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Involvement of miR164- and miR167-mediated target gene expressions in responses to water deficit in cassava

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Abstract

Cassava (*Manihot esculenta* Crantz) is an important crop and it is significantly affected by water stress. The computational analysis of *cis*-regulatory elements in promoter regions of 21 drought-responsive miRNA gene families and 35 miRNA-target genes in cassava indicated some elements relevant to drought stress responses. To investigate the role of miRNAs and target genes in responses to a water deficit in cassava in more detail, *in vitro* plantlets were subjected to an imitated water deficit by 40 % polyethylene glycol. Using RT-qPCR, the differential expression of the cassava *miR164*/*MesNAC* and *miR167/MesARF6/8* were observed to be associated with changes in the leaf shape, stomatal closure, and relative water content. The modified 5'-RNA ligase-mediated rapid amplification of cDNA-end (5'RLM-RACE) experiment confirmed *MesNAC* and *MesARF8* as the *in vivo*-target genes of miR164 and miR167, respectively, in cassava leaf. The possible functions of miR164 and miR167-target genes in response to water deficit are discussed.

Additional key words: *cis*-acting regulatory elements, drought, *Manihot esculenta,* microRNAs, polyethylene glycol, transcription factors.

Introduction

Drought is one of the most important problems that impairs plant growth and development, and leads to significant losses in crop yield and productivity. To survive, plants respond and adapt to a drought stress by changes at molecular, morphological, biochemical, and physiological levels (Chaves *et al*. 2003). Stomatal closure is an early response to acute water deficit (Arve *et al*. 2011). With continuing drought, changes in lateral root development have also been observed (Xiong *et al*. 2006). Many studies have attempted to search for the genes controlling lateral root development which is related to an important agronomic trait for drought tolerance (Xie *et al*. 2000, Jeong *et al*. 2010). To date, a number of drought responsive genes have been identified in various plant species, such as *Arabidopsis* (Seki *et al*. 2002), rice (Rabbani *et al*. 2003), and cassava (Lokko *et al*. 2007, Utsumi *et al*. 2012). However, the regulation of gene expression is a complex process, especially at the transcriptional level (Floris *et al*. 2009, Kaufmann *et al*. 2010). With the discovery of microRNAs (miRNAs), the role in gene regulation at the post-transcriptional level mediated through the function of miRNAs under a stress response has been indicated (Reinhart *et al*. 2002, Sunkar *et al*. 2012).

In plants, miRNAs control a variety of biological processes, such as development and responses to phytohormones and stresses (Liu and Chen 2009, Sunkar *et al*. 2012, Eldem *et al.* 2013, Jin *et al*. 2013, Liu *et al.* 2013). In particular, a number of studies show that miRNAs are associated with a drought stress response (Zhou *et al*. 2010, Khraiwesh *et al*. 2012). With increasing number of drought-responsive miRNAs and target genes identified to date, an attention has been paid to the investigation of the regulatory role of miRNAs in plant stress response mechanisms. Although all plants seem to possess the miRNA-directed gene modulation during a drought stress response, this process has been observed only in some plant species. For example,

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Abbreviations: miRNA - microRNA; 5'RLM-RACE - 5'-RNA ligase-mediated-rapid amplification of cDNA-end; RWC - relative water content.
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miR169 is up-regulated in rice under drought stress (Zhou *et al*. 2010), whereas the down-regulation of *miR169* is observed in *Arabidopsis thaliana* and *Medicago truncatula* (Li *et al*. 2008, Trindade *et al.* 2010, Wang *et al.* 2011). In *Arabidopsis*, the accumulation of *miR159* was observed in response to dehydration and to abscisic acid (ABA) as well. Moreover, an independent study found that a transcription regulator, auxin response factor 10 (ARF10), targeted by *miR160*, is also responsive to ABA. Under drought stress, several miRNAs of *Arabidopsis* including *miR157, miR167, miR168, miR171, miR319, miR393, miR396, miR397*, and *miR408*, are up regulated (Sunkar 2010). Plants prefer to restrict the proliferation of their horizontal roots in response to drought stress due to ABA/auxin antagonism. The possible link between the *miR393*-mediated attenuation of auxin signaling and the response of roots to drought stress was proposed (Chen *et al.* 2012). Using the next generation sequencing technology, the potato *miR171* and its role in drought stress has been identified (Hwang *et al*. 2011). Therefore, more in-depth and detailed characterizations of drought-responsive miRNAs in each plant are needed.

No apical meristem, *Arabidopsis* transcription activation factor, cup-shaped cotyledon (NAC) and ARF6/ARF8 are classes of specific transcription factors that control diverse developmental processes and abiotic stress responses in plants. A number of NAC domain proteins are implicated in abiotic stress responses, such as drought and salinity (Nakashima *et al*. 2012). The stress related transcription factor NAC1, involved in lateral root development in *Arabidopsis*, has a complementary site with *miR164* and thus can be regulated by *miR164*. Rice over-expressing stress-responsive NAC1 (SNAC1) in guard cells withstands a severe drought stress due to increased stomatal closure and/or a higher ABA sensitivity to prevent water loss (Hu *et al*. 2006). Apart from NAC, many ARFs are regulated by miRNAs. *ARF6* and *ARF8* were predicted as targets for *miR167* which is differentially expressed under drought stress in many plant species, such as tobacco, maize, and *Arabidopsis* (Liu *et al*. 2008, Frazier *et al*. 2011). Based on the importance of these miRNA-target genes during drought stress, their expression profiles reveal their involvement in plant development and in drought stress responses.

As an important source of sugars and renewable energy, cassava ranks as one of the most important crops in the world. Although cassava can tolerate a drought stress, it has been reported that its biomass is significantly affected (El-Sharkawy 2004, Vandegeer *et al*. 2013). According to this, the identification of miRNAs that modulate drought-response mechanisms should contribute to the improvement of its drought tolerance. However, drought stress-related miRNAs in cassava have not been revealed. To investigate the role of miRNAs in responses to water deficit in cassava, *in vitro* plantlets were subjected to a water deficit, and analyses of morphological and physiological changes as well as the expression of *miR164* and *miR167* and their target genes were carried out. The knowledge obtained from this study should provide us better understanding the regulatory role of miRNA during water deficit in cassava.

Materials and methods

Cassava (*Manihot esculenta* Crantz) cv. Kasetsart 50 (KU50), which is widely cultivated in South-East Asia due to a high adaptability in any growing environment and a high yield, was used in this study. *In vitro* cassava plantlets were cultured in a Murashige and Skoog (MS) medium supplemented with 2 % (m/v) sucrose, 0.6 % (m/v) agar, pH 5.6, under a 16-h photoperiod, an irradiance of 300 μmol m^{-2} s^{-1}, and a temperature of 26 ± 1 °C. To create a water deficit, the eight-week-old plantlets were carefully transferred from the semi-solid medium to a liquid medium and left overnight. The plantlets were subsequently transferred to a liquid medium containing 40 % (m/v) PEG 6000 (having an osmotic potential of -1.8 MPa according to Michel *et al.* 1973), and incubated for 2, 6, 12, and 24 h. The control groups were treated in the same way, but without PEG. The experiment was performed in parallel for five biological replicates. After the treatment, root and leaf tissues were harvested, immediately frozen in liquid nitrogen and stored at -80 ºC for further analyses.

To study the effects of the water deficit on stomata, leaves of 8-week-old plants undergoing the water deficit stress were collected and stained with toluidine blue. The characteristics of guard cells were visualized by light microscopy (*Olympus B202*, Tokyo, Japan).

Leaf water status was monitored by the determination of the relative water content (RWC) as described in Barr *et al.* (1962). Briefly, leaf discs were randomly selected from fully expanded leaves. The leaf mass was determined immediately after cutting (FM). The leaf discs were then rehydrated by floating on water at 26 ± 1 °C for 4 h (TM). Subsequently, the leaf discs were oven dried at 80 ºC for 24 h (DM), and RWC [%] was calculated as (FM - DM)/(TM - DM) × 100.

Total RNA was isolated from cassava tissues using a *Concert*™ plant RNA reagent kit (*Invitrogen*, Carlsbad, USA) according to the manufacturer's instructions. Total RNA was quantified using a *ND-1000* (*Nanodrop Technologies*, Wilmington, USA), and assessed for quality by electrophoresis on a 1 % (m/v) denaturing agarose gel. RNA samples were stored at -80 ºC until further analyses and subsequently treated with *DNaseI* (*Ambion*, Austin, USA). For analysis of miRNA, the reverse transcription was carried out using 100 ng of total RNA and a *Ncode*™ miRNA first-strand cDNA synthesis kit together with quantitative real-time-PCR (RT-qPCR) kits (*Invitrogen*). Briefly, total RNA was polyadenylated using poly(A) polymerase and ATP, and then cDNA was

synthesized from the tailed miRNA population using *SuperScript™ III* reverse transcriptase and a specially designed universal RT primer. For miRNA-target genes, first-strand cDNA was synthesized from 1 μg of total RNA using the *SuperScript*™ III first-strand synthesis system for RT-qPCR (*Invitrogen*) following the manufacturer's protocol.

To analyze the miRNAs and target gene expressions, RT-qPCR was carried out in nine technical replicates using the *StepOnePlus™* real-time PCR system and a *Power SYBR® Green* PCR master mix (*Applied Biosystems*). PCR conditions were: 95 ºC for 10 min followed by 40 cycles at 95 ºC for 15 s and at 60 ºC for 1 min. The cDNA amplification was monitored as fluorescence signals in real time during instrument run. The primers for miRNA and target gene amplifications were designed as described by Patanun *et al.* (2012) and successfully validated (Table 1 Suppl.). In order to confirm the expressions of miRNAs and their target genes, primers used in RT-qPCR need to have a high specificity. Based on the melting or dissociation curves, the PCR amplification of the genes of interest with selected primer pairs was observed to produce a single type of amplicon (Fig. 1 Suppl.).

The expressions of *U6 snRNA* and *18S rRNA* were performed on small RNA and total RNA libraries from leaves and roots of cassava under the water deficit, respectively. Their expression stability was measured by calculating ΔCt in a set of cDNA from both tissues. As expected, the stable expressions of both *U6 snRNA* and *18S rRNA* were observed (Fig. 2 Suppl.). The transcriptions of genes *U6 snRNA* and *18S rRNA* were used to normalize the transcriptions of miRNA and the target genes. The relative expressions of miRNAs and the target transcripts were manually calculated by the comparative C_T method ($2^{-\Delta\Delta CT}$).

A *GeneRacer* kit (*Invitrogen*) was used for the modified 5'-RNA ligase-mediated-rapid amplification of cDNA-end (5'RLM-RACE) to identify products of miRNA directed cleavage. Briefly, decapped mRNA was directly ligated to an RNA oligo adapter. The ligated mRNA was then reverse transcribed using *SuperScript*™ *III* reverse transcriptase (*Invitrogen*). To amplify the cleavage products of miRNA-target genes, the *GeneRacer*™ 5'primers provided in a kit and 3'gene specific primers (Table 1 Suppl.) were used for PCR followed by nested PCR (Fig. 3 Suppl.). The nested PCR products were gel purified using a *HiYield*™ *Gel*/PCR fragments extraction kit (*RBC Bioscience*, Taipei, Taiwan) and then were cloned. The inserts of expected sizes were sequenced (*Macrogen*, Seoul, South Korea).

The significance of different testing RT-qPCR within the groups of samples (the control and water deficit) was determined using one-way *ANOVA*. The independent sample *t*-test was performed to compare the difference between the control and water deficit plants at each time point. The results were considered statistically significant when $P < 0.05$. All statistical analyses were carried out using the *SPSS* (*v. 18.0.0*) software.

To identify putative *cis*-regulatory elements, the upstream sequence of up to 3 000 bp of the mature miRNA sequences and target genes of cassava were derived from *Phytozome* (http://www.phytozome.net/cassava). These sequences were used to predict the *cis*-acting element in *PlantCARE* (http://bioinformatics.psb.ugent.be/webtools/plantcare) and to analyze drought responsive *cis*-elements and other stress responsive *cis*-elements.

Results

To address the possible role of drought responsive miRNAs, we analyzed the occurrence and distribution of putative *cis*-regulatory elements corresponding to drought stress on the promoter region of interesting miRNAs and their target genes. In this study, the promoter regions of 21 from 25 miRNA gene families and 35 miRNAs-target genes in cassava could be obtained. The upstream sequences of up to 3 000 bp of mature miRNAs and target genes coding sequences were analyzed for known stress-responsive *cis*-elements to reveal regulatory elements relevant to abiotic and biotic stresses. It should be noted that the putative core promoter (TATA-box) and conserved sequences (CAAT-box), which signal the binding site for the transcription factor, were found in all the miRNAs and target genes.

Several known stress responsive elements observed in the miRNAs promoter regions included the *cis*-elements involved in anaerobic induction (ARE), abscisic acid (ABA) response (ABRE), ABA and VP1 response (CE3), fungal elicitor response (W1-box, TTGACC), cold- and dehydration-response (C-repeat/DRE), heat stress response (HSE), defense and stress response (TC-rich repeats, ATTTTCTTCA), low temperature response (LTR), MYB binding site involved in drought-induction (MBS), anoxic specific induction (GC-motif, CCCCCG), and wound-responsive element (WUN motif). Moreover, several regulatory elements associated with phyto-hormones could be identified including the part of auxin-responsive element (AuxRE), auxin response (TGA-element), MeJA-responsiveness (CGTCA-motif), ethylene-responsive element (ERE), gibberellins response (GARE-motif), gibberellin-responsive element (P-box, CCTTTTG), and salicylic acid responsive (TCA-element) (Table 2 Suppl.).

Based on the frequency of elements found in the upstream region of the drought responsive miRNA genes of cassava, the first three common elements are HSE, TC-rich repeats, and MBS (Fig. 1*A* and Table 2 Suppl.). Moreover, ABRE, ARE, CGTCA-motif, TCA-element, and TGACG-motif were also detected at slightly lower frequency. Besides the miRNA genes, the *cis*-elements in the promoter region of the drought responsive miRNA-

target genes of cassava were also determined. Among the putative *cis*-elements (Table 3 Suppl.), several are known as stress-responsive elements found in the promoter region of miRNAs. ARE, HSE, and ABRE were the most prevalent *cis*-elements found in the target genes (Fig. 1*B*).

Under the control conditions, the eight-week-old cassava plantlets exhibited fully developed mature leaves. To create a water stress, 40 % PEG was added to the

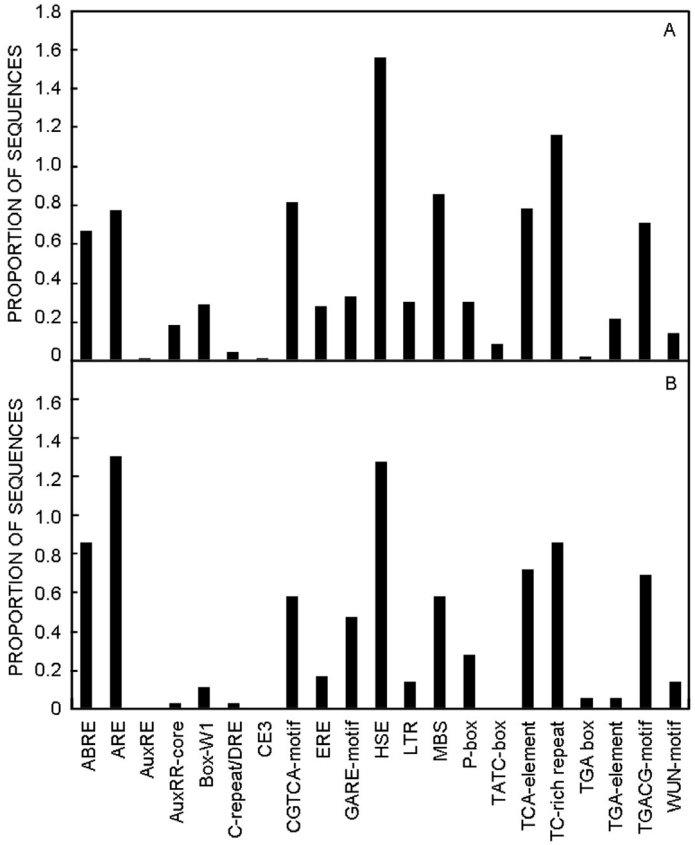

Fig. 1. *Cis*-regulatory elements (3 000 bp) present on the upstream sequence of the putative promoter region of all drought responsive miRNAs (*A*) and miRNA-target genes (*B*) in cassava. The proportion of regulatory elements on the Y-axis represents the number of each distinct regulatory element per miRNAs and target genes.

Table 1. The relative water content (RWC) of detached cassava leaves subjected to a PEG treatment. Means ± SE from three independent experiments. The *asterisks* indicate RWC values significantly different from the control (the *t*-test). Significant differences according to the Tukey's test ($P < 0.05$) was marked by different letters.

Treatment	0 h	2 h	6 h	12 h	24 h
Control	97.31 ± 1.98 a	92.83 ± 2.03a	90.95 ± 2.05a	96.86 ± 0.50a	92.54 ± 1.80a
PEG	-	71.84 ± 0.78b*	49.99 ± 1.37c*	68.68 ± 1.56b*	67.52 ± 4.84b*

media for 2, 6, 12, and 24 h. The first sign of a visible morphological change was wilted leaves which appeared after 2 h and the effect was more pronounced as the stress continued (Fig. 2*A*). Under the water stress, stomatal closure occurred to cut down the transpiration loss of water and so to conserve moisture in the plants. In the cassava plantlets, the partial closure of the stomata was observed after 2 h followed by the complete closure after 6 h. Then, the stomata slightly opened at 12 and 24 h of the water-stress. In contrast, stomata of plants under the control conditions remained completely open throughout the 24 h period (Fig. 2*B*). RWC is an appropriate measure of a plant water status, and under the control conditions, it was maintained at 88 - 97 %, whereas under the water stress it gradually decreased to 50 % after 6 h. However, RWC increased to about 68 % after 12 and 24 h, respectively.

In order to address the role of miRNAs in the water deficit, the candidate miRNAs and their target genes were tested for their expression profiles by using RT-qPCR. Under the water stress, the expression patterns of *miR164* and *miR167* in leaves were different from those in roots. In the leaves, a significant down-regulation of *miR164* was observed, whereas the up-regulation in the roots was

detected (Table 2). The expression of *miR167* in the leaves was rapidly up-regulated from 2 to 12 h, and down-regulated after 24 h in comparison with the control Nevertheless, *miR167* in the roots was sharply up-regulated 24 h after the exposure to the water deficit (Table 3).

To investigate whether the putative miR164 and miR167-target genes were negatively correlated with miRNAs in response to the water deficit, the expression of these miRNAs and target genes in leaves and roots of the cassava plantlets under the water stress treatment were determined. In the leaves, the expression of *MesNAC* was strongly increased after 2 h and remained at

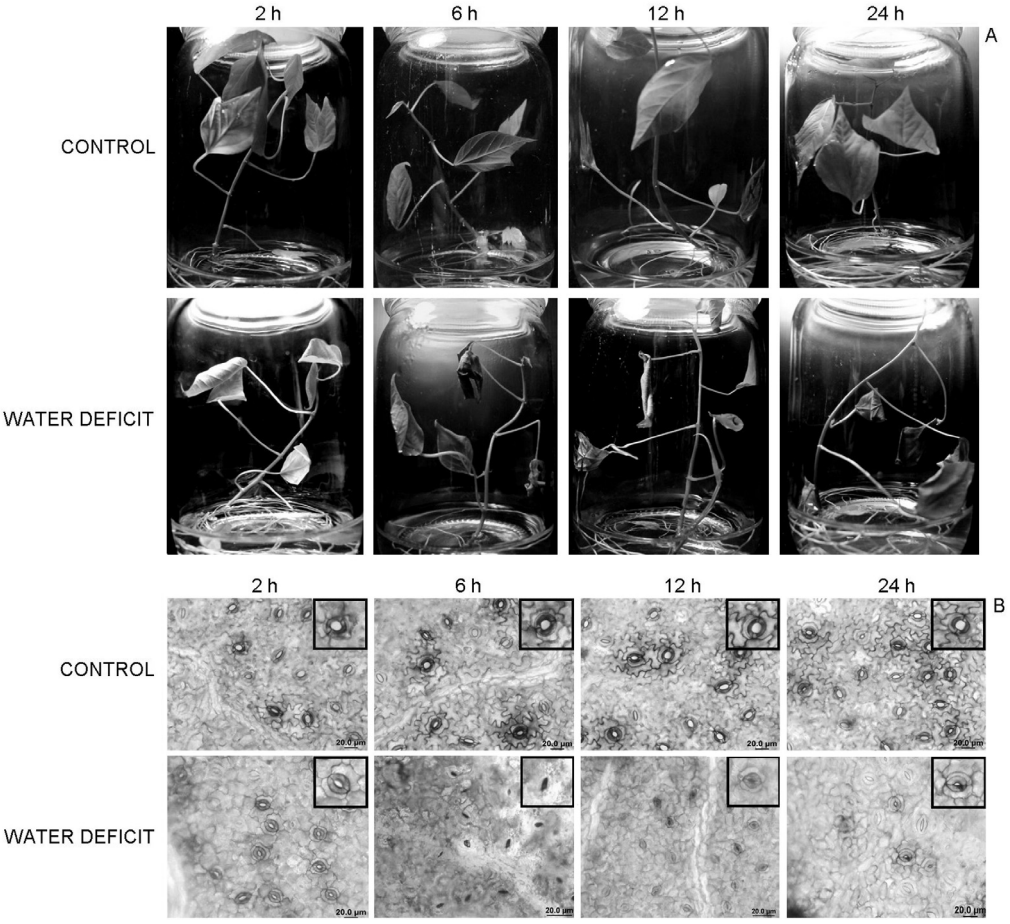

Fig. 2. *A* - The effect of water deficit on morphology of cassava plantlets. Eight-week-old cassava plantlets were transferred to a control MS medium and MS medium containing 40 % (m/v) PEG 6000 (the water deficit treatment) for 2, 6, 12, and 24 h. *B* - The stomatal response of cassava to the water deficit. The stomata were observed under a light microscope.

Table 2. The RT-qPCR analysis of *mes-miR164* and its target gene *MesNAC* in leaves and roots of cassava plantlets under the water deficit induced by PEG for 2 to 24 h. The relative expression was normalized by small reference RNA, *U6 snRNA* and a housekeeping reference gene, *18S rRNA*. The expression was set at 1.00 for the control at 0 h. Means ± SE from nine technical replicates. The *asterisks* indicate values significantly different from the control (the *t*-test). Significant differences according to the Tukey's test ($P < 0.05$) were marked by different letters.

Gene	Tissue	Treatment	0	2	6	12	24
miR164	leaf	control	1.00a	0.85 ± 0.06a	0.93 ± 0.02a	0.93 ± 0.04a	0.52 ± 0.04b
		PEG	-	0.13 ± 0.00fg*	0.06 ± 0.00h*	0.13 ± 0.00g*	0.15 ± 0.00f*
	root	control	1.00a	0.21 ± 0.01d	0.99 ± 0.03a	0.40 ± 0.01c	1.84 ± 0.07b
		PEG	-	2.40 ± 0.23g*	0.46 ± 0.03h	2.72 ± 0.19g*	8.53 ± 0.48f*
MesNAC	leaf	control	1.00a	0.16 ± 0.01d	0.19 ± 0.00d	0.57 ± 0.03b	0.24 ± 0.01c
		PEG	-	143.69 ± 2.44g*	88.62 ± 3.70h*	109.94 ± 8.65h*	246.11 ± 8.12f*
	root	control	1.00a	1.14 ± 0.04b	0.66 ± 0.02c	0.31 ± 0.01d	0.72 ± 0.05c
		PEG	-	22.79 ± 0.82f*	2.73 ± 0.07h*	0.29 ± 0.01i	21.08 ± 0.18g*

Table 3. The RT-qPCR analysis of *mes-miR167* and its target genes *MesARF6* and *MesARF8* in leaves and roots of cassava plantlets under the water deficit (2 to 24 h). The relative expression was normalized by small reference RNA, *U6 snRNA* and a housekeeping reference gene, *18S rRNA*. The expression was set at 1.00 for the control at 0 h. Means ± SE from nine technical replicates. The *asterisks* indicate values significantly different from the control (the *t*-test). Significant differences according to the Tukey's test ($P < 0.05$) was marked by different letters.

Gene	Tissue	Treatment	0	2	6	12	24
miR167	leaf	control	1.00a	1.44 ± 0.03b	0.96 ± 0.02a	0.51 ± 0.02c	0.42 ± 0.02d
		PEG	-	4.75 ± 0.10g*	3.19 ± 0.29h*	5.80 ± 0.12f*	1.04 ± 0.07i*
	root	control	1.00a	0.32 ± 0.04cd	0.14 ± 0.17d	0.58 ± 0.02ac	2.00 ± 0.26b
		PEG	-	0.28 ± 0.04g	0.36 ± 0.05g*	0.40 ± 0.04g*	3.37 ± 0.06f*
MesARF6	leaf	control	1.00a	0.92 ± 0.02c	0.49 ± 0.02c	1.26 ± 0.04b	0.77 ± 0.07d
		PEG	-	4.47 ± 0.08h*	5.72 ± 0.15g*	6.50 ± 0.26fg*	4.78 ± 0.75f*
	root	control	1.00a	0.11 ± 0.00c	1.35 ± 0.08b	1.46 ± 0.01b	1.19 ± 0.15ab
		PEG	-	49.85 ± 4.13f*	0.08 ± 0.01g*	0.10 ± 0.01g*	0.36 ± 0.17g*
MesARF8	leaf	control	1.00a	0.52 ± 0.01e	0.62 ± 0.01d	1.38 ± 0.03c	1.22 ± 0.04b
		PEG	-	0.01 ± 0.00h*	0.03 ± 0.00i*	0.11 ± 0.00g*	0.13 ± 0.00f*
	root	control	1.00a	1.07 ± 0.04b	1.00 ± 0.04a	1.18 ± 0.02c	1.17 ± 0.03c
		PEG	-	0.08 ± 0.00g*	4.17 ± 0.12f*	0.16 ± 0.00g*	0.04 ± 0.01g*

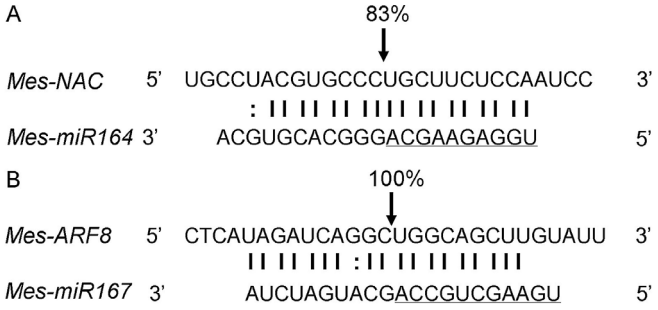

Fig. 3. Mapping *NAC* (*A*) and *ARF8* (*B*) mRNA cleavage sites by 5' RNA ligase-mediated RACE (5'RLM-RACE). The top strand represents a miRNA-complementary site present on its target mRNA, whereas the bottom strand is the miRNA strand. The underlined letters depict the 1st- 10th nucleotide position of miRNA sequences. Perfectly complementary bases are connected with the *solid line*s, and G:U wobble pairing is indicated by the *dotted lines*. The *arrows* indicate the site where cleavage occured, and the numbers represent the percentage of the target mRNA clones with sequences perfectly complementary to the miRNA.

the high level until 24 h (Table 2). The *MesARF6* transcript in the leaves was up-regulated already after 2 h and further increased with the stress duration. In contrast, the expression of *MesARF8* was significantly down-regulated upon the water stress (Table 3). In the roots, the *MesNAC* expression was up-regulated after 2 h, then it significantly decreased after 6 and 12 h, and increased again after 24 h (Table 2). The *MesARF6* expression in the roots was also induced after 2 h and then disappeared after 6 h, whereas the expression of *MesARF8* was significantly down-regulated after 2 h, but then was extremely up-regulated after 6 h and again sharply down-regulated after 12 h (Table 3).

To verify whether the predicted targets of miRNAs were authentic and cleaved *in vivo*, 5'RLM-RACE was carried out. miRNAs had high complementarity to their targets and generally directly cleavaged the target mRNA at the position between 10th and 11th nucleotides from the 5'end of the miRNA-targeting sequence. Since the cleaved mRNA products in plants have 5' phosphate, it can be directly ligated to an RNA adaptor. To confirm that *MesNAC*, *MesARF6*, and *MesARF8* identified in cassava leaves were the real target of *mes-miR164* and *mes-miR167*, respectively, the 5'RLM-RACE cleavage assays was carried out using RNA isolated from leaf tissues. In the 5'RLM-RACE, the nested PCR was carried out using nested gene specific primers for the precise amplification of target. The specific bands of *MesNAC, MesARF6*, and *MesARF8* were detected at 197, 296, and 236 bp (Fig. 3 Suppl.). The amplified products from the nested PCR reactions were gel purified, ligated to a cloning vector, and sequenced. These results are consistent with the *MesNAC* transcript being a direct target of *mes-miR164*, whereas *mes-miR167* could guide the cleavage of *MesARF8* (see Fig. 3). We thus propose that in cassava leaves, *MesNAC* and *MesARF8* were identified as *in vivo* targets of *mes-miR164* and *mes-miR167*, respectively.

Discussion

Plant responses to the environment are complicate processes and could occur at many different levels: morphological, physiological, cellular, and molecular. Water stress reduces the cell water potential and elevates the concentrations of solutes inside cells and extracellular spaces. Water deficit leads to growth inhibition, ABA and proline accumulations, and rapid stomatal closure (Chaves *et al*. 2003, Lisar *et al*. 2012). In this study with cassava plantlets, an imitated drought stress was created by addition of 40 % PEG to a culture medium. After a certain period, the cassava plantlets exhibited physiological and morphological changes including leaf wilting, stomatal closure, and a decrease in leaf RWC. The changes in stomatal opening are considered as part of the early response mechanism to minimize water loss by transpiration (El Sharkawy *et al*. 1984, Alves *et al*. 2004, Utsumi *et al*. 2012).

Despite a large number of miRNAs have shown the potential involvement for responses to water deficit conditions in plants by computational and high-throughput sequencing small RNA, only a few miRNAs and their target mRNAs have been functionally characterized during water deficit in *Arabidopsis*, rice, poplar, and *Medicago*. Recently, a number of drought responsive genes have been identified in cassava (Utsumi *et al.* 2012), but the link between miRNAs and water deficit has not been reported. In the present study, the 138 conserved miRNAs representing 25 drought-responsive miRNA families were identified, and the target gene of each individual miRNA was also predicted by using computational approaches. *Cis*-regulatory elements function as molecular switches allowing organisms to response to environmental stress signals at the transcriptional level (Yamaguchi-Shinozaki *et al*. 2006). The presence of Heat shock element (HSE), TC-rich repeat, and MYB binding site involved in drought-inducible motifs (MBS) in the drought-responsive miRNA promoters was observed. MBS are binding sites for MYB transcription factors which play a major role in abiotic stress responses and especially regulate gene expression under drought stress and in an ABA signaling pathway (Abe *et al*. 1997, 2003). Several conserved *cis*-acting elements in the promoters of the drought-responsive miRNAs and target genes were observed in association with plant hormones. Drought stress responses are strongly regulated by phytohormones (Shinozaki *et al*. 2000). An ABA responsive element (ABRE) signals for gene expression upon drought stress in many plant species (Maruyama *et al*. 2012). Recent microarray analyses revealed that ABRE is the most conserved *cis*-element in dehydration-inducible promoters in *Arabidopsis,* rice, and soybean (Maruyama *et al*. 2012). The presences of MBS and ABRE may thus indicate a cross-talk between a plant drought response and the ABA signaling pathway mediated by miRNAs. Methyl jasmonate (MeJA) responsive elements (CGTGCA; TGACG) are important for the regulation of plant development and response to biotic and abiotic stresses. The increased content of MeJA probably also mediates gene expression under drought stress (Creelman *et al*. 1995).

The expression patterns of *miR164*, *miR167*, and their target genes during the water stress were studied. Among several stress-responsive elements in the *miR164/ MesNAC* promoter regions, one was identified as typical ABA-responsive element. ABA roles in plant adaptation to drought stress are well known (Daszkowska-Golec *et al*. 2013). The ABA responsive, MeJA responsive, and ethylene responsive elements were found in the promoter region of *miR164/MesNAC*. Similarly, various *cis*-elements for phytohormone responses also existed in *miR167/MesARF8.* A MeJA responsive element was observed on both *miR167* and *MesARF8*, whereas an ethylene responsive element, which is involved in the transcriptional activation of drought-responsive genes, was found only in the *MesARF8* promoter sequences. The results therefore suggest that some drought-responsive miRNAs and target genes were regulated by several phytohormones at the transcriptional level. We also confirmed the differential expression profiles of *miR164*, *miR167*, and their target genes in response to the water deficit in cassava leaves and roots. Since this observation largely agrees with previous studies in various plants (Li *et al.* 2011, Elden *et al.* 2012, Sunkar *et al.* 2012), it could be explained by the fact that the cassava has its own tissue specific miRNA expression profile under water deficit. Here, the possible role of *miR164* and *miR167* directing their respective target genes expression, which are NAC transcription factor (*MesNAC*) and auxin responsive factor (*ARF6, ARF8*), in response to water deficit in cassava was demonstrated.

NAC transcription factors are induced by abiotic stresess including drought and salinity in *Arabidopsis* (Takasaki *et al*. 2010). Additionally, a possible involvement of *miR164* in regulating the post-transcriptional processing NAC transcription factors has been reported (Guo *et al*. 2005). The *miR164* from different plants is differentially regulated by cold, salt stress, pathogen, and nematode infection (Khraiwesh *et al*. 2012), as well as in development of *Arabidopsis* leaves (Nikovics *et al*. 2006). ABA is produced and rapidly accumulated in response to drought stress in many plants (Shinozaki *et al*. 2000, Xiong *et al*. 2002) including cassava (Alves *et al.* 2004). In addition, there are reports indicating a cross-talk among ABA, jasmonate, and ethylene signaling in drought responses (Wilkinson *et al*. 2012, Daszkowska-Golec *et al*. 2013). Additionally, the increasing expression of *MesNAC* may also be regulated at the transcriptional level by ABA. Moreover, the NAC responsive genes that encode proteins with recognized roles in a drought stress response, *ERD*, *LEA*, *RD26*, and *R2R3 MYB*, increase during drought stress in cassava (Lokko *et al*. 2007, Utsumi *et al*. 2012). The *R2R3-MYB* is specifically

localized in guard cells to promote closing stomata under drought stress (Cominelli *et al*. 2005, 2010, Liang *et al*. 2005). Taken together, we hypothesize that during the water deficit, the down-regulation of *miR164* together with ABA signaling led to the up-regulation of *MesNAC* which in turn directed the *R2R3 MYB* expression to promote stomatal closure and conservation of water.

The *miR167* target auxin response factors 6 and 8 (*ARF6* and *ARF8*) and *miR167* expressions in the cassava leaves were regulated upon the water deficit. The up-regulation of the *miR167* expression was detected and led to the reduction of the *MesARF8* mRNA level, whereas the expression of *MesARF6* was up-regulated. As result, *MesARF6* and *MesARF8* responded to the water-limited conditions through independent- and dependent-regulation of *miR167*, respectively. *miR167* has been reported to be induced during drought stress in *Arabidopsis* (Liu *et al*. 2008) and tobacco (Frazier *et al*. 2011). There is a report indicating that a drought stress can induce a jasmonic acid synthesis in soybean (Hamayun *et al*. 2010). Consistent with this, MeJA responsive elements were found on the promoter of a cassava *miR167* gene.

In the roots, the expression of *miR164* was up-regulated upon the water deficit. However, its target gene, *MesNAC*, expression was not in the reverse correlation with the *miR164* since the amount of the *MesNAC* transcript was not reduced but increased 2 and 24 h after the onset of the water deficit. This may suggest the possibility that *miR164* was involved in the translational inhibition of *MesNAC*, as in the case of *miR172* which negatively regulates its target gene *APETALA2* at the translational level rather than a direct mRNA cleavage during flowering time in *Arabidopsis* (Chen *et al*. 2004). Moreover, the up-regulation of *MesNAC* might be regulated through a *cis*-regulatory element on its promoter region such as ABA responsive element and coupling elements in response to the water deficit. The role of *MesNAC*, which is transcriptionally up-regulated or may be translationally inhibited in roots upon water deficit, needs to be further investigated. *miR164* is a negative regulator of *AthNAC1* which in turn affects lateral root development in *Arabidopsis* (Guo *et al*. 2005) and maize (Li *et al*. 2012). In cassava, *MesNAC* but not *NAC1* was predicted to be the target of *miR164*, although *miR164* and *MesNAC* did not show a negative correlation. The regulation of *miR164/Mes*NAC in lateral root formation should be further studied.

Unlike in the leaf tissues, the expressions of *miR167* and its targets *MesARF6* and *MesARF8* in the cassava roots were inversely regulated. It has been shown that *miR167* participates in drought stress in *Arabidopsis* (Liu *et al*. 2008). In *Arabidopsis*, *arf6* and *arf8* single mutants show development of more lateral roots than the wild-type under an osmotic stress (Kinoshita *et al*. 2012). *ARF8* was reported to positively regulate the expression of *GH3* gene which functions in the lateral root inhibition (Nakazawa *et al*. 2001). Together with these results, it is possible that *miR167* is involved in reactions to water deficit by mediating *ARF6* and *ARF8* functions. Therefore, we hypothesize that this regulation would allow the adaptation of lateral roots in response to water stress.

In conclusion, this study demonstrates the behavior of *miR164* and *miR167* and their target genes during the water stress of cassava. The results suggest the possible dual role of *miR164* and *miR167* to regulate their target genes *MesNAC* and *MesARF8*, respectively, leading to water deficit responses, such as stomatal closure, a reduction in leaf expansion, and lateral root development.

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