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BRIEF COMMUNICATION

Cassava microRNAs and storage root development

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Abstract

Cassava storage roots serve as an outstanding source of starch that is commonly utilized for nourishment and industrial applications. Despite the extensive studies, which indicated diverse important roles of miRNAs as post-transcriptional regulators of gene expression, the potential contribution of microRNAs to storage root development in cassava are sparse. Here, we characterized the key miRNAs and auxin content in two main types of cassava roots, fibrous roots and storage roots. The differential expression pattern of miRNAs and their mRNA targets, *miR164/NAC* and *miR167/ARF6, ARF8,* revealed the correlation in storage root development. A higher content of indole-3-acetic acid was observed in storage roots in contrast with fibrous roots, and the possible role was discussed. Altogether, this first finding suggested the roles of *miR164/miR167* in the molecular mechanism underlying cassava storage root development.

Additional key words: auxin, fibrous roots, *Manihot esculenta*, miR164, miR167, RLM-RACE.

Cassava (*Manihot esculenta* Crantz.) is a perennial plant of the *Euphorbiaceae* family. Up to 500 million people around the world consume cassava starch from its huge storage roots where starch makes 70 - 90 % of total dry mass (Nuwamanya *et al*. 2008). Cassava starch can be also used in paper, textile, pharmaceutical, and cosmetic industry, as well as an alternative energy resource (Fu et al. 2016).

Usually, cassava is propagated by stem cuttings. The adventitious roots called fibrous roots (FRs) emerge from the stem base and function in water and nutrient absorption. Then, some of them start to bulk and increase in their diameter due to secondary root growth. These become storage roots (SRs) whose function is dedicated to store cassava starch (Hillocks *et al.* 2002). The molecular mechanisms involved in the initiation of storage root formation in cassava are not fully understood. Several transcription factors were found to be involved in the SR formation of *Callerya speciosa* (Xu *et al.* 2016). In potato, the regulation of gibberellin content as well as fluctuation in day length determine tuberization (Kloosterman *et al.* 2007). In recent years, glycolysis/gluconeogenesis, ethylene, gibberellin, cytokinins, auxins, and protein folding have been suggested to associate with cassava SR development (Sojikul *et al.* 2010, 2015, Yang *et al.* 2011, Saithong *et al.* 2015, Naconsie *et al.* 2016)

To date, several studies reported that microRNAs

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Abbreviations: ARF - auxin response factor; ELISA - enzyme-linked immunosorbent assay; FR - fibrous roots; IAA - indole-3-acetic acid; KU50 - Kasetsart 50; miR - micro RNA; NAC - NAM, ATAF1/2, and CUC2 proteins; 5' RLM-RACE - 5' RNA ligase-mediated rapid amplification of cDNA ends; SR - storage roots.
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(miRNAs) with approximately 21 nt in length have a regulatory function in gene expression in several physiological processes (Jones-Rhoades *et al*. 2006, Brodersen *et al.* 2008) including stress responses (Alptekin *et al*. 2017). Many of plant miRNAs are evolutionarily conserved and the regulation of gene expression by miRNAs existed from the earliest stages of plant evolution. Although many of plant miRNA genes are conserved across species, the size and genomic organization significantly varied, which may cause spatial and temporal differences in target gene regulation (Budak and Akpinar 2015). Root development is critical for plant growth, and miRNAs have been reported to participate in this complex genetic networks. Various miRNAs, such as *miR160, miR164, miR167*, and *miR390* have recently been found to be essential for plant root growth and development in several plants (Meng *et al*. 2010).

The NAC proteins are one of the large families of plant-specific transcription factors, which have been implicated in diverse processes including developmental programs. The NAC domain was identified from consensus sequences of *Petunia* no apical meristem (NAM) and *Arabidopsis* NAC transcription factor (ATAF1/2) and cup-shaped cotyledon (CUC2) proteins. Transcripts of *NAC1* targeted by *miR164* were demonstrated to provide a homeostatic mechanism to a down-regulated auxin signal for lateral root development in *Arabidopsis* (Guo *et al.* 2005). The indole-3-acetic acid (IAA) regulates biological processes by controlling gene expression *via* DNA-binding auxin response factors (ARFs). The ARFs are components that confer specificity to auxin response through selection of target genes as transcription factors. The ARFs bind to auxin response DNA elements in the promoters of auxin-regulated genes and regulate transcription of these genes. The IAA was suggested to act as a controlling key for modulating adventitious rooting in petunia cuttings through AUX/IAA proteins (Druege *et al.* 2014). It has been reported in *Arabidopsis* that the formation of adventitious roots are regulated by auxin-related miRNAs through various ARF transcription factors (Guilfoyle 2007). The knock out mutant lines *arf6* and *arf8* produce less adventitious roots compared to a wild type, whereas their over-expressing lines develop more suggesting a role of ARF6 and ARF8 as positive regulators of adventitious root formation. Therefore, *miR167*, which targets *ARF6* and *ARF8* transcriptions, serves as a negative regulator of adventitious root development in *Arabidopsis* (Gutierrez *et al.* 2009). By contrast, the number of adventitious roots in rice significantly decreases in an auxin resistance mutant, in which *miR167* expression is repressed suggesting a positive effect of *miR167* on rice adventitious root formation (Meng *et al.* 2009).

So far, only little information of cassava miRNAs has been reported, including a genome-wide scan for conserved miRNAs (Patanun *et al.* 2013) and recent high-resolution small RNA sequencing to identify novel miRNAs (Khatabi *et al.* 2016), but no report has focused on miRNAs and cassava SR formation. To decipher the regulation of miRNA genes during cassava SR development, *miR164* and *miR167* and their target genes were investigated in FRs and SRs of the 8- and 12-week-old plants. Furthermore, the actual cleavage sites on mRNA targets were confirmed by using modified 5' RNA ligase-mediated rapid amplification of cDNA ends (5' RLM-RACE) and IAA content in the root tissue.

Cassava (*Manihot esculenta* Crantz) cv. Kasetsart 50 (KU50), which is one of the most popular cultivars especially in Southeast Asia, was grown at the Rayong Field Crop Research Center, Thailand. Plants were grown from stem cuttings for 8 and 12 weeks and then roots were harvested and gently washed with running-tap water. Three samples of each FR ($0.1 \leq 0.5$ cm diameter) and SR (>1.0 cm diameter) were collected from three healthy plants. In SRs, the cortex was removed and the parenchyma storage tissue was cut into small pieces. In FRs, the epidermis layer was peeled off, and remaining tissue was cut into small pieces. The tissues were quickly frozen in liquid nitrogen and stored at -80 °C. Quantitative determination of IAA in cassava root extracts was performed using enzyme-linked immunosorbent assay (ELISA) by the anti-IAA monoclonal competitive antibody binding method (for detail see the Supplement).

Total RNA was isolated from the frozen roots by using *Concert*™ *Plant RNA Reagent* (*Invitrogen*, Carlsbad, USA) according to the manufacturer's instructions. Twenty cubic millimeters of RNase-free water was added to dissolve the RNA. The contaminating genomic DNA was removed by using a *DNA-free*™ kit (*Ambion*, Austin, USA). The total RNA samples were quantified using a *ND-1000* (*Nanodrop Technologies*, Delaware, USA), RNA quality was checked by 1 % (m/v) agarose gel electrophoresis, and then, the samples were stored at -80 °C until use. In order to construct the miRNA library, the first-strand cDNA synthesis for miRNA amplification was synthesized from 100 ng of total RNA using *Ncode*™ miRNA first-strand cDNA synthesis and real time quantitative PCR kits (*Invitrogen*) according to the manufacturer's instructions. Further, the first-strand cDNA for the miRNA-target gene library was prepared from 1 µg of total RNA by using the *Superscript*™ *III* PCR cDNA synthesis system (*Invitrogen*). The resulting cDNA was stored at -20 °C or used immediately for PCR.

Validation of selected miRNA was performed using end-point real time PCR. A cDNA of the miRNAs from the storage root samples was amplified with a miRNA-specific primer and an adaptor primer (Table 1 Suppl.). Real-time quantitative PCR of mature miRNAs and miRNA-target genes was performed (primers are listed in Table 1 Suppl.; for detail of the method see the Supplement). A miRNA-target prediction and validation was performed by modified 5' RLM-RACE as previously described by Patanun *et al.* 2013. An oligonucleotide adapter having 5' adenylated and 3' blocked was ligated to the 3' end of the cleaved RNA followed by PCR amplification using nested gene specific primers. Nested PCR was used to increase the specificity and sensitivity of RACE products of the 5' end. A primary PCR using a gene specific primer GSP1 was performed to generate a gene-specific RACE product. Consequently, the nested PCR reaction with the nested gene specific primer GSP2 was performed. After PCR amplification, the specific band was detected. The real time PCR product was further purified, cloned, and sequenced. After sequencing, analysis of the miRNA cleavage product was carried out (see the Supplement).

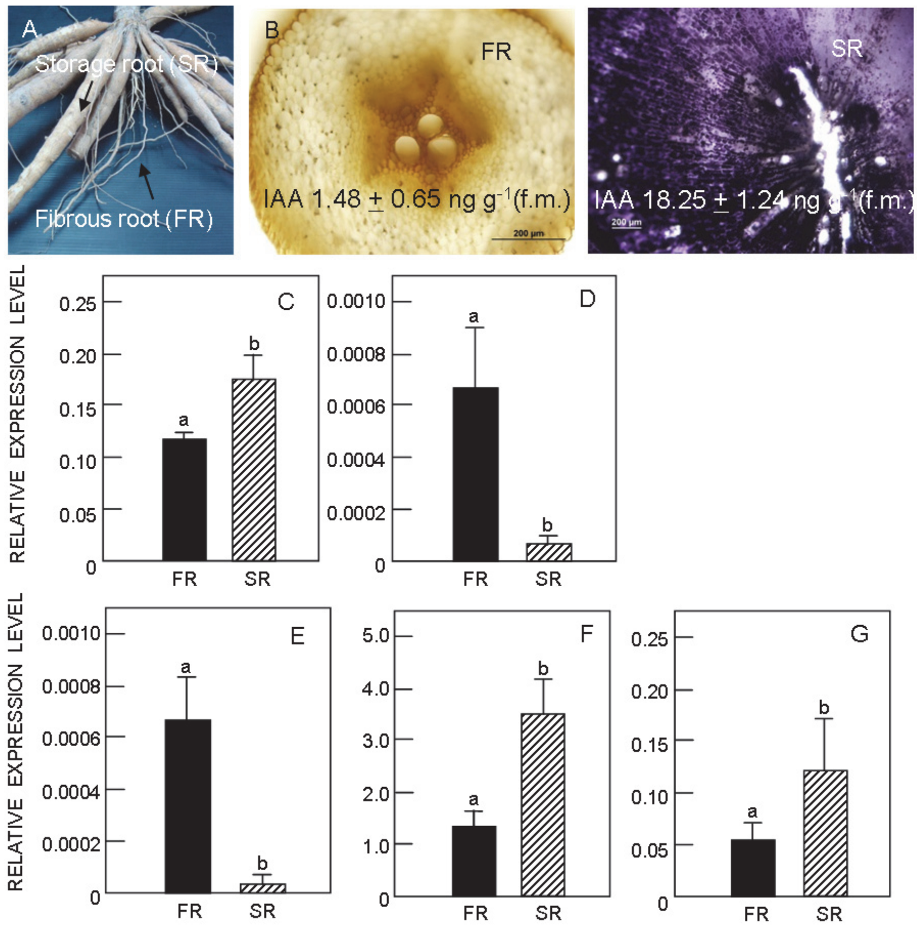

Fig. 1. Relative expressions of miRNAs and their target genes in a fibrous root (FR) and storage root (SR) measured by real-time quantitative PCR. *A* - FR and SR; *B* - a cross section and iodine-stained FR and SR showing IAA content. Relative expressions of *miR164* (*C*), *NAC* (*D*), *miR167* (*E*), *ARF6* (*F*), and *ARF8* (*G*) were investigated in FR and SR of eight-week-old cassava plants. Means ± SEs, $n = 9$, different letters indicate significant differences at $P \leq 0.05$.

In plants, miRNA has been studied extensively for over a decade (Budak and Akpinar 2015). An interest in cassava miRNAs has drawn attention in recent years, and during years 2011-2013, 169 cassava miRNAs belonging to 34 miRNA families were identified using a computational approach (Amiteye *et al.* 2011, Patanun *et al.* 2013). Subsequently, small RNA sequencing from several laboratories showed many conserved and new miRNAs with their predicted potential miRNA target genes. They are involved in regulation of many important physiological processes (Pérez-Quintero *et al.* 2012, Chen *et al.* 2015, Khatabi *et al.* 2016, Rogans and Rey 2016) including starch biosynthesis (Chen *et al.* 2015) and in responses to stresses (Ballen-Taborda *et al.* 2013), *e.g.*, drought (Phookaew *et al.* 2014), chilling (Xia *et al.* 2014) or anthracnose disease (Pinweha *et al.* 2015). Our previous investigation of miRNAs *via* homology search revealed that *miR164* and *miR167* families in cassava consist of four and seven members, respectively (Patanun *et al.* 2013). Although *miR164* and *miR167* had been previously predicted, there was a need to confirm the existence of these miRNAs in cassava cv. KU50. For validation of the selected miRNAs, we used the end-point RT-PCR, and the result was a ~80 nt PCR product (Fig. 1 Suppl.). Then, the PCR products were cloned and sequenced result showed that *miR164* was 5'-UGGAGA

AGCAGGGCACGUGCA-3' and *miR167* was 5'-UGA AGCUGCCAGCAUGAUCUA-3'. Nucleotide sequences were searched for homology against miRNAs in the *miRBASE 21* database. The cassava *miR164* and *miR167* shared high similarities to *miR164* and *miR167* from other plant species such as *Arabidopsis* and rice (Fig. 2 Suppl.). Comparison to the predicted cassava miRNA based on homology search revealed a few mismatch nucleotides at the 3' end of identified miRNA sequences indicating unique miRNA sequences in cv. KU50 (Fig. 3 Suppl.). The results also imply that the primers used in this experiment were highly specific for amplifications of *miR164* and *miR167* transcripts in expression analysis.

Due to the complementarities among miRNAs and their particular target genes, homology-based search facilitated miRNA target gene prediction. It was claimed that miRNA target genes are well-conserved among divergent plant species (Chorostecki *et al.* 2012). Consistent with the previous findings, the results reveal that *NAC* transcription factors were targets of *miR164*, whereas the target genes of *miR167* were *ARF6* and *ARF8* (Table 2 Suppl.). In plants, pairing miRNA and its target mRNA causes cleavage and subsequent mRNA degradation (Lai *et al.* 2004). Using this advantage, the primers for real-time PCR of the target genes were designed on each side of the miRNA-target sequence covering the complementary sequence of miRNA so that only intact, un-cleaved transcripts would be amplified. An U6 spliceosomal RNA (*U6* small nuclear RNA) and *18S* rRNA were used as reference genes to ensure that a higher or lower expression results from the real expression of miRNAs and target genes, respectively. The experiments were performed using the total RNA from FRs and SRs of 8- and 12-week-old cassava plants. The high expression of *miR164* was restricted to cassava SRs; on the other hand, *miR167* was highly expressed in FRs (Fig. 1 and Fig. 4 Suppl.). These results suggest the possible role of *miR164* and *miR167* in physiological process and further support the existence of different miRNAs in different cassava root types.

The reciprocal expression pattern of miRNAs and their target genes were observed in FRs and SRs of eight-week-old cassava plants (Fig. 1). The cross sections of FRs and SRs were stained with a iodine solution, and starch accumulation was observed in SRs (Fig. 1*B*). The *NAC* was up-regulated in FRs and down-regulated in SRs suggesting the negative correlation of *miR164* and its target gene in the cassava roots (Fig. 1*C,D*). The expression of *miR167* decreased during SR development, whereas transcriptions of its target genes *ARF6* and *ARF8* were up-regulated suggesting the negative correlation of *miR167* and its target genes during SR root development (Fig. 1*E-G*). Interestingly, the expression of miRNAs and their target genes measured in FRs and SRs of 12-week-old cassava plants also revealed the similar profiles as found in 8-week-old cassava plants (Fig. 4 Suppl.), thus supported the inverse expression patterns of *miR164, miR167*, and their target genes during SR development. In addition, IAA was found to differently accumulate in the cassava root system. Content of IAA was higher in 8- and 12-week-old SRs [18.25 ± 1.24 and 19.63 ± 1.12 ng g^{-1}(f.m.)] as compared to FRs [1.48 ± 0.65 and 1.45 ± 0.54 ng g^{-1}(f.m.)], respectively.

Basically, lateral roots function as an anchorage as well as in improving water and nutrient uptake. The lateral roots in *Arabidopsis* may be equivalent to the FRs in cassava. It has been reported that transmitting auxin signals through NAC1 promotes lateral root development in *Arabidopsis.* Overexpressing *miR164* in *Arabidopsis* shows a low accumulation of *NAC1* transcripts, which attenuates auxin signaling leading to a reduced lateral root emergence (Guo *et al.* 2005). The 1 000 bp upstream promotor region from the 5'end of miRNA precursor sequence of each predicted cassava miR164 isoforms were previously checked for a regulatory motif. Interestingly, a TGA-element, which is an auxin-responsive element has been found in the *mes-miR164a* promotor region (Patanun *et al.* 2013).

In *Arabidopsis*, a *NAC* family gene was demonstrated to play an intermediary role in auxin-induced development of lateral roots (Xie *et al.* 2000). Cytokinins and auxins have been involved in thickening of storage roots (tuberization) in sweet potato, whose storage roots develop from some of fibrous roots similarly as observed in cassava (Noh *et al.* 2010). The signal transduction pathway of auxin was also reported to play a great role in tuberous root initiation in *Brassica rapa* (Li *et al.* 2015). It should be noted that in cassava, *NAC*, but not *NAC1,* was found to be the target of *miR164* though *miR164/NAC* showed a negative correlation with SR development. The function of NAC1 was not clearly characterized in cassava tuberization yet. Recently, 96 *NAC* genes have been identified in the cassava genome; those can be categorized into 16 subgroups (Hu *et al.* 2015). Nevertheless, we obtained a direct evidence that *NAC* was cleaved by *miR164* using a modified 5'RLM-RACE. A 66.67 % of cloned sequences showed a cleavage site corresponding to an expected region. This result further confirms that *NAC* was regulated by *miR164* in cassava through mRNA degradation.

To promote the expressions of auxin-responsive genes, ARFs bind to auxin-response elements within the promotor region of auxin-responsive genes. A high auxin content leads to destruction of Aux/IAA repressors by the function of transport inhibitor response1 (TIR1). Then, the activated ARFs modulate the expressions of the auxin-responsive genes. In contrast, in the absence of threshold auxin content, Aux/IAA-ARF interaction occurs, thus impedes auxin-responsive gene expressions (Guilfoyle 2007). In sweet potato, the IAA content is high during the early stage of SR development (Noh *et al.* 2010). It has been reported that auxin-associated miRNAs firmly modulate lateral root formation in *Arabidopsis via* a complex mechanism that involves various ARFs including ARF6 and ARF8, which are positive regulators. In other words, *miR167*, which targets *ARF6* and *ARF8*, is a negative regulator of lateral root development (Gutierrez *et al.* 2009), which is similar to the situation that occurred in the present study in cassava. Nevertheless, the auxin-related miRNA regulatory pathways are not possibly fully conserved in phylogenetic distant species, as *miR167* in rice showed a positive regulation of lateral and adventitious root development (Khan *et al.* 2011).

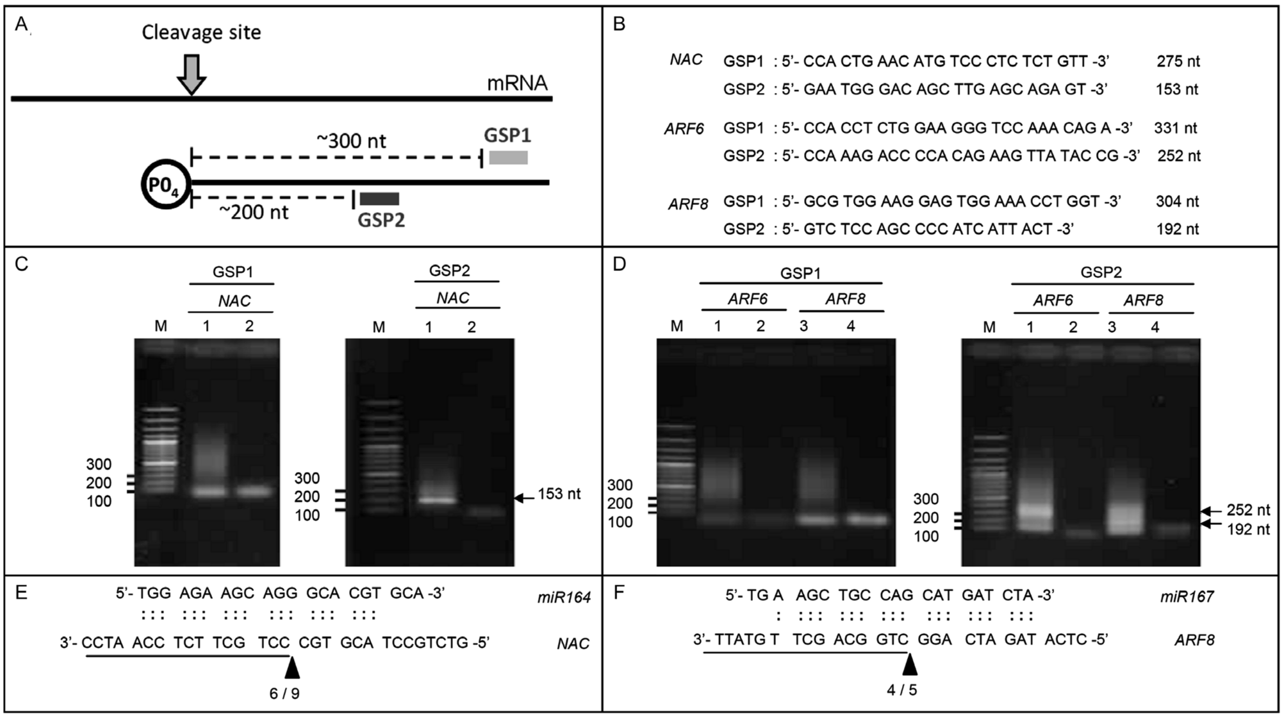

Fig. 2. The 5' RNA ligase-mediated rapid amplification of cDNA ends for validation of a direct cleavage of *NAC, ARF6*, and *ARF8* by *miR164* and *miR167,* respectively. *A* - The cleaved RNA was nested PCR amplification using gene specific primers GSP1 and GSP2. *B* - GSP1, GSP2, and an expected nested-PCR product of *NAC*, *ARF6*, and *ARF8. C* - Nested-PCR product of *NAC* (*lane 1*), negative control (*lane 2*), and marker (*lane M*). *D* - Nested PCR product of *ARF6* (*lane 1*), *ARF8* (*lane 3*), negative controls (*lanes 2* and *4*), and marker (*lane M*). *E* - Number of sequences representing the same cleavage site found per total sequenced clones; the *arrow* indicates a cleavage site on *NAC* which is located at the 10th nucleotide-position of *miR164*. *F* - Number of sequences representing the same cleavage site found per total sequenced clones; the *arrow* indicates a cleavage site on *ARF8* which is located at the 10th nucleotide-position of *miR167*.

The modified 5'RLM-RACE results reveal that six out of nine clones showed the 5'cleaved end of *NAC* exactly mapped to the 10th nucleotide position of mature *miR164*; it is the predicted cleavage site of *miR164*. On the other hand, four out of five clones of *ARF8* was found to contain a specific cleavage site corresponding to the mature *miR167*, whereas surprisingly, *ARF6* did not show any cleavage site corresponding to *miR167* (Fig. 2). This result suggests that *ARF6* might not be targeted by *miR167* in cassava. A similar finding has also been discovered in *Arabidopsis*, in which Ru *et al.* (2006) found that *miR167* directly cause *ARF8* transcript degradation, but not *ARF6*. Fewer base-pairs between *ARF6* and *miR167* might affect the cleavage of the *ARF6* mRNA. These findings indicate that both *NAC* and *ARF8* were exactly targeted by *miR164* and *miR167*, respectively, suggesting the role of *miR164* and *miR167*, at least in part, in SR development.

In conclusion, based on our finding on the higher IAA content in SRs as compared to FRs in both 8- and 12-week-old cassava plants, this study leads to the hypothesis on possible roles of miRNAs and their target genes in cassava SRs development in auxin-dependent manner (Fig. 5 Suppl.). The higher auxin accumulation in SRs may promote *miR164* transcription resulting in *NAC* degradation, and reduction of NAC consequently reduce lateral roots emergence. In contrast to the situation in FRs, where IAA content is pretty low, similar to the case of the FRs of sweet potato (Noh *et al.* 2010), *miR164* was found to be down-regulated and contributed to the high transcription of *NAC*, which is required for normal fibrous root development. Meanwhile, a high expression of *miR167* in FRs down-regulated *ARF* transcription. The low content of IAA and Aux/IAA proteins repressed ARF function, and so auxin-responsive genes were not transcribed thereby resulting in suppression of SR formation. On the other hand, the high IAA content in SRs permitted ARF function allowing auxin-responsive genes to turn on, thus promoting primary thickening growth of SRs. Due to the benefits of cassava SRs for various applications, cassava study will provide us fundamental knowledge to manipulate this plant according to desired qualities.

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