Review Article

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

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 and tolerant to most abiotic stresses such as drought. Further, 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 that are activated during drought stress have been characterized, cassava tolerance to drought stress mediated by non-coding RNAs (ncRNAs) such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) has not been extensively described. 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 discuss the omics-methodologies used in diverse research to discover miRNAs and lncRNAs and their over-arching functions in several morpho-physiological and molecular mechanisms in cassava under drought stress. The review identifies candidate miRNAs and lncRNAs that could potentially be used to improve cassava performance under drought stress either via molecular breeding or transgenic systems.

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]. Non-coding RNAs (ncRNAs) have recently received attention from the scientific community for their roles in genetic regulation and modification of plant responses to drought stress, as well as their potential application for boosting crop productivity under drought stress.

Eukaryotic transcriptomes are made up of ncRNAs that have little or no protein-coding potential but are nevertheless functional [5]. Plants have small ncRNAs or microRNAs (miRNAs) (18-30 nucleotides), medium-sized ncRNAs (31-200 nucleotides), and long ncRNAs (lncRNAs) (>200 nucleotides) [5]. These ncRNAs perform critical regulatory roles in plant growth and development as well as adaptability to environmental stresses by altering gene expression at the transcriptional and post-transcriptional levels [5, 6]. Recent research has demonstrated that lncRNAs and miRNAs have a role in drought stress responses via abscisic acid-mediated regulation, auxin and ethylene signaling, osmoprotection, calcium signaling, and antioxidant scavenging [7, 8, 9]. Furthermore, lncRNAs and miRNAs have a role in plant water deficit response via complex cellular pathways encompassing chromatin modulation, target mimicry, transcriptional regulation, phytohormone signaling, and directly regulating drought-responsive genes [2]. Thus altering the levels of lncRNAs and miRNAs 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

Long non-coding RNAs (lncRNAs) are a varied and widely expressed class of RNAs that play important functions in regulation of gene expression. Although lncRNAs are transcripts with more than 200 nucleotides that do not encode proteins [13], recent research has shown that they play key roles in plant responses and adaptability to abiotic stresses such as drought [14, 15]. The lncRNAs participate in drought stress response in plants by leveraging 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 in rice [22], maize [16], foxtail millet [23], switch grass [17], banana [24], and *Brassica juncea* [25] have been discovered and described. For instance, over-expression of lncRNAs increased rice grain yield [26] and also improved drought tolerance in *Arabidopsis thaliana* [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* improved cassava's tolerance to drought stress 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 including *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] using a combination of genomics and transcriptomics identified novel putative cassava lncRNAs that may be involved in the post-transcriptional regulation of stress-induced transcription factors (TFs) such as the zinc-finger, *WRKY*, and nuclear factor Y gene families in cassava response to drought stress. They identified 47 and 51 lncRNAs that were respectively up- and down regulated under drought stress. They discovered *Manes.09G025200*, a nuclear factor Y subunit A9 (NF-YA9) gene that is the target of another novel lncRNA, *ncM17949* [28]. *NF-YA9* mRNA and lncRNAs were also found and documented in autotetraploid cassava following drought stress treatments by Xiao et al. [29]. *NF-Y* has previously been shown to provide drought stress resistance in maize and rice [30, 31]. Suksamran et al. [28] discovered enriched functions of 802 target genes of 98 lncRNAs under drought conditions in cassava. These genes were involved in the regulation of stomatal opening and ABA synthesis, a response commonly linked with drought response in plants. Candidate lncRNAs including *ncP12197* have been predicted to bind directly to Manes.06G154600 that encodes the SLAC1 protein, which is thought to regulate guard cells for stomatal opening or closure during drought stress [11]. Another candidate, *ncM15664* decreased while the expression of its predicted target gene, *Manes.18G037900* / ABA-responsive elements-binding factor 2 (*ABF2*) was significantly elevated 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 and other four lncRNAs (*lincRNA64, lincRNA350, lincRNA182* and *lincRNA392*) whose expression were suppressed under drought. Upon drought stress, lncRNA *TCONS_00060863* regulated ABA biosynthesis while *TCONS_00097416* was involved in ethylene signaling pathways [10]. A number of lncRNAs were reported acting as putative target mimics of already 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*, ethylene signaling, *AP2/EREBP* TFs, Zinc Finger TFs, proline-rich extensin-like receptor kinase and leucine-rich repeat protein kinases, which functions in root hair elongation or extension [10], a trait important for drought response. Furthermore, co-expression network analysis predicted lncRNA functions 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 response [10].

Xiao et al. [29] used strand-specific RNA-Seq to uncover autotetraploid-specific lncRNAs that differed in expression in drought stressed cassava leaves. In this study, drought tolerance was mediated by lncRNAs which inhibited expression of photosynthetic genes and up regulated subtilisin-like proteases, which enhanced both stomatal density and activity of UDP-glucosyltransferase. Further co-expressed network analysis revealed two lncRNAs (*LNC_001148* and *LNC_000160*) that modulate drought stress tolerance by controlling stomatal density in autotetraploid cassava via co-expressed target genes encoding subtilisin-like proteases [29]. Under drought stress, autotetraploid conditions reduce transpiration by reducing stomatal density, stomatal aperture size, or stomatal closure, and decreasing H_2O_2 levels [29].

Wu et al. [7] used the same strand-specific RNA-Seq. technique to identify 194 lncRNAs in cassava that were differently expressed between ABA and PEG-induced dehydration stress treatment. Trans-regulatory co-expression networks revealed that ABA-specific lncRNAs were primarily involved in receptor kinase signaling, hormone metabolism, and cell wall modification, whereas PEG-specific DE lncRNAs were primarily involved in jasmonate metabolism, biotic and abiotic stress, calcium signaling, and ion transport [7]. Furthermore, four lncRNAs (*TCONS_00129136*, *TCONS_00122745, TCONS_00088201*, and *TCONS_00067612*) were identified as putative targets of cassava functionally well-known miRNAs (*miR156* and *miR159*) involved in ABA- and drought-response, implying their roles in cassava drought response via ABA-dependent pathways with 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 over expressed and suppressed under the drought stress conditions [32]. These lncRNAs were linked to hormone signal transduction, secondary metabolite biosynthesis, and the sucrose metabolism pathway in drought-stressed cassava. 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.

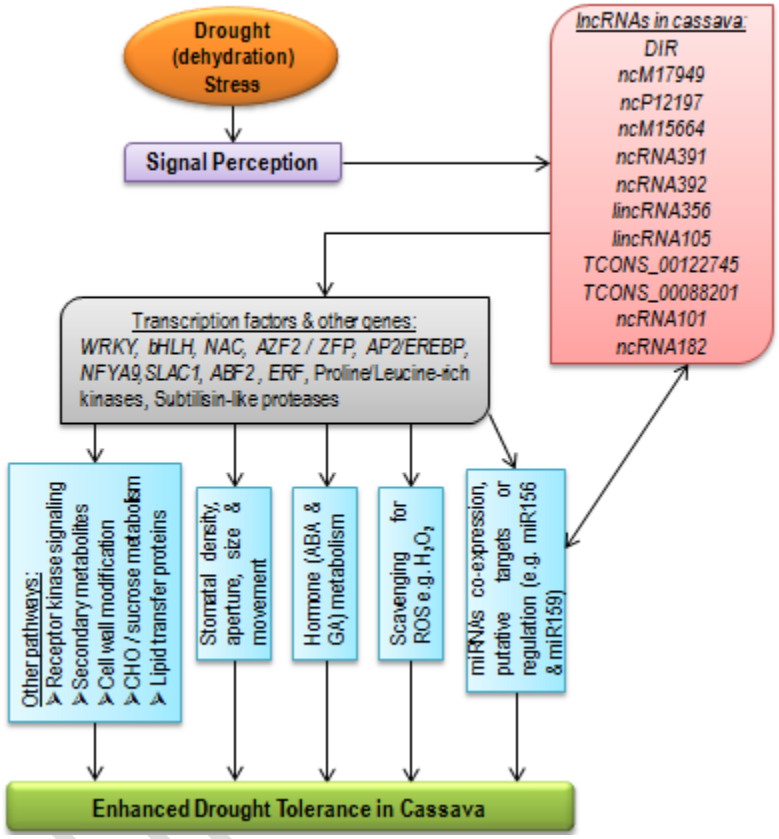

Figure 1: Illustration of *lncRNAs* pathways in cassava response to drought stress (based on literature review for other plant species and cassava)

MicroRNAs (miRNAs)

MiRNAs are single-stranded non protein-coding short RNAs that are 20 to 24 nucleotides long and play important roles in post-transcriptional gene silencing in a variety of organisms [33, 34]. MiRNAs regulate growth, development, organogenesis, phytohormone signaling, and adaptive responses to both biotic and abiotic stresses in

plants [35]. MiRNAs have a crucial function in regulating and enhancing plant adaptation and tolerance to environmental fluctuations and stresses [2, 36, 37]. MiRNAs have been demonstrated to be key modulators of drought tolerance in plants, influencing the cleavage of multiple drought responsive genes and thereby inhibiting their translation [38]. Drought alters the expression of miRNAs, which affect the expression of numerous drought-responsive genes, transcription factors, and phytohormones [2, 39]. Several plant species, including Arabidopsis [40], sugarcane [41], soybean [42], tomato [43], wheat [44], sorghum [45], maize [46], and rice [38, 47], contain functional drought sensitive miRNAs. Under drought stress conditions, for example, up-regulated miR171f was involved in elongation of root growth and development of rice plants [22], down-regulated miR167 led to up-regulation of Phospholipase D, a gene involved in controlling ABA response and stomatal movement in maize [9], down-regulated miR159 triggered expression of transcription factors (*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]. Previously, miR156 was classified as a mediator of stomatal movements thus regulating water relations and stomatal functioning.

Similarly, Khatabi et al. [49] used high resolution sequencing of short endogenous RNA libraries from the leaf, stem, callus, and male and female flower tissues to identify miRNAs in cassava. Several of these miRNAs targeted transcription factor (TF) families linked to drought resistance genes. 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]. They also discovered miR160, miR167, and miR393 that target auxin response factor genes. Cassava tolerance to drought stress is attributable, at least in part, to the modulation of auxin receptors by miR393 [49, 50].

Zeng et al. [51] combined computational prediction with drought stress-based experimental research in a genome-scale comprehensive study of miRNAs in the *Euphorbiaceae.* They discovered 48 miRNAs in two cassava cultivars that were either up- or down-regulated in response to drought stress. 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]. Phookaew et al. [52] used RT-qPCR to detect differences in the

expression of cassava miR164 (target *MesNAC*) and miR167 (targets *MesARF6* and *MesARF8*), which were linked to changes in leaf shape, stomatal closure, and relative water content in cassava during water deficit treatment.

Ballén-Taborda et al. [53] applied high-throughput sequencing and bioinformatics to identify miRNA gene targets involved in post-transcriptional abiotic stress regulation that might be used to improve drought tolerance in cassava. For the 60 conserved miRNA sequences, they discovered 134 possible target genes for drought tolerance. 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 (*bHLHs*) DNA-binding protein, miR828 targeted L-aspartate oxidase and miR164 targeted Auxin-response family protein [33, 53]. Furthermore, the predicted targets of non-conserved miRNA sequences included a protein phosphatase that was homologous to an ABA-induced gene in Arabidopsis thaliana (HAI3), gibberellin oxidase, PAL, and several WRKY TFs, including WRKY33, which functions in ROS detoxification. The discovered miRNAs and miRNA gene targets in this study may play a role in drought-induced post-transcriptional regulation and could be used to genetically engineer drought-resistant cassava genotypes [53]. Figure 2 below is a summary representation of miRNA-mediated regulation of drought stress response in cassava as reviewed above

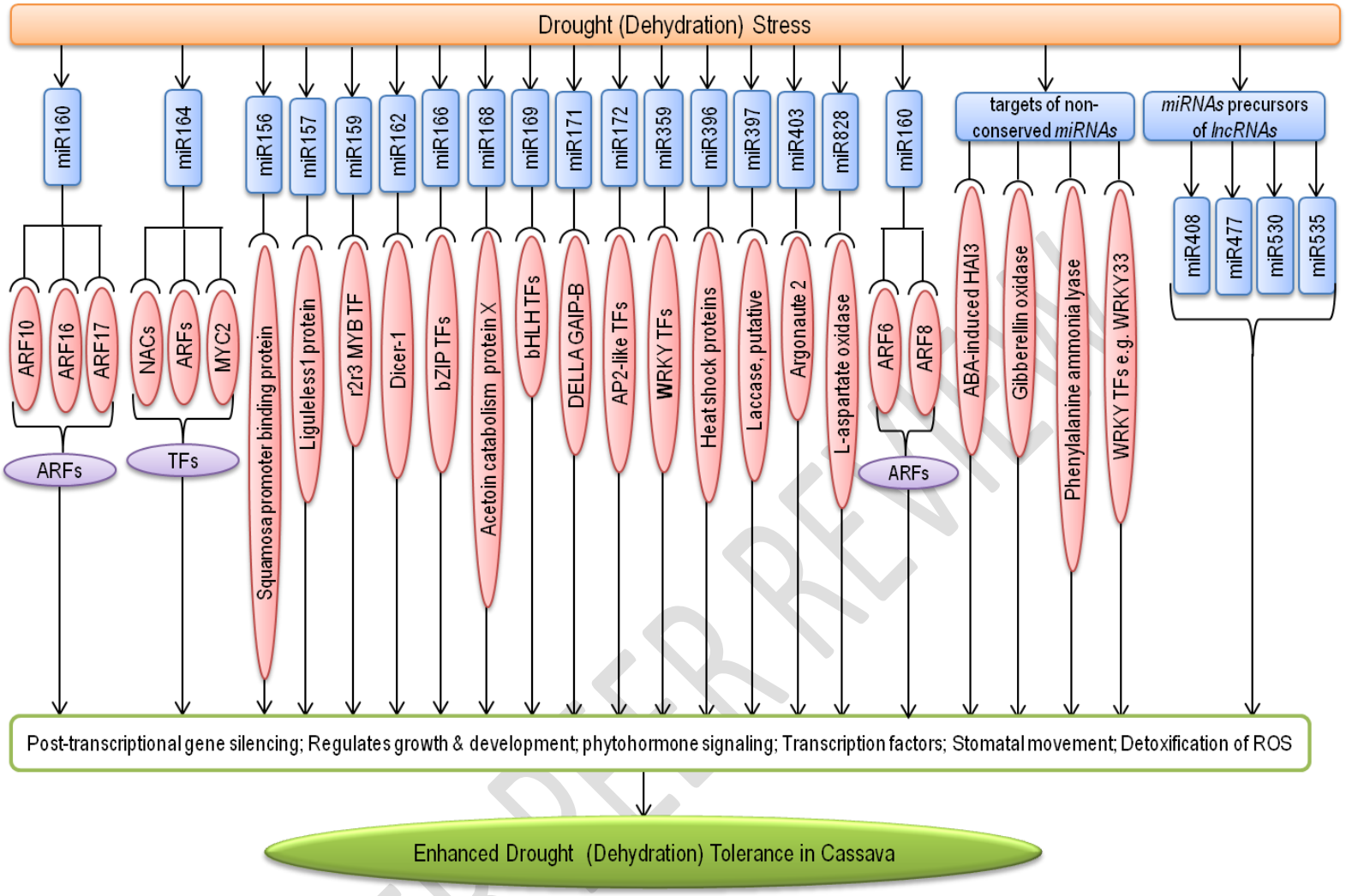

Figure 2: Summary of miRNAs and their target genes in cassava response to drought stress. The model is adopted and edited from figure 1 in Nadarajah and Kumar (2019).

Interactions between lncRNAs and miRNAs

Several miRNAs in cassava under drought circumstances have been identified as lncRNA target mimics. Indeed, Ding et al. [10] proposed that lncRNAs in cassava might function via miRNAs in response to drought stress. For example, they discovered 11 drought-specific differentially expressed lncRNAs that operate as probable target mimics of 24 miRNAs in cassava under drought stress. Some of these included miR156, miR164, miR169, miR172, and miR395 all involved in abiotic stress response [10, 54]. MiR156 targets Squamosa-promoter binding protein-le (SPL) genes, miR164 targets *MYC2* genes, and miR172 targets AP2-like transcription factor, whose expression is promoted by SPL genes, while miR169 and miR398 target *MYC2* and *CSD2* genes, 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. Under drought stress, Xiao et al. [29] found 21 possible miRNA precursors of lncRNAs in cassava, including miR162, miR166, miR408, miR477, miR530, miR171, miR159, miR535, miR169, and miR167. Differential expression of these miRNAs and their target genes has been linked to plant drought resistance. Under drought stress conditions, for example, miR169 modulates stomatal opening and reduces the rate of transpiration. According to Li et al. [55], miR169 targeted *NF-YA* family genes, and over-expression of *NF-YA5* and *NF-YA3* or down-regulation of miR169 improved drought stress tolerance in Arabidopsis and soybean, respectively. The miR169 negatively regulated *MeNF-YA3* in cultivated cassava cultivars, and the lower miR169 expression in the wild cassava progenitor indicated that the progenitor was more resistant to drought stress than the cultivar.

Li et al. [32] found 12 lncRNAs as precursors to 11 known cassava miRNAs, including miR156g, miR160d, miR166h, miR167g, and miR169d, which may be involved in stress response. They also discovered 16 lncRNAs that might function as miRNA mimics when bound by conserved miRNAs including miR164, miR169, miR2275, and miR1446 [32]. Among these miRNAs, the expression of miR169 and miR2275 was down-regulated compared to miR164, whose expression was considerably up-regulated by drought stress [32]. Drought stress elevated the production of lincRNA340, which was followed by an increase in miR169-targeted *NF-Y* genes after drought treatment. Drought stress also enhanced the abundance of lincRNA119, which was associated with increased mRNA abundance of relevant miR2275 targets such as *Manes.02G04600* and *Manes.14G106000* [32]. The *lncRNA, TCONS_00068353* also served as a target mimic for miR156k and miR172c, allowing them to modulate many abiotic stress-responsive genes [32].

Conclusion

The review has highlighted the roles of lncRNAs and miRNAs in enhancing cassava's adaptive response to drought stress including regulating transcription and co-expression of various target drought responsive genes that are involved in cassava leaf and root growth and development, photosynthetic pathways, stomatal movement, density and aperture sizes, transpiration and relative water contents, phytohormones (ABA, ethylene, gibberellins etc) synthesis and signaling, scavenging of reactive oxygen species and accumulation of secondary metabolites among others (Fig. 1 and Fig. 2). Candidate lncRNAs that were identified included *DIR, ncM17949, ncP12197, ncM15664, ncRNA391, ncRNA392, ncRNA356, ncRNA105, ncRNA101, ncRNA182, TCONS_00060863, TCONS_00097416, TCONS_00060863, TCONS_00122745, TCONS_00088201* and *LNC_000160* (Fig. 1). Similarly miRNAs with a role in drought

tolerance in cassava included *miR160, miR164, miR156, miR157, miR159, miR162, miR166, miR171, miR172, miR359, miR396, miR397, miR403* and *miR828* (Fig. 2).The review has also summarized the interactions between these lncRNAs and miRNAs under drought stress.

Data availability

No data was used or analyzed for this article.

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