blot, and then further screened miRNA target genes after selected target miRNA through prediction by using bioinformatics,which based on the miRNA target genes database, and finally researched on regulation function of miRNA to target gene by inhibition of miRNA expression and overexpression. There are some advantages and disadvantages in several detection methods of plants miRNA expression in this paper, therefore, this is a main trend for the development and improvement of plant miRNA detection to further develop new generation of PCR of high efficiency, high sensitivity and specify, and which can be performed popoularly, and to develop the new generation of probe material of high-throughput, high sensitivity and specificity. It requires the continuous improvements of traditional test methods, and need to develop more advanced, more effective microRNA detect methods, in order to search for functional microRNA from plants genome database, detect their expression and regulation mechanism of miRNA on the target genes, their mutual signaling transduction pathways, and then identify genetic approach and biological significance of miRNA. Once a series of questions about miRNA detection is to be solved, it will be of great help to research on gene regulation and function at the molecular level for plant breeding, and also further accelerate the research pace of functional genomics and proteomics, and even promote the development of systems biology and bioinformatics.

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