Review Article

A Review of Non-Coding RNAs Associated with Drought Stress Response in Cassava (*Manihot esculenta* Crantz)

Abstract

Cassava is a tuberous root crop that offers food and nutrition security for vulnerable populations especially in the developing world. The crop is climate-resilient, widely adaptable to varied environments, tolerant to most abiotic stresses such as drought. It is easy to propagate and can produce significant yield under low input levels compared to other major crops. Cassava's inherent tolerance to drought stress has been linked with various morpho-physiological and molecular mechanisms. Although major molecular pathways and genes activated under drought stress have been described, cassava drought stress tolerance mediated by non-coding RNAs (ncRNAs) such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) have not been exhaustively elucidated. This review therefore consolidates recent progress that has been made towards the discovery and characterization of miRNAs and lncRNAs in cassava response to drought stress. The review details the omics-approaches used in various studies to discover miRNAs and lncRNAs and their over-arching functions in several physiological and molecular mechanisms in cassava under drought.

Keywords: cassava; climate-resilience; food security; drought stress; miRNAs; lncRNA

Introduction

Agricultural drought occurs as a result of sub-optimal or below normal precipitation or a deficit in soil moisture that negatively affects plant growth, development and production. This subsequently damage crops leading to reduced yield or total crop failure. Plants' response to drought stress can be categorized as avoidance, tolerance or resistance, escape and recovery from stress [1, 2]. These mechanisms can be observed through changes in plant characteristics at ecological, morphological, biochemical, physiological and molecular levels [2]. The changes are genetically programmed and regulated via differential expression patterns of drought responsive genes [2, 3]. Within the complex and diverse molecular pathways, numerous genes are activated for signal transduction, synthesis of phytohormones, transcription factors and protein kinases, metabolism, osmotic regulation or adjustments, protein modification and conversion, accumulation of metabolites and scavenging for reactive oxygen species among others [3, 4]. Recently, the roles of non-coding RNAs (ncRNAs) in genetic regulation and modulation of plant

responses to drought stress and their potential application for improving crop production under drought stress have gained attention from scientific community.

Eukaryotic transcriptomes consists of ncRNAs that have minimal or no-protein coding capacity but are functional [5]. Categories of ncRNAs that have been discovered and characterized in plants includes small ncRNAs (18–30 nucleotides) such as microRNAs (miRNAs), medium-sized ncRNAs (31–200 nucleotides) and long ncRNAs (lncRNAs) (>200 nucleotides) [5]. Irrespective of these differences, ncRNAs play essential regulatory roles in plant growth and development and adaptations to environmental stresses by modulating gene expression at transcriptional and post-transcriptional levels [5, 6]. For instance recent studies have shown involvement of lncRNAs and miRNAs in drought stress responses through abscisic acid-mediated regulation, auxin and ethylene signaling, osmoprotection, calcium signaling and scavenging of antioxidants [7, 8, 9]. Further, lncRNAs and miRNAs participate in plant response to water deficit through a complex cellular pathways involving chromatin modulation, target mimicry, transcriptional regulation, hormonal signaling and by directly regulating drought-responsive genes [2]. Thus modulation of lncRNAs and miRNAs levels in plants offers a possibility for engineering climate-resilient crops [6] such as cassava.

Regulations of drought responsive genes by lncRNAs and miRNAs have recently been reported in cassava [7, 10], a drought tolerant crop that can produce high yield and sustain food and nutrition security especially in the arid and semi-arid regions of the developing world where smallholder farmers are particularly affected by climate change [11, 12]. Cassava's response to drought stress at molecular levels increasingly involves lncRNAs and miRNAs. This review therefore consolidates recent data on the discovery and functions of lncRNAs and miRNAs in cassava under drought stress conditions. The omics-approaches deployed in discovery of these lncRNAs and miRNAs are highlighted and their roles in other crops or plants under drought are compared with cassava. Potential candidate lncRNAs and miRNAs that can be used to enhance productivity of drought susceptible cassava varieties through breeding are also listed.

Long non-coding RNAs

The long non-coding RNAs (lncRNAs) are a diverse and widely expressed class of RNAs with key roles in regulation of gene expression. Although lncRNAs are transcripts with more than 200 nucleotides that do not encode proteins [13], recent studies have indicated their critical roles in plant responses and adaptation to abiotic stresses [14] such as drought [15, 16]. The lncRNAs participates in drought stress response in plants by capitalizing on their co-expression networks with microRNAs (miRNAs), protein-coding genes and transcription factors [10, 17, 18], recruiting complex mechanisms based on antisense transcription-mediated modulation, chromatin modulation, or directly regulating the transcription of various drought-responsive genes [19, 20, 21]. Drought-responsive lncRNAs have been identified and described in rice [22], maize [16], foxtail millet [23], switch grass [17] and banana [24] and *Brassica juncea* [25]. Over-expression of lncRNAs increased grain yield in rice [26] and enhanced drought tolerance in *Arabidopsis* [27]. Recent research has also characterized functions of

lncRNAs in cassava response to drought stress. For example, Dong et al. [14] cloned one lncRNA from cassava referred to as *DROUGHT-INDUCED INTERGENIC lncRNA* (*DIR*), whose expression was induced by drought stress. *DIR* enhanced drought stress tolerance in cassava under transgenic experiments. Over-expression of *DIR* further altered expression of several genes involved in response to stimulus and secondary metabolites pathway [14]. Specifically, the *DIR*-mediated drought stress response strongly induced up regulation of transcription factors such as WRKY (*Manes. 03G009300*), bHLH (*Manes. 16G101000*) and NAC (*Manes. 16G172900*); transporters such as NAD *dependent epimerase* (*Manes. 02G009300*) and lipid transfer protein (*Manes. 01G074600)* as well as AZF2 (*Manes. 07G061700*), a gene that encodes a zinc finger protein [14].

Suksamran et al. [28] integrated genomics and transcriptomics-based approaches to identify novel putative cassava lncRNAs that might be involved in post-transcriptional regulation of stress-induced transcription factors (TFs) such as zinc-finger, WRKY and nuclear factor Y gene families in response to drought stress. Under drought stress, 47 and 51 of the lncRNAs were expressed at elevated and lower levels, respectively. For drought stress, *Manes.09G025200*, annotated as a nuclear factor Y subunit A9 (*NF-YA9*) encoding gene was predicted to be a target of another novel lncRNAs, *ncM17949* [28]. Expression of *NF-YA9* mRNA and lncRNAs in autotetraploid cassava under drought stress has been reported [29]. *The NF-Y* previously conferred drought stress tolerance in maize and rice [30, 31]. Under drought conditions in cassava, Suksamran et al. [28] reported enriched functions of the 802 target genes of 98 lncRNAs including genes involved in the regulation of stomatal opening and ABA that have been associated with drought response in plants. Candidate lncRNAs such as *ncP12197* was predicted to bind directly with *Manes.06G154600* coding for *SLAC1* protein which is reportedly involved in regulating guard cells for stomatal opening/closure during drought [11] while *ncM15664* decreased and predicted its target, *Manes.18G037900* / ABA-responsive elements-binding factor 2 (*ABF2*) increased significantly under drought conditions [28].

Ding et al. [10] identified 124 drought responsive lncRNAs in cassava leaves and roots subjected to PEG-induced dehydration stress using strand-specific RNA-Seq. technology. The qRT-PCR validation revealed three lncRNAs (lincRNA101, lincRNA391 and lincRNA356) that were up-regulated while four lncRNAs (lincRNA64, lincRNA350, lincRNA182 and lincRNA392) were down regulated under drought stress treatments. The *TCONS_00060863* and *TCONS_00097416* lncRNAs were involved in regulation of ABA and ethylene signaling pathways, respectively, under drought stress [10]. Some of these lncRNAs were identified acting as putative target mimics of known miRNAs in cassava and others as *cis-acting lncRNA-mRNA* pairs that regulated expression of their neighboring genes such as those encoding SAUR-like auxin-responsive, melatonin responsive, 8-hydroxylase involved in ABA catabolism, ethylene signaling, *AP2/EREBP* TFs, Zinc Finger TFs, proline-rich extensin-like receptor kinase and leucine-rich repeat protein kinase required for root hair elongation [10]. Further, co-expression network analysis predicted functions of lncRNAs with genes involved in cell cycle and cell organization, cell wall, calvin cycle and light reaction, major CHO metabolism,

secondary metabolism, signaling receptor kinase, hormone metabolism (such as ABA and GA) and abiotic stress [10]. Xiao et al. [29] also applied strand-specific RNA-Seq technique to identify autotetraploid-specific *lncRNAs* that were differentially expressed in drought stressed leaves. The lncRNAs were involved in drought tolerance through down-regulation of photosynthetic genes and up-regulation of subtilisin-like proteases which increased stomatal density and increased UDP-glucosyltransferase. Specifically, co-expressed network analysis indicated two lncRNAs (*LNC_001148* and *LNC_000160*) mediated drought tolerance by regulating stomatal density in autotetraploid cassava via co-expressed target genes encoding subtilisin-like proteases [29]. Autotetraploidy reduces transpiration by lesser extent increasing of stomatal density, smaller stomatal aperture size, or greater stomatal closure, and reducing accumulation of H_2O_2 under drought stress [29].

Using the same strand-specific RNA-Seq. approach, Wu et al. [7] identified 194 lncRNAs that were differentially expressed between ABA and polyethylene glycol (PEG) treatment in cassava. Trans-regulatory co-expression network revealed that ABA-uniquely-responsive lncRNAs were primarily participated in receptor kinases signaling, hormone metabolism, and cell wall modification while PEG-uniquely-responsive DE lncRNAs were mainly involved in jasmonate metabolism, biotic and abiotic stress, calcium signaling, and transport [7]. Further, four lncRNAs (*TCONS_00129136, TCONS_00122745, TCONS_00088201* and *TCONS_00067612*) were identified as putative targets of cassava functionally well-known miRNAs (such as *miR156* and *miR159*) involved in ABA- and drought-response, suggesting their roles in cassava drought response via ABA-dependent pathways with the participation of miRNAs regulation [7]. Li et al. [32] carried out a genome-wide identification and functional prediction of cold and/or drought-responsive lncRNAs in cassava. They reported a total of 318 lncRNAs involved in cold or drought stress with nearly 10 and 8% of the lncRNAs strongly induced and repressed respectively by drought treatment. Specifically, five lncRNAs (*ncRNA101, ncRNA391, ncRNA356, ncRNA28* and *ncRNA105*) and four lncRNAs (*ncRNA64, ncRNA350, ncRNA182* and *ncRNA392*) were respectively up-regulated and down regulated under the drought stress conditions [32]. These lncRNAs were associated with hormone signal transduction, secondary metabolites biosynthesis and sucrose metabolism pathway in cassava under drought stress. Figure 1 below provides a summary of lncRNAs identified in cassava and their effects on several physiological and molecular pathways as well as other genes that ultimately contributes to cassava's drought stress tolerance.

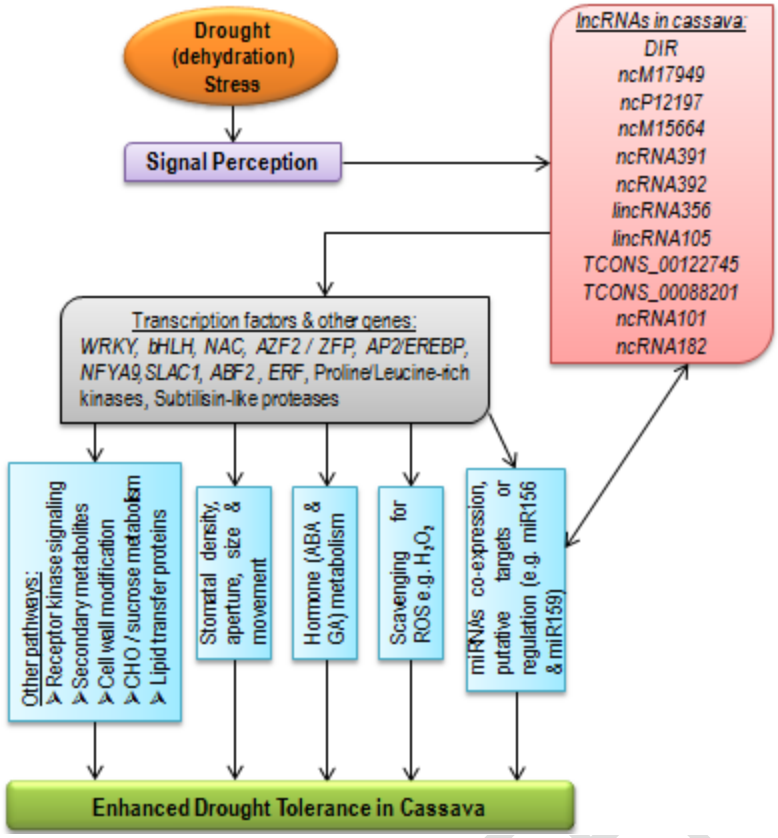

Figure 1: Illustration of *lncRNAs* pathways in cassava response to drought stress (based on literature review for other plant species and cassava)

MicroRNAs (miRNAs)

MiRNAs are single stranded 20 - 24 nucleotide long non protein-coding small RNAs that play important roles in post-transcriptional gene silencing in many organisms [33, 34]. In plants, miRNAs play a regulatory role in growth, development, organogenesis, and phytohormone signaling and adaptive responses to biotic and abiotic stresses [35]. MiRNAs play an important role in regulating and promoting plant adaptation and tolerance to fluctuations and adverse environmental conditions [2, 36, 37]. Studies have shown that miRNAs are important modulators of drought tolerance in plants where they influence the cleavage of several drought responsive genes and hence inhibit their translation [38]. The miRNAs themselves show altered expression in response to drought and control expression of several drought-responsive genes, transcription factors and phytohormones [2, 39]. Drought responsive miRNAs have been identified and characterized in several plant species including *Arabidopsis* [40], sugarcane [41], soybean [42], tomato [43], wheat [44], sorghum [45], maize [46] and rice [38, 47]. For instance under drought stress conditions, up-regulated miR171f was involved in progression of root growth and development of rice plants [22], down regulation of miR167 led to up-regulation of Phospholipase D, a gene involved in controlling ABA response and stomatal movement in maize [9], down regulation of miR159 triggered

expression of transcription factors (such as HD-ZIP, ARF and GA-MYB) which contributed to greater adventitious and lateral root formation [2] and miR156 interacted with the ABA-dependent strigolactone signaling pathways to enhance drought tolerance in tomatoes [48]. The miR156 was identified as a mediator of stomatal movements thus regulating water relations and stomatal functioning.

Similarly, Khatabi et al. [49] identified miRNAs in cassava using high resolution sequencing of small endogenous RNA libraries from the leaf, stem, callus and male and female flower tissues. Several of these miRNAs targeted transcription factor (TF) families of genes associated with drought tolerance. These included mes-miR166j that targeted basic-leucine zipper TF; mes-miR319f that targeted MYB1; mes-miR156k that targeted Squamosa promoter-binding protein-like; mes-miR169a that targeted transcription-repair coupling factor; miR477 that targeted sequence-specific DNA binding transcription factor and TEOSINTE BRANCHED 1 CYCLOIDEA and PCF TF targeted by mes-miR319f [49]. Additionally they also identified miR160, miR167 and miR393 that targeted auxin response factor genes. Cassava tolerance to drought stress is at least partly due to miR393 regulation of auxin receptors [49, 50]. Zeng et al. [51] used a genome-scale systematic study of miRNAs in *Euphorbiaceae* by combining computational prediction and drought-based experimental analysis. They reported 48 miRNAs in two cassava varieties that were either up- or down regulated under drought stress treatment. Among them included miR156 (*Squamosa* promoter-binding protein), miR157 (LIGULELESS1 protein), miR159 (r2r3-myb transcription factor), miR160 (Auxin response factor), miR162 (dicer-1), miR164 (NAC domain-containing protein), miR166 (DNA binding protein), miR167, miR168 (Acetoin catabolism protein X), miR171 (DELLA protein GAIP-B), miR395 (WRKY), miR396 (heat shock protein binding protein), miR397 (laccase, putative) and miR403 (Argonaute 2) [51]. Using RT-qPCR, Phookaew et al. [52] observed differential expression of the cassava *miR164* (target *MesNAC*) and miR167 (targets *MesARF6 & MesARF8*) which were associated with changes in the leaf shape, stomatal closure, and relative water content in cassava under water deficit treatment.

Ballén-Taborda et al. [53] applied high-throughput sequencing and bioinformatics for identification of *miRNAs* gene targets involved in post-transcriptional abiotic stress regulation that could prove useful in engineering cassava for drought resistance. They identified 134 potential target genes for drought tolerance for the 60 conserved miRNA sequences. For example, miR156 targeted genes in the *squamosa* promoter-binding family, miR159 targeted a MYB-like regulatory protein, *miR160* targeted Chitinase-A and several Auxin response factors (*ARF10, ARF16* and *ARF17*), *miR166* was associated with basic-leucine zipper (*bZIP*), miR169 targeted basic helix-loop-helix (bHLH) DNA-binding protein, miR828 targeted L-aspartate oxidase and miR164 targeted Auxin-response family protein [33]. Additionally, the predicted targets of non-conserved miRNA sequences included a protein phosphatase that was homologous to an ABA-induced gene in *A. thaliana* (*HAI3*), gibberellin oxidase, phenylalanine ammonia-lyase (PAL) and several *WRKY* TFs such as *WRKY33* which functions in detoxification of ROS. The miRNAs and miRNA gene targets identified in this study may play a role in drought-induced posttranscriptional regulation and could be utilized in

engineering cassava for drought resistance [53]. Figure 2 below is a summary representation of miRNA-mediated regulation of drought stress response in cassava as reviewed above

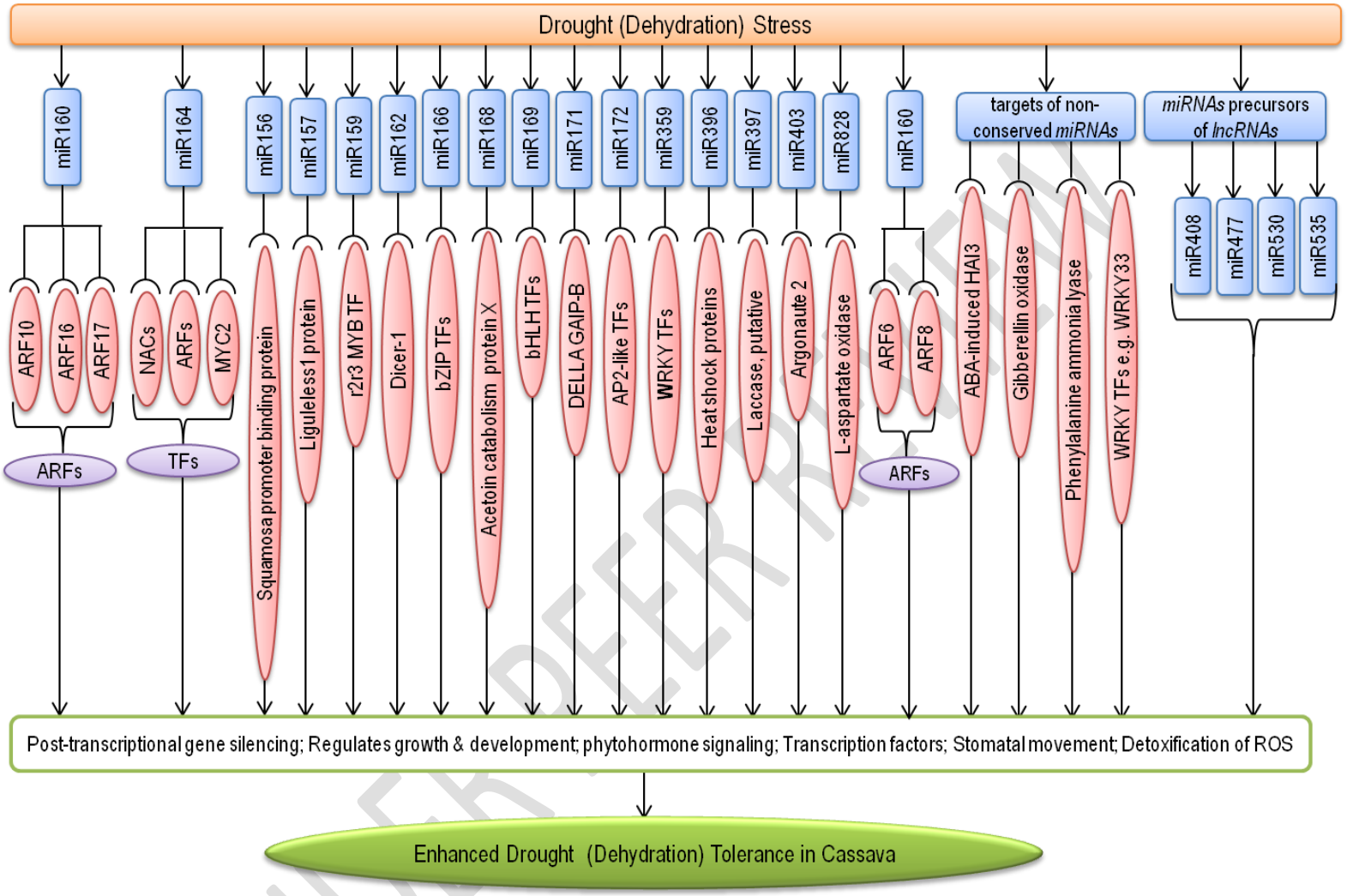

Figure 2: Summary of miRNAs and their target genes in cassava response to drought stress. The model is adopted and edited from figure 1 in Nadarajah and Kumar (2019).

Interactions between lncRNAs and miRNAs

Several miRNAs in cassava under drought conditions have been mimic targets of lncRNAs. Indeed Ding et al. [10] suggested that lncRNAs might function through miRNAs in response to drought stress in cassava. For instance they identified 11 drought-specific differentially expressed lncRNAs acting as putative target mimics of 24 miRNAs in cassava under drought stress of which miR156, miR164, miR169, miR172 and miR395 were all indicated to be involved in abiotic stress response [10, 54]. MiR156 targeted Squamosa-promoter binding protein-le (SPL) genes, miR164 targets MYC2 genes and miR172 targets AP2-like transcription factor while its (miR172)

expression was enhanced by the SPL genes and MYC2 and CSD2 genes were targeted by miR169 and miR398 respectively [10]. Wu et al. [7] identified four lncRNAs that contained binding sites for two miRNAs (miR156 and miR159) that responds to both ABA and drought stress. Xiao et al. [29] also identified 21 potential miRNA precursors of lncRNAs in cassava under drought stress in cassava including miR162, miR166, miR408, miR477, miR530, miR171, miR159, miR535, miR169 and miR167. Differential expression of these miRNAs and their targeted genes has been associated with drought stress tolerance in plants. For example miR169 in regulates stomatal opening and transpiration rate under drought stress conditions. Li et al. [55] reported that miR169 targeted *NF-YA* family genes with over-expression of *NF-YA5* and *NF-YA3* or down-regulation of miR169 enhanced drought stress tolerance in *Arabidopsis* and soybean respectively. The miR169 negatively regulated *MeNF-YA3* in cultivated cassava cultivars with the lower miR169 expression recorded in the wild cassava progenitor showed the progenitor had higher tolerance to drought stress compared to the cultivars [31].

Li et al. [32] identified 12 lncRNAs as 11 known cassava miRNAs precursors including miR156g, miR160d, miR166h, miR167g and miR169d that may be involved in stress response. They also found 16 lncRNAs that may act as miRNA mimics bound by conserved miRNAs such as miR164, miR169, miR2275 and miR1446 [32]. Of these, miR164 was significantly up-regulated by drought stress compared to miR169 and miR2275 that exhibited decreased expression. Out of this mimicry, expression of lincRNA340 was induced by drought stress with a subsequent increase of miR169-targeted NUCLEAR FACTOR Y (NF-Y) genes after drought treatment. Drought stress also induced lincRNA119 that was positively correlated with increased mRNA abundance of corresponding miR2275 targets such as *Manes.02G04600* and *Manes.14G106000* [32]. The *lncRNA, TCONS_00068353* also acted as a target mimic for miR156k and miR172c to control several abiotic stress-responsive genes [32].

Conclusion

The review highlights roles of these ncRNAs including regulating transcription of various drought responsive genes involved in cassava leaf and root growth and development, photosynthetic pathways, stomatal movement and density, transpiration and relative water contents, phytohormones synthesis and signaling, scavenging of reactive oxygen species and accumulation of secondary metabolites among others. Potential application of candidate miRNAs and lncRNAs in improving cassava performance under drought stress is also explored.

Data availability

No data was used or analyzed for this article.

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