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Role of microRNAs in regulating drought stress tolerance in maize

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Abstract
Maize is one of the most important crop all over the world. One of the major abiotic stress is drought that reduces the productivity of maize. Plants have many mechanisms to combat with the serious drought effects and one of the defense mechanism is the reprogramming the gene expression by microRNAs. miRNAs are the non-coding 20-22 nucleotide length that have emerged as important regulators of gene expression at post-transcriptional level. In this study the differential expression of microRNAs in drought tolerant lines was detected. Seven families of microRNAs have been selected for study. The target genes of the miRNAs have been found out by using online tools psRNA Target and RNA hybrid. A total of seven microRNAs targeting 16 mRNAs were predicted. The hypothesis that selected microRNAs differentially expressed to drought stress was validated by qRT-PCR. Under drought stress, the differential expression of microRNAs regulates the expression of their target genes, resulting in multiple responses of physiological and biochemical pathways relative to drought tolerance of maize. miR160, miR164, miR166, miR393, miR529, miR169 and miR2275.families may play more important role. The different members of the same family may play similar regulation effects in most cases.

Keywords: Drought, stress tolerance, micro RNA, target genes, *Zea mays*

Introduction
Drought is one of the most common environmental stresses affecting growth, development and yield of plants (Ceccarelli and Grando, 1997) [6]. Understanding plant tolerance to drought is important for the improvement of crop productivity (Lawlor, 2013) [17]. In molecular terms, many genes have been implicated in drought tolerance (Shinozaki and Yamaguchi- Shinozaki, 2007) [20]. But many of the transgenic plants overexpressing the drought genes did not exhibit significant improvement or not at all improvement for drought.
miRNAs are small non-coding RNAs 22-24 nucleotide length that are present in plants and have function to regulate gene expression by sequence- specific interaction with target mRNAs. microRNA expression has found to be altered during drought stress thus focusing light on drought response mechanism which can potentially used in development of new drought tolerant crops (Chen *et al*., 2012) [7]. Due to Drought stress the up- or down-regulated miRNAs both are potentially relevant for engineering plant tolerance against drought, as miRNA target genes probably include genes that contribute both positively or negatively to tolerance.
Enhancing the accumulation of target(s) contributing to drought tolerance could be achieved either by overexpressing target genes, or by silencing the corresponding miRNA (Sunkar *et al*., 2007) [21]. There are several bioinformatics tools available for miRNA prediction and they have facilitated the way for the discovery of miRNAs in various plant species (Yang and Li, 2011) [24]. Plant miRNAs are highly complementary to the target mRNAs, which allow easy and fast identification of their putative targets with confidence. (Jones-Rhoades *et al*., 2004) [15]. Using the bioinformatics tools, has helped us in finding many target genes involved in a wide diversity of functions in various plant species (Axtell and Bowman, 2008) [1]. Recent researches have demonstrated that miRNAs control gene expression at posttranscriptional levels by targeting the primary transcripts for cleavage or translational repression (Rogers and Chen, 2013) [19]. In this present study, the differential expression of genes which are tolerant to drought and bioinformatics tool was used for the prediction of target genes.

Material and methods
A. Plant growth experiment
Four maize inbred lines, SKV 671 and CML 22 (drought tolerant) and SKV 569 and SKV 1442(drought susceptible) were used for this study. The plants were regularly irrigated for 15 days after sowing and then they were subjected to drought stress.

The stress was imposed on these plants for 5 days by withholding the water (Kakumanu *et al*., 2012) [16] The control plants were watered daily (figure 1). After 5 days of severe stress plant samples were collected for stress treated and control plants for further expression analysis.

Fig 1: Stressed and control plants.

B. miRNA Selection

miRNAs mature sequences for *Zea mays* were downloaded from miRBase Registry Database (http://www.mirbase.org/), Release 21: June 2014. After literature survey Seven families with their subfamilies were selected based on their role in drought response in other crops but not in maize.

C. Target Gene Prediction.

The miRNA-mRNA duplex was predicted using web-based as well as standalone miRNA target finding tools: psRNA Target (http://plantgrn.noble.org/psRNATarget/) (Dai and Zhao, 2011) [9], RNA hybrid (Rehmsmeier *et al*., 2004) [18]. The potential targets of maize miRNAs were predicted using both the tools with default parameters. The GNOMON id for the mRNA gene was downloaded from NCBI Genome database by GNOMON gene prediction tool.

D. Functional annotation of Target genes.

The sequences of the predicted genes were submitted to Blast2go (online tool) for their functional annotation (Conesa *et al*., 2005) [8].

Results and Discussion

Drought-responsive microRNAs

In the present study we found fourteen microRNAs in seven microRNA families which were detected to be differentially expressed on significant level at post-transcriptional level on facing drought condition. miRNA160, miRNA164, miRNA166, miRNA169, miRNA2275 and miRNA529 responded well to drought in root samples and leaf samples but the expression pattern was more significant in root samples. The gene miRNA2275 responded very well to drought stress which is newly found in maize, but in peach and almonds it has been found that it has role in regulating drought. Theconserved miRNA targets were verified by qRT-PCR, and target annotation showed that these targets were involved in multiple biological processes, including transcriptional regulation and response to stimulus (Table 1).

Table 1: Drought related miRNAs and their families with their targets and functional annotation.

miRNA	Target mRNAs	Annotations
zma-miR160f	gnl|GNOMON|46030063.m	gdsl esterase lipase at5g45910-like
zma-miR164b	gnl|GNOMON|46030063.m	psbp domain-containing protein chloroplastic-like
zma-miR164f	gnl|GNOMON|18192014.m	Hypothetical protein
zma-miR164h	gnl|GNOMON|46106013.m	wound responsive protein
zma-miR166a-3p	gnl|GNOMON|35860043.m	tpa: homeobox lipid-binding domain family protein
zma-miR166b/c/d/g/h/i-3p/e/f	gnl|GNOMON|15104054.m	partial
zma-miR166j/k/n-3p	gnl|GNOMON|8472093.m	Homeobox-leucine zipper protein athb-15-like
zma-miR166l/m-3p	gnl|GNOMON|52446103.m	rolled leaf 1
	gnl|GNOMON|1168013.m	tpa: homeobox lipid-binding domain family protein
zma-miR169f-3p	gnl|GNOMON|13694074.m	protein msp1
zma-miR169i/j/k-5p	gnl|GNOMON|74364063.m	nuclear transcription factor y subunit a-3
zma-miR169l-5p	gnl|GNOMON|74364063.m	nuclear transcription factor y subunit a-3
zma-miR2275a-3p	gnl|GNOMON|55702013.m	mitochondrial protein
zma-miR393a/c-5p	gnl|GNOMON|39086093.m	protein transport inhibitor response 1-like
zma-miR529-5p	gnl|GNOMON|13750094.m	tpa: squamosa promoter-binding (sbp domain) transcription factor family protein isoform 1

Seven drought tolerant miRNA families were validated in this study. Different samples of root and leaves of same microRNA family were identified to have similar expression profiles and two miRNA genes, miRNA164 and miRNA166 were found to have more than one targets. Many of the miRNA genes were down-regulated resulting in the drought tolerant genes are speculated to correspond to the up-regulated expression of their target genes. These target genes are implied to improve the tolerance of maize from drought stress. miRNA160, miRNA164, miRNA166, miRNA169, miRNA393, miRNA529 is tolerant to drought in other plant species (Ding *et al*., 2013) [11]. miRNA2275 plays specific role in regulating drought (Esmaeli *et a*l., 2016) [12]. Several Auxin response factors (ARFs) such as ARF10, ARF16 andARF17 have been confirmed as target genes for miRNA160. miRNA160 have been reported to play major roles in drought and ABA response in plants (Ding *et al*., 2013) [11]. miRNA393 also plays important role in Auxin signaling. The target of miR393 encodes TIR1 (transport inhibitor response 1), an auxin receptor in Arabidopsis. The TIR1 enzyme is a positive regulator of auxin signalling by promoting the degradation of Aux/ IAA proteins through ubiquitination (Dharmasiri and Estelle, 2002) [10]. Xia *et al*. (2012) [23]. miRNA166 is drought-responsive miRNAs that were previously characterized as crucial for cell development. It post-transcriptionally regulates class-III homeodomain-leucine zipper (HD-Zip III) transcription factors, which helps in lateral root development, axillary meristem initiation, and leaf polarity (Boualem *et al*., 2008) [5]. miRNA166 was downregulated in response to drought in maize in present study and similar results was found in barley. These results clears that miR166-mediated post-transcriptional regulation is

an important regulatory pathway involved in the regulation of architecture of root and drought response. miRNA 169 is down-regulated in the present study and over-expression of this helps in reduced stomatal opening and drought tolerance which is in accordance with the findings of (Zhang *et al*., 2011) [29] in tomatoes in which miRNA169 is down-regulated. miRNA529 is down-regulated in the present study the identification of differentially expressed novel plant miRNAs and their target genes, and involvement of miRNAs in the process of drought response and/or tolerance in maize and in Oryza sativa such results have been published (Zhou *et al*., 2010) [32]. miRNA164 plays important role in regulating drought stress and it is down-regulated in present study. Several studies in Arabidopsis and rice have shown that miR164 cleaves NAC mRNAs that modulate plant developmental processes and responses to abiotic stress according to Zhang (2014) [31].

Different Pathways adapted by miRNAs for Drought tolerance.

1. miRNA 160

miRNA 160 is involved in Abscisic acid response and auxin response factor (Ding *et al*., 2013) [11]. It is one of the major stress hormone produced in plants under water deficit conditions.

2. miRNA 164

It is found that miRNA164 plays very important role in regulating the post-transcriptional processing of NAC transcription factors (Guo *et al*., 2005) [13]. NAC proteins play very important role in combating abiotic stress in various plants.

3. miRNA166

It is reported to be drought stress responsive gene and known to regulate (*HD-ZIP III*) Homeodomain- leucine zipper transcription factor class III. They are very important for lateral root development, axillary meristem initiation and leaf polarity (Hawker and Bowman, 2004) [14].

4. miRNA 169

It is one of the largest miRNA families that is conserved in all plants. It has a big role in plant proper development and response to environmental stress. As it is a conserved family which regulates a homologous target it behaves in a contradictory way in different plant species that is because of differences in plant developmental process, growth conditions and stress intensity (Ding *et al*., 2013) [11].

5. miRNA 393

Zhang *et al*., (2007) [28], identified by their study that miRNA 393 is drought induced. miRNA 393 encodes the target gene TIR 1 (transport inhibitor response 1) an Auxin receptor in Arabidopsis.

6. miRNA 529

It is downregulated in response to abiotic stress and plays important role to make the plant tolerant (Bakshi *et al*., 2016) [2]. miRNA 529a-5p is predicted to target the transcript from only SPL genes (*for Squamosa Promoter binding protein Like*). A group of *SPL* genes including *SPL14* has post-transcriptional regulation by miRNA 529.miRNA 529 was downregulated in rice and it has target genes cDNA squamosa promoter-binding-like protein, zinc finger family protein, U-box domain containing protein (Zhou *et al*., 2010) [31]. It has possible involvement in flower development (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004) [15, 22].

7. miRNA 2275

miRNA 2275 is present in peach and monocots but has not been reported in other land plant species (Barakat *et al*., 2012) [3]. Some studies conducted on miRNA 2275 reported up-regulation of this miRNA under cold stress in peach and rice respectively (Barerra and Figueroa, 2012 and Barakat *et al*., 2012) [4, 3].

Conclusions

To gain insight into the molecular mechanisms of maize plant response towards drought stress, the conserved microRNAs were examined. The effect of miRNAs on plant biochemical pathways were observed and novel miRNAs in maize were identified to enhance drought stress. Recently a majority of plant researches are going on with focus on microRNAs. The findings in this research provide us with valuable information for functional characterization of miRNAs response to drought stress.

microRNAs regulate the expression of their target genes by up-regulating or down-regulating them which results in various physiological and biochemical responses of maize to drought stress. MicroRNA families miRNA160, miRNA164, miRNA166, miRNA169, miRNA393, miRNA529 and miRNA2275 play very important role in regulating drought stress. These data provide us with the way for the future studies and continued efforts to find more miRNAs and confirm the functions of miRNAs in drought.

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