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Computational prediction, identification, and expression profiling of microRNAs in banana (*Musa* spp.) during soil moisture deficit stress

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SUMMARY
Soil moisture deficit stress is one of the most important abiotic stresses limiting the production and productivity of banana (*Musa* spp.). Plant responses to drought stress are regulated at both the transcriptional and post-transcriptional levels. As post-transcriptional gene regulators, microRNAs (miRNAs) regulate gene expression during drought stress. The present study aimed to identify drought-responsive miRNAs in an expressed sequence tag (EST) library of drought-stressed banana and to study their expression in a drought-tolerant banana cultivar, 'Saba', subjected to soil moisture deficit stress. *In silico* prediction resulted in the identification of three miRNAs (miR156, miR169, and miR2118) whose expression was confirmed using real-time qPCR assays with the respective complementary DNAs (cDNAs) synthesised in multiplex stem-loop-primed reverse-transcription reactions. The selection of a suitable reference miRNA for this study was carried out using six genes, which included the traditional *Musa* reference gene for 25S rRNA. miR399 was selected as the reference miRNA, based on its constant level of expression in various tissues and sample types. Additional regulatory roles for miR169 on expression of the dehydrin and aquaporin genes in banana were investigated using qPCR assays in drought-stressed leaf samples collected at different times during stress. The results suggested that miR169, miR156, and miR2118 were up-regulated during soil moisture deficit stress, and also that miR169 had an indirect, transcription-level role in regulating dehydrin and aquaporin gene expression. The aquaporin gene exhibited more prominent expression compared to dehydrin in banana during drought stress.

Soil moisture deficit stress negatively affects plant growth and development in banana, leading to a sharp decline in fruit productivity. The effect depends on the stage of the crop, as well as the duration and intensity of the stress (Ravi and Uma, 2010). Moisture stress is a complex trait involving physio-chemical processes in which a large number of biomolecules are involved (Bartels, 1996). High variability in the nature of soil moisture deficit stress, and our limited understanding of its complexity, have made it difficult to identify specific physiological traits for screening. This has limited the genetic improvement of banana for drought tolerance. Efforts to phenotype bananas and plantains for their responses to soil moisture deficit stress are awaiting field validation (Ravi and Uma, 2010). In addition to phenotyping, better knowledge of the molecular mechanisms involved are essential for drought tolerance-related studies.

Yoshimura *et al*. (2008) and Mahajan and Tuteja (2005) reported that plant responsive mechanisms depend on specific gene regulatory networks and transcription factors, which are themselves regulated by the expression of miRNAs (Zhou *et al*., 2010). Several plant miRNAs are known to regulate responses to plant development and environmental stresses, including drought, salinity, and nutrient deficiency (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Lu *et al*., 2008; Bari *et al*., 2006; Chiou, 2007; Zhao *et al*., 2007; Liu *et al*., 2008; Ding *et al*., 2009). In general, miRNAs are transcribed from a distinct class of genes and regulate the expression of other genes at the post-transcriptional level. They negatively regulate target gene expression (Zhao *et al*., 2007) through two mechanisms: transcriptional repression or post-transcriptional degradation via direct binding.

Sunkar *et al*. (2012) and Kantar *et al*. (2011) showed that the expression profiles of protein-coding genes fluctuate greatly in response to drought stress due to the expression of specific miRNAs. Approx. 25% of the miRNAs identified to date have been found in stress-induced ESTs. Despite our knowledge of the mechanisms of expression of genes or loci associated with drought, studies on the roles of miRNAs in drought-tolerance have been limited. Zhou *et al*. (2010) and Sunkar *et al*. (2008) emphasised the importance of the molecular characterisation of the miRNAs involved in tolerance to drought stress, while Figueroa *et al*. (2011) suggested that expression profiling of miRNAs under different physiological conditions was essential to design any crop improvement strategy.

Recent advances in sequencing technologies, the accumulation of genomic information on many plant species, and powerful bio-informatics tools for the prediction of miRNAs, have resulted in the identification of many miRNAs in both model and non-model plants. miRNAs from diverse plant species such as *Arabidopsis*, rice, maize, wheat, cowpea, oil palm, soybean (Joshi *et al*., 2010), and common bean (Huertero *et al*., 2009) have been identified or characterised. In banana, 235 miRNAs in 37 families have been identified (D'Hont *et al*., 2012),

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but their roles in abiotic stress responses, especially during moisture-deficit stress in banana, remain unclear. As most known mature miRNAs are evolutionarily conserved in the plant kingdom, the use of bio-informatics tools will facilitate the identification of *Musa* miRNAs that are homologous or orthologous to those in other plant species (Floyd and Bowman, 2004; Wang *et al*., 2004; Zhang *et al*., 2006). We therefore attempted to identify miRNAs among the drought-stressed *Musa* ESTs available in the public domain and to measure their differential expression in a drought-tolerant banana cultivar, 'Saba' (ABB) that was subjected to soil moisture deficit stress.

MATERIALS AND METHODS

Drought imposition

Preliminary screening under field conditions led to the identification of 'Saba' (ABB) as a drought-tolerant banana cultivar (Ravi *et al*., 2013), and this was used in the present study. Fifty uniform-sized (1.0 kg), 3 month-old healthy suckers were collected and planted in cement pots (48 cm × 38 cm) filled with 35 kg of soil mixture [a 1:1:1 (v/v/v) mix of sand, red soil, and farmyard manure].

The plants were maintained at 100% field capacity (FC) continuously for 90 d. Progressive soil moisture deficit stress was imposed on one set of 25 plants by withholding water for 24 d. While the other set, maintained at FC, served as the controls. The duration of the period of growth under drought stress was referred to as mild (1 – 7 d after the application of stress; DAS), medium (8 – 20 DAS), or severe (21 – 24 DAS). The moisture content of the soil declined gradually to < 30% of FC by the end of the stress period (24 d). This coincided with the gradual emergence of the typical symptoms of moisture-deficit stress such as a reduction in the rate of leaf emergence, rolling of the existing leaves, and drying of the lower leaves by 15 DAS.

Leaf samples from three drought-stressed plants were collected individually at different time intervals from 0 – 24 d. In control plants, triplicate leaf samples were collected on day-0 and on day-24 and pooled for analysis. The samples were frozen in liquid nitrogen and stored at –70ºC until total RNA was prepared.

Computational prediction of miRNAs

Drought stress-associated miRNAs were data-mined from the drought-stressed *Musa* ESTs available in public databases. A total of 5,494 *Musa* spp. ESTs were retrieved from the NCBI databases in April 2012 (http://www.ncbi.nlm.nih.gov/nucest/?term=musa-drought+stress), processed by removing vector, poly(A), and other contaminating sequences, and assembled using the online software available at http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::cap3. The resultant contigs and singlets were used as queries for BLAST searches on the plant microRNA database (PMRD; http://bioinformatics.cau.edu.cn/PMRD/) to predict conserved orthologous miRNAs. The sequences thus identified were also used for *de novo* predictions of the secondary structures and stabilities of potential miRNA sequences using Mfold-3.2 software (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi), applying the criteria proposed by Ambros *et al*. (2003). In addition, two further miRNAs, one from *Arabidopsis* and one from maize, were included for the identification of suitable candidates as reference miRNAs.

Stem-loop reverse transcription and miRNA amplification

Total RNA was extracted from drought-stressed and control banana leaf samples using the Spectrum™ Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO, USA). Each reverse transcription reaction was performed in 20 µl using the RevertAid First-Strand cDNA synthesis kit (Fermentas, Hanover, MD, USA) with 2 µg of total RNA (DNase-treated) as template. The reaction mixture also included the multiplex miRNA stem-loop RT primers for miR156, miR169, miR2118, miR396, miR399, and a primer for 25S rRNA. Pulsed-temperature conditions were adopted as reported by Tang *et al*. (2007). The reaction conditions for cDNA synthesis were as follows: 60 cycles of 42ºC for 30 s, 45ºC for 30 s, and 50ºC for 1 s. The reaction was stopped by incubating the mixture at 70ºC for 5 min. A 0.5 µl aliquot of the cDNA reaction was then used as a template for miRNA amplification by PCR using the miRNA-specific forward and common reverse primers (Table I). The temperature profile used was an initial denaturation for 5 min at 94ºC, followed by 45 cycles of 94ºC for 5 s, 60ºC for 5 s, and 72ºC for 8 s. The amplicons were resolved by

TABLE I

miRNAs and their corresponding primer sequences for stem-loop reverse transcription and specific miRNA-specific amplification using PCR

Name	Primer sequenc (5' ➛ 3')
miR156	ugacagaagagagugagcaca
RT‡	TCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGCTC
FP	GCGGCGGTGACAGAAGAGAGT
miR169	uagccaaggaugacuugccug
RT	TCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGGCA
FP	GCGGCGGTAGCCAAGGATGA
miR396	uuccacagcuuucuugaacuu
RT	TCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAGTTC
FP	GCGGCGGTTCCACAAGCTTTC
miR2118	uugccgauuccucccaucccua
RT	TCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAGGGA
FP	GCGGCTTGCCGATTCCTCC
miR399	ugccaaaggagaauugcccug
RT	TCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGGGC
FP	TGCCAAAGGAGAATTGCCCTG
Universal-RP	GTGCAGGGTCCGAGGT

‡RT, reverse transcription primer; FP, miRNA-specific forward primer; Universal RP was the common reverse primer.

electrophoresis in 3% (w/v) agarose gels. Stem-loop RT primers, and miRNA-specific forward and universal reverse primers were designed according to Chen *et al.* (2005).

Selection of a suitable miRNA as a reference for expression analysis during drought stress

Real-time quantitative PCR (qPCR) for the detection of copy numbers of miRNAs was carried out using two banana cultivars, 'Saba' (drought-tolerant), and 'Monthan' (moderately drought-tolerant), based on preliminary evaluations by Ravi *et al.* (2013). The conditions used for drought stress (24 DAS) were similar to those described for expression profiling. In addition, samples of five different tissues (leaf, bract, bud, corm, and sheath) of 'Grand Naine', grown under field conditions, were analysed. qPCR using a Roche Light Cycler 480 (Roche Diagnostics, Mannheim, Germany) was carried out using six primers (five miRNA primers and primers for the traditional *Musa* reference gene for 25S rRNA). The mean cycle crossing point (cP) values of triplicate samples of the different tissues were analysed using GeNorm in GenEX software (Schlotter *et al.*, 2009) to select the best reference miRNA to be used for normalisation during miRNA quantification.

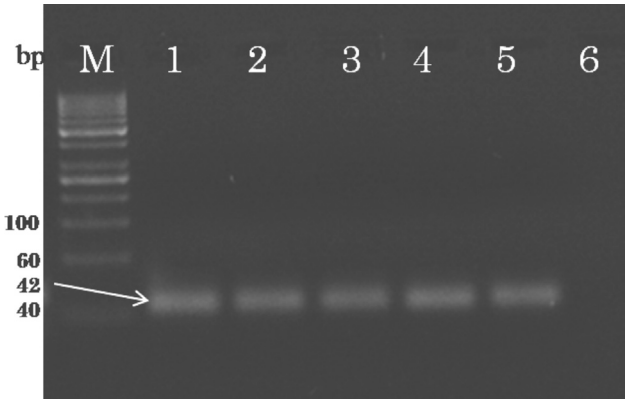

FIG. 1
MicroRNA amplicons separated in a 3% (w/v) agarose gel in TAE buffer. Lane M, 10 bp DNA ruler; lane 1, miR156; lane 2, miR169; lane 3, miR2118; lane 4, miR 396; lane 5, miR399; and lane 6, negative control (PCR reaction mix without template).

RESULTS AND DISCUSSION

Computational prediction of miRNAs

Approx. 1% of predicted genes contain miRNAs and these can be identified either through direct cloning of small RNAs (Sunkar and Zhu, 2004) or by computational strategies (Adai *et al.*, 2005). An EST database is a good and cost-effective resource for the identification of miRNAs. Twenty-six percent of ESTs containing miRNAs are related to biotic or abiotic stress responses (Zhang *et al.*, 2005). In the present study, an attempt was made to predict *Musa* miRNAs in the ESTs from drought-stressed banana tissues available in the public domain (5,494 sequences; NCBI EST database). These ESTs were processed and searched against the plant microRNA database (http://www.bioinformatics.cau.edu.cn/PMRD/).

Based on the highest level of homology with known

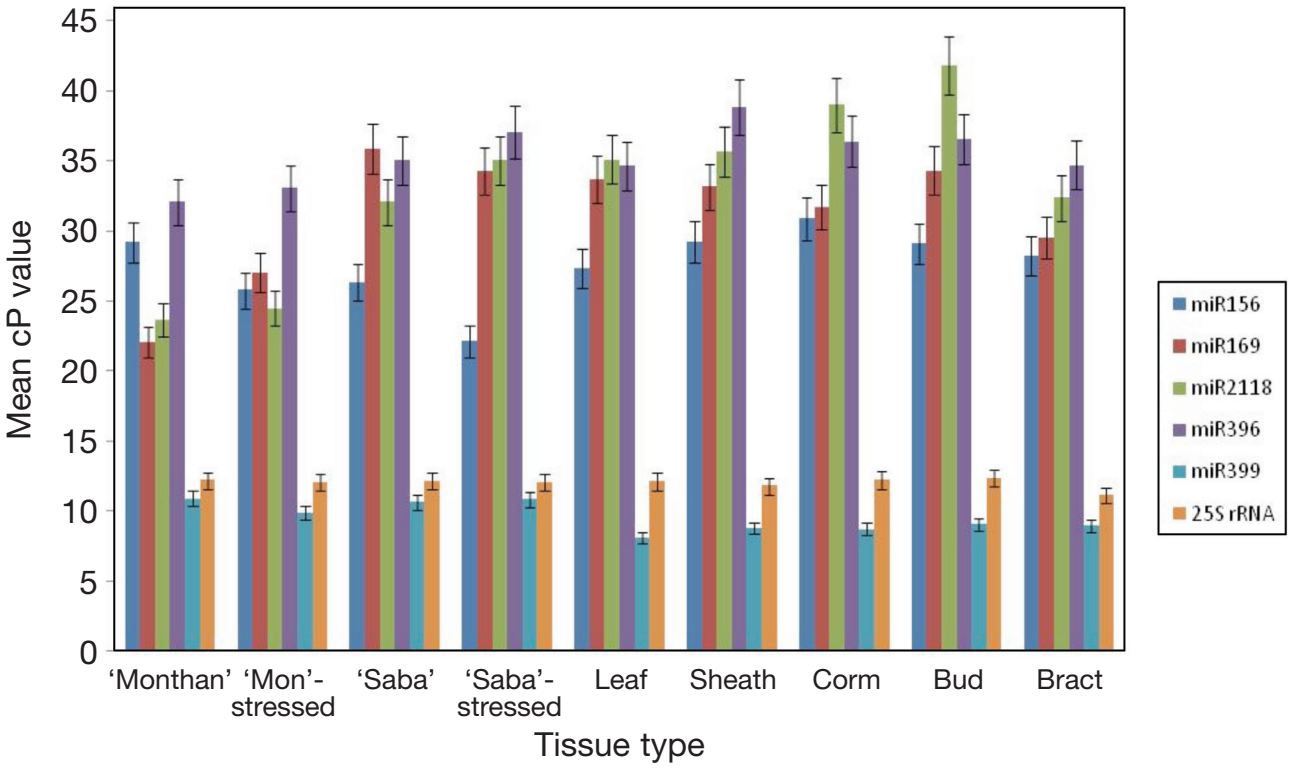

FIG. 2
Expression of miRNAs and 25S rRNA in the leaves of two banana cultivars and in different tissues (leaf, sheath, bud, bract, and corm) of 'Grand Naine'. Samples were also from the leaves of control ('Monthan'), drought-stressed 'Monthan' ('Mon'-stressed), control 'Saba', and drought-stressed 'Saba' ('Saba'-stressed). Mean cycle crossing point (cP) values (n = 3) were from real-time qPCR assays and represent the number of qPCR cycles required to detect expression of the target genes (miRNA or 25S rRNA).

plant miRNAs, only three *Musa* miRNA candidates were selected. These miRNAs were miR156, miR169, and miR2118, and their respective resource ESTs were gi|146226345, gi|197637388, and gi|197654740. These miRNAs exhibited 100% homology to microRNAs from *Arabidopsis* and rice. miRNAs have been reported to be evolutionarily conserved within the plant kingdom (Sunkar and Zhu 2004; Reinhart *et al*., 2002). The miRNAs predicted from drought-stressed banana ESTs confirmed earlier reports on miRNA169 and miRNA 2118 in drought-stressed rice ESTs (Zhou *et al*., 2010), and on miRNA 156 in drought-stressed switchgrass (Sun *et al*., 2012). All the predicted miRNAs were validated in drought-stressed banana leaf tissues using stem-loop RT and miRNA-specific forward and universal reverse PCR primers (Figure 1), followed by DNA sequencing. The expected size of the amplicons (42 bp; Chen *et al*., 2005) and sequence similarity confirmed the presence of the respective miRNAs.

Identification of suitable reference miRNA candidates

Real-time qPCR has been shown to be a sensitive and specific method by which to discriminate even single nucleotide differences between miRNAs (Liu *et al*., 2005). Identification of a suitable reference miRNA is important when studying the relative levels of expression of miRNAs by normalisation. To date, no reference miRNA has been identified for use in banana. Hence, we attempted to select the best candidate among the three miRNAs identified in the present study, along with two additional miRNAs, one each from maize and *Arabidopsis*.

The five selected miRNAs, plus the *Musa* reference gene for 25S rRNA, were used for gene expression studies in samples of various banana tissues (e.g., leaf, sheath, root, corm, bud, and bract) of 'Grand Naine', along with drought-stressed leaf samples from the cultivars 'Saba' and 'Monthan' (Figure 2). The expression of miRNAs from *Arabidopsis* and maize in banana confirmed that miRNAs are highly conserved across genera, in agreement with reports by Sunkar and Zhu (2004) and Reinhart *et al*. (2002).

The mean cP values for all five miRNAs combined from the different tissues, as well as in the drought-stressed samples, were compared using the GeNorm tool in GenX software. The mean cP values (M-values) of measures of the stability of gene expression for the five miRNAs were 2.45 (miR156), 3.22 (miR169), 3.92 (miR2118), 1.91 (miR396), 1.03 (miR399), and 1.03 (25S rRNA). The least variable was miR399 (1.03), as for 25S rRNA (1.03). Thus, the qPCR results indicated that miR399 exhibited a stable level of expression across various tissues, as well as under normal or drought-stressed conditions (Figure 2). Hence, miR399 was selected to use for normalisation during quantification of the levels of expression of the other miRNAs expressed under drought stress.

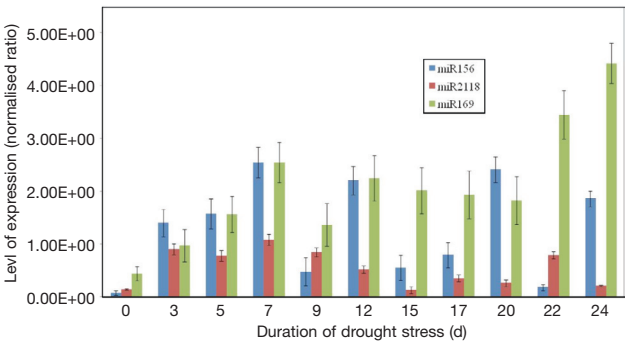

FIG. 3
Levels of expression of miR156, miR2118, and miR169 at different times during 24 d of soil moisture-deficit stress in the banana cultivar, 'Saba'. Levels of expression are presented as normalised ratios based on the relative quantification method using miR399 as the reference genes.

Differential expression of miRNAs during drought stress

To identify the expression profiles of three miRNAs (miR156, miR169, and miR2118) under soil moisture deficit stress, triplicate samples of leaves from the drought-tolerant cultivar, 'Saba', were collected at different time intervals during drought stress. Real-time qPCR was carried out to study the patterns of expression of miRNAs using the relative quantification method. Mean cP values for the triplicate samples were considered for normalisation against two endogenous controls (miR399 and 25S rRNA). The overall pattern of expression showed that all three miRNAs were over-expressed during drought stress compared to the controls (Figure 3). The trend in expression of all three miRNAs (Figure 3) varied depending on the severity of the drought stress imposed. During the first week of drought stress (i.e., mild stress), miR156 and miR169 consistently showed induced expression. Although, up-regulation of miR169 was maintained under medium stress (up to 20 DAS), its level of expression declined subsequently. However, its peak of expression occurred under severe stress (20 – 22 DAS). Unlike miR169, expression of miR156 increased consistently until 20 DAS. During the first week of drought stress, all three miRNAs were up-regulated, indicating that they could be part of an early transcriptional control mechanism for drought tolerance. The expression of all miRNAs varied with the three stages of drought stress (mild, medium, and severe). Mild and severe drought stress induced miR169 and miR156, but not miR2118, while medium stress (8 – 20 DAS) gave no consistent pattern of expression. Such inconsistent expression could be due to balancing the expression of target genes and miRNAs to maintain the physiology of the plant under soil moisture deficit stress. The levels of expression of miR156 and miR2118 were high on day-7, while expression of miR169 reached its highest level on day-24. Expression of miR169 was more significantly up-regulated than miR156 and miR2118 as the drought stress progressed. Similar up-regulation of miR169 in response to drought (Zhao *et al*., 2007) has been reported in rice. However, miR169 was also reported to be down-regulated during drought stress in rice (Zhou *et al*., 2010), while, in *Medicago truncatula*, it showed no change in expression (Trindade *et al*., 2010). This suggests a dynamic, crop-dependent pattern of expression of miR169 during drought stress.

Compared to miR169, miR156 is considered to be an important miRNA for drought tolerance as it plays a vital role in multiple biological processes (Sun *et al*., 2012). Over-expression of miR156 led to a prolonged vegetative phase and an enhanced accumulation of

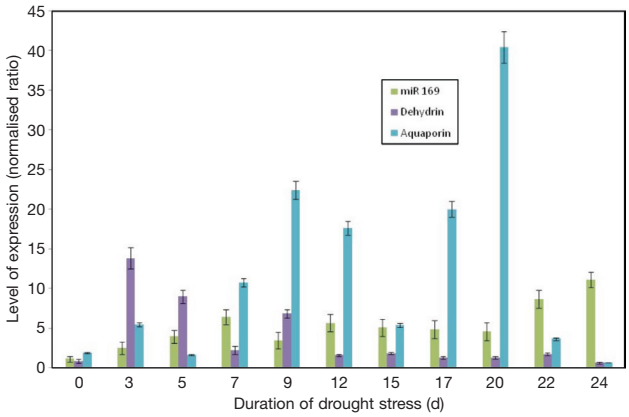

FIG. 4

Comparisons of the level of expression of miR169 and possible target genes for dehydrin (*DHN*) and aquaporin (*AQN*) at different durations of soil moisture-deficit stress in the banana cultivar, 'Saba'. The relative levels of expression of miR169, *DHN*, and *AQN* were measured by qPCR.

biomass in switchgrass (Fu *et al*., 2012). It was also found to regulate the gene coding for the most conserved plant protein, the squamosa-like promoter binding protein, associated with drought and salt stress. In the present study, miR156 was over-expressed during soil moisture deficit stress, as reported in *Arabidopsis* (Shen *et al*., 2010), indicating that it is essential for regulating plant developmental processes under moisture deficit conditions.

The up-regulation of miR2118 reported in the present study also agreed with the findings of Wang *et al*. (2011) in *M. truncatula*. miR2118 has been claimed to be a novel miRNA involved in biotic and abiotic stress responses (Kulcheski *et al*., 2011) and has been reported to target mRNAs for proteins associated with disease resistance (Wang *et al*., 2011). Similar results have been reported in cowpea (Figueroa *et al*., 2011) and switchgrass (Sun *et al*., 2012). Drought-induced expression of miR2118 was observed in the present study, but the inconsistent pattern of expression did not allow us to draw any conclusions regarding its role. Nevertheless, up-regulation of miR2118 may indicate its possible involvement in inducing drought tolerance.

Comparisons of the qPCR data with the expression of known genes for drought tolerance

Independent qPCR assays were carried out to measure the levels of expression of two known candidate genes involved in drought tolerance, those for dehydrin (*DHN*) and aquaporin (*AQN*). These values were then compared with the levels of expression of miR169, which exhibited significant up-regulation under drought stress. qPCR-based relative quantification indicated the up-regulation of all three genes (*DHN*, *AQN*, and for miR169) compared to non-stressed plants, with varying levels of expression based on the severity of the stress (Figure 4). As the drought stress progressed from mild to medium, up-regulation of *AQN* occurred, while a declining trend was observed during severe drought stress. In the case of *DHN*, up-regulation was observed only at 3 DAS, then declined to 12 DAS, with stable expression until 22 DAS, and a further decline thereafter. Both miR169 and *AQN* exhibited up-regulation during low and medium drought stress. During severe stress, miR169 continued to be over-expressed. Both miR169 and *DHN* were up-regulated during mild and medium stress, while, at 24 DAS, miR169 was highly up-regulated, coinciding with a decline in the expression of *DHN*. miR169 was up-regulated throughout low, medium, and severe drought stress, with a peak during severe stress at 20 – 24 DAS. Unlike miR169, the level of expression of *AQN* declined during severe stress, while the level of expression of *DHN* did not exhibit any specific trend. The lowest level of expression of *DHN* was observed during severe drought stress. Interestingly, we found that over-expression of miR169 during severe drought stress coincided with the lowest levels of expression of both *DHN* and *AQN*.

An inverse relationship between the levels of expression of miR169 and *AQN* and *DHN* might be due to direct or indirect gene regulation by miR169. Previous reports have shown that a gene for the transcription factor, nuclear factor Y (NFY), was the most important target for miR169 (Li *et al*., 2008; Zhou *et al*., 2010). In general, NFY binds to the CCAAT box region of promoters (Zhang *et al*., 2011). The presence of this binding site in the *DHN* and *AQN* promoters has been reported by Deng *et al*. (2006) and by Tungngoen *et al*. (2009), respectively. Thus, miR169 might be indirectly involved in the regulation of both *DHN* and *AQN*, through NFY gene expression under severe drought stress. The inverse relationship observed between expression of miR169 and both the *AQN* and *DHN* genes suggests that miR169 might control the expression of NFY and eventually the expression of *AQN* and *DHN* by reducing transcription, or by a post-transcriptional degradation mechanism.

In conclusion, consistent up-regulation of miR169 has been shown to be critical for drought-tolerance in banana. Among the possible candidate genes, induced expression of *AQN* appeared to have dominant role over *DHN* in the drought-tolerant banana cultivar, 'Saba'.

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