MeSPL9 attenuates drought resistance by regulating JA signaling and protectant metabolite contents in cassava

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Abstract

Key message Analysis of drought-related genes in cassava shows the involvement of MeSPL9 in drought stress tolerance and overexpression of a dominant-negative form of this gene demonstrates its negative roles in drought stress resistance.

Abstract Drought stress severely impairs crop yield and is considered a primary threat to food security worldwide. Although the SQUAMOSA promoter binding protein-like 9 (SPL9) gene participates extensively in numerous developmental processes and in plant response to abiotic stimuli, its role and regulatory pathway in cassava (Manihot esculenta) response to the drought condition remain elusive. In the current study, we show that cassava SPL9 (MeSPL9) plays negative roles in drought stress resistance. MeSPL9 expression was strongly repressed by drought treatment. Overexpression of a dominant-negative form of miR156-resistant MeSPL9, rMeSPL9-SRDX, in which a 12-amino acid repressor sequence was fused to rMeSPL9 at the C terminus, conferred drought tolerance without penalizing overall growth. rMeSPL9-SRDX-overexpressing lines not only exhibited increased osmoprotectant metabolites including proline and anthocyanin, but also accumulated more endogenous jasmonic acid (JA) and soluble sugars. Transcriptomic and real-time PCR analysis suggested that differentially expressed genes were involved in sugar or JA biosynthesis, signaling, and metabolism in transgenic cassava under drought conditions. Exogenous application of JA further confirmed that JA conferred improved drought resistance and promoted stomatal closure in cassava leaves. Taken together, our findings suggest that MeSPL9 affects drought resistance by modulating protectant metabolite levels and JA signaling, which have substantial implications for engineering drought tolerant crops.

Introduction

Drought dramatically cuts crop yields and becomes a major threat to food security due to reduction in arable land area and reduced water availability worldwide (Ray et al. 2015). With the increase in the population of the earth, it is paramount to develop better-adapted crops in water-limiting environments to meet the requirement of world’s food security and sustainability in future (Foley et al. 2011). Luckily, as sessile organisms, plants have evolved various defensive strategies to get through drought stress. Until recently, enormous progresses have been achieved to elucidate the responding mechanism of plants to drought stimulus and diverse genes enhancing drought tolerance have been introduced into crops, which is crucial for generating plants with increased drought resistance. It is also well documented that plants combat with drought stress by mobilizing transcriptional alteration of a number of genes, resulting in accumulation of key proteins, detoxification...
enzymes, phytohormones, or various metabolites, which contribute directly or indirectly to prevent plant cells from dehydration-induced damage (Fabregas et al. 2018; Krassen-sky and Jonak 2012; Seki et al. 2007). These studies reveal that drought tolerance is a complex trait concurrently mediated by a series of genes and pathways.

The root crop cassava (Manihot esculenta) belongs to the Euphorbiaceae family and is the major food and bioenergy crop in the world. It is particularly important to the resource-poor farmers in arid regions, such as America, Asia, and Africa, owing to its relatively strong resistance to water- and nutrient-limited environments (EI-Sharkawy 2004; Okogbenin et al. 2013). However, harsh or persistent drought also significantly depresses the growth and development of cassava plants, thereby restricting their economic yield (EI-Sharkawy 2004). Our previous work reveals the involvement of the activation of jasmonic acid (JA) signaling pathway in drought-tolerant mechanisms of cassava (Li et al. 2017a). The phytohormone JA, as well as its metabolically active derivative JA-isoleucine (JA-Ile), is a critical signaling molecule, which orchestrates plant responses to biotic and abiotic stimuli (Riemann et al. 2015). Therefore, transgenic modulation of JA levels represents an attractive avenue to enhance the drought resistance of crops.

The essential components in JA biosynthesis and signaling have been widely investigated in plants. JA biosynthesis starts in the plastids and utilizes α-linolenic acids of chloroplast membranes as precursor. Oxygenation of α-linolenic acids by the enzyme 13-lipoxygenases (LOXs) to produce 13S-hydroperoxylacetylenic acids (13-HPOTs), which is followed by the action of allene oxide synthases (AOSs) forms 12, 13S-epoxy-octadecatrienoic acids (12,13-EOTs). These are substrates for allene oxide cyclases (AOCs) that generate 12-oxophytodienoic acids (OPDAs). In the following, OPDAs are transported into peroxisomes where they are reduced by OPDA reductases (OPRs) and subsequently activated by CoA ester prior to undergoing three rounds of β-oxidation steps to form JA (Acosta and Farmer 2010; Wasternack and Hause 2013). Several reports suggest that endogenous JA and its precursor contents increased in plant cells under drought stress (Fu et al. 2018; Gupta et al. 2017; Savchenko et al. 2014). Exogenous application of JA and overexpression of the JA biosynthetic enzyme gene LOX both could confer enhanced tolerance to drought conditions in plants; downregulation of the JAZ receptor also leads to drought-tolerant phenotypes (Fu et al. 2018; Xing et al. 2020). It has been proposed that JA improves drought tolerance by controlling stomatal aperture and increasing the antioxidant capacity of plants under osmotic stress (Riemann et al. 2015; Xing et al. 2020). Although the contributions of JA in plant response to environmental stresses have been suggested, the exact function of JA in cassava under drought stress condition remains disputed.

In cassava, previous studies have widely identified drought-responsive genes and characterized molecular mechanisms by using transcriptome profiling and genetic approaches. For example, one study documented that the contents of proline, malondialdehyde (MDA), and soluble sugars were all drastically increased during the PEG-simulated drought stress (Fu et al. 2016). Functions of drought-responsive genes such as dehydration-responsive element binding proteins (DREB) (An et al. 2017), heat shock protein (HSP) (Wei et al. 2020), and MYB (Ruan et al. 2017) have been verified and well characterized. Cassava plants have been conferred drought resistance using genetic approaches, however, this is accompanied reduced overall plant growth (An et al. 2017). Thus, identification of key genes that enhance stress tolerance without changing the architecture of plants is crucial for breeding crops with boosting growth in rain-fed environments. Here, we describe the characterization of one member of SQUAMOSA promoter binding protein-like (SPL) gene family, called MeSPL9, the downregulation of which can improve drought tolerance without affecting plant growth in cassava.

SPL proteins constitute a highly conserved plant-specific transcription factor family and play a key role in affecting plant development and optimizing plant response to stresses. A total of 16 SPL genes are identified in Arabidopsis, 10 of which are targets of miR156 (Guo et al. 2008). The role of miR156-targeted SPLs has been extensively explored by examining the phenotypes of plants expressing miR156-resistant versions of these genes under the control of the constitutively expressed CaMV 35S promoter, or their own promoters (Wang et al. 2008). These overexpression phenotypes suggest that SPL proteins regulate a network of target genes and mediate various processes in plant development and physiology, encompassing the timing of vegetative phase change (Wu et al. 2009; Wu and Poethig 2006), anthocyanin biosynthesis (Gou et al. 2011; Wang et al. 2020), and root regeneration in tissue culture (Ye et al. 2020). On top of their functions in plant development, emerging evidence has proved that SPLs also serve as the main governing factors in response to environmental stresses. Studies have shown that miR156-mediated silencing of SPLs improved drought stress resilience by reducing water loss, while promoting leaf gas exchange and abscisic acid (ABA) sensitivity (Feyissa et al. 2019; Visentin et al. 2020). Transgenic Arabidopsis with increased miR156 expression silenced SPLs displayed enhanced tolerance under drought and salt conditions (Stief et al. 2014). In rice, miR156 overexpression inhibited the expression of miR156-targeted SPLs, resulting in enhanced resistance to NaCl and osmotic stresses (Cui et al. 2015).

In this study, we show that overexpression of a dominant-negative mutant MeSPL9-SRDX modulate multiple drought stress-related traits in cassava plants. Although the traits regulated by the miR156-SPL pathway are closely associated
with plant development, we discovered that downregulation of MeSPL9 could enhance drought tolerance without penalizing overall plant growth. Our detailed transcriptomic and metabolite profiling showed that MeSPL9-SRDX overexpression triggered JA accumulation and production of osmoprotectants (i.e., proline, soluble sugar, and anthocyanin), which are transcriptionally regulated by MeSPL9 in cassava during drought condition. The findings would help in understanding the role of MeSPL9 in plant response to drought stimulus and can be used as a tool in marker-assisted breeding to generate drought-resistant cassava and potentially other crops.

**Materials and methods**

**Plant transformation and phenotype evaluation**

The full-length coding sequence (CDS) of cassava MeSPL9 was cloned and mutated by PCR using Site-directed Mutagenesis Kit (Sangon Biotech) with specific primers harboring the miR156-targeted site (Table S3), following the manufacturer’s protocols. The PCR fragment was confirmed by sequencing and subcloned into the pCAMBA1301 binary vector under control of the CaMV 35S promoter, in which MeSPL9 was fused with the EAR motif repressor domain (SRDX). The plasmid was introduced into Agrobacterium tumefaciens strain LBA4404, which was then transformed into cassava cultivar TMS60444 using friable embryogenic callus-mediated method (Zhang et al. 2000). The stem cuttings of wild type (WT) and transgenic lines were cultured in MS plates and placed in a chamber (26 ± 2 °C, 16/8 h light/dark cycle) for three weeks. The seedlings were then planted in pots with high-quality soil substrates (Peatsoil: vermiculite, 1:1) for drought treatments, or in Wenchang Plantation for Transgenic Crops, Hainan, China, for phenotype observation. The performance of at least ten plants per transgenic line and WT was recorded regularly till harvest.

**Southern blotting and quantitative real-time PCR (qRT-PCR)**

Total DNA was extracted from leaves of WT and transgenic lines by the CTAB method. Approximately 20 µg of isolated DNA were digested with EcoRI and separated on 0.8% (w/v) agarose gels, followed by transferring to a positively charged nylon membrane (Roche, Mannheim, Germany). Then the membrane was hybridized with the DIG-labeled hygromycin phosphotransferase (HPT) probe. After hybridization, membrane washing and signal detection were performed by using the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche, Mannheim, Germany), following the manufacturer’s protocols.

Total RNA isolation, cDNA synthesis, and qRT-PCR were conducted as previously described (Li et al. 2017b). Briefly, the differential gene expression between samples was assessed by the 2−△△CT method (△CT = Ct[Target]-Ct[Reference], and △△CT = (△CT)Exp.−(△CT)Control) (Livak and Schmittgen 2001), with MeACTIN (Manes.13G08430) used as the reference gene. Significance of differences was determined by Student’s t test. *P < 0.05; **P < 0.01; ***P < 0.001. All samples were measured three times, and the experiments included three independent biological replicates. The primers are listed in Table S3.

**JA and drought treatments**

Three-week-old seedlings of WT and transgenic lines were planted in pots and grown in greenhouse (26 ± 2 °C, 16/8 h light/dark cycle) for two weeks under a fully watered regime. For JA treatment, methyl JA (MeJA, Sigma-Aldrich) was dissolved in DMSO to 50 mM and diluted to a final concentration of 10 µM with liquid MS (Murashige and Skoog) medium. WT seedlings were withhold water and were sprayed with mock (liquid MS) or 10 µM of MeJA once per week until the plants gradually dry out. For drought treatment, water was withheld for three weeks until the WT and transgenic lines exhibited obvious symptoms of damage. Each treatment contained at least six plants per line, and all treatments included three biological replicates.

**Proline quantification**

Four-week-old transgenic and WT lines grown in MS medium were divided into two groups (control and drought treatment); each independent sample consisted of at least five plants and all treatments included three biological replicates. After the plants were treated with 20% PEG6000 for 6 h, approximately 1 g of shoot tissues was collected for each sample and extracted with various reagents corresponding to different assays. Proline content was determined by the sulfosalicylic acid–acid ninhydrin method as previously described (Cheng et al. 2019).

**Determination of water loss rate and stomatal aperture**

To determine the water loss rate, fully expanded, healthy leaves of 3-month-old WT and transgenic plants (six leaves per repeat) were collected and immediately weighed (Fresh Weight, FW). Afterward, the leaves were placed in plastic trays at 26 °C with 55% relative humidity. The weight (dried weight, DW) of the leaves was measured in assigned intervals. The water loss rate (%) was calculated as (FW-DW)/FW × 100%. The experiment was conducted in triplicate.
Stomatal aperture was measured as previously described (Xing et al. 2020). Fully expanded mature leaves of transgenic and WT plants were incubated in stomatal opening solution for 2 h under light conditions. Afterward, the leaves were soaked in different solutions, including 20% PEG 6000 and 10 μM JA, for 2 h prior to observation. The stomatal aperture was represented by averaging at least 60 stomata ratios (width/length).

Quantification of endogenous hormones and soluble sugars

Plant hormones, such as salicylic acid (SA), ABA, JA, and JA-Ile, were determined in the shoots of 4-week-old WT and transgenic lines under control and drought stress conditions. Drought stress was imposed by withholding water for 2 weeks in a greenhouse. Three replicates of samples were collected for each line, immediately frozen in liquid nitrogen, and stored at −80 °C. A quantitative analysis of these endogenous hormones was conducted by high-performance liquid chromatography-mass spectrometry (HPLC–MS) as previously described (Fabregas et al. 2018). The standards ABA, SA, JA, and JA-Ile were purchased from Sigma-Aldrich (America).

Soluble sugar determination was undertaken according to the method described previously (Maloney et al. 2015). Briefly, shoot tissues (100 mg) were collected and homogenized in liquid nitrogen, dissolved in 6 ml of methanol/chloroform/water (12:5:3, v/v), and incubated at −20 °C for 30 min. Crude extracts were centrifuged at 6000 rpm and 4 °C for 10 min, and the supernatant was collected. The pellets were washed and resuspended with methanol/chloroform/water (12:5:3, v/v). After centrifugation, the second supernatant was added to the first, 5 ml of distilled water was added to the pooled supernatants and phases were partitioned. Aqueous phase that contained the soluble sugars was collected and dried. The soluble sugars were dissolved in distilled water and quantified by HPLC. The concentration of each component was measured with regression equations from calibration curves derived from external standards.

RNA sequencing (RNA-seq) analysis

Young leaves and shoot tips from four-week-old WT and rMeSPL9-SRDX lines grown under control and drought (20% PEG, 3 h) conditions were harvested. Two biological replicates consisting of five independent plants for each sample were conducted. Total RNA isolation, library construction, and deep sequencing were performed on the NovaSeq™ 6000 platform (Illumina) according to the manufacturer’s protocols at the Guangzhou Gene Denovo Biotechnology Co., Ltd. (Guangzhou, China). After removing the adapters and low-quality bases, clean reads were aligned to the cassava genome assembly with Hierarchical Indexing for Spliced Alignment of Transcripts (HISAT) (Kim et al. 2015), followed by transcript construction using Stringtie (Pertea et al. 2015). The Fragments Per Kilobase per Million (FPKM) value was calculated for each unigene by RSEM (RNA-Seq by Expectation–Maximization) (Li and Dewey 2011). The differentially expressed genes (DEGs) were identified by using DESeq2 (Love et al. 2014), with FDR (False discovery rate) < 0.05. The RNA-seq data have been deposited to the National Center for Biotechnology Information (NCBI) under the accession number PRJNA693998.

Statistical analyses

All results were presented as mean ± standard deviation (SD). Data were analyzed by one-way analysis of variance (ANOVA) and t test in SPSS (IBM, New York, NY) and Microsoft Office Excel, considering *P < 0.05, **P < 0.01, and ***P < 0.001 as significance.

Accession numbers

MeSPL9 (Manes.09G032800), MeLOX2 (Manes.07G002400), MeLOX6 (Manes.10G149000), MeAO (Manes.14G098200), MeAOS (Manes.18G031800), MeOPR2 (Manes.03G039700), MeOPR3 (Manes.18G120500), MeENO1 (Manes.01G023200), MeGPT2 (Manes.17G036800), MePK (Manes.17G052300), MeGAPC (Manes.12G015500), MeSPS (Manes.18G109400), MelNVA (Manes.02G035900).

Results

Identification and expression pattern of MeSPL9 in cassava

Bioinformatic analysis of the cassava genome identified 21 full-length SPLs, which is greater than the that (16) reported in Arabidopsis (Guo et al. 2008). All of the deduced MeSPL proteins, with lengths ranging from 148 to 1044 amino acids (aa), contained the conserved SBP domain (Table S1). A phylogenetic tree involving 21 MeSPLs and 16 AtSPLs was constructed based on the neighbor-joining (NJ) method. The topology of the tree demonstrated that these SPLs were sparsely distributed in a majority of clades, and clustered into seven distinct groups, namely Group 1 to Group 7 (Fig. 1a). The MeSPLs were named MeSPL1 to MeSPL15 after the corresponding AtSPLs with highest sequence similarity. Remarkably, 10 AtSPLs are post-transcriptionally targeted by miRNA156 (Wu et al. 2009). Their closest homologs in cassava, including MeSPL3a/b/4/5, MeSPL9/
MeSPL2a/2b, and SPL6a/6b/6c/13a/13b, are all proved to be cleaved by mes-miR156 (Chen et al. 2015; Li et al. 2020).

To explore the potential function of SPL genes, expression level changes of all 21 SPLs in cassava were analyzed based on our available RNA-Seq data (Li et al. 2017a). The results showed that MeSPL9 was downregulated especially by drought (Fig. 1b). The spatial expression pattern analysis revealed that MeSPL9 was preferentially expressed in leaves, stems, and shoot apex, while lower expression levels could be detected in roots (Fig. 1c). The qRT-PCR was applied to further verify whether the MeSPL9 expression was related to drought stress. The results were overall in line with transcriptome data showing suppressed MeSPL9 expression under drought conditions (Fig. 1d). The above results demonstrate that MeSPLs may play different roles in cassava under drought conditions, and lower MeSPL9 expression is favored in cassava response to drought stress.

**Generation and phenotype analysis of rMeSPL9-SRDX transgenic cassava plants**

We tried to generate MeSPL9 knockdown mutant plants using the chimeric repressor gene-silencing technology, in which mes-miR156-resistant MeSPL9 (rMeSPL9) was
fused with a 12-aa SRDX under the control of constitutive 35S promoter (Fig. 2a) (Hiratsu et al. 2003). Previous studies demonstrated that this chimeric version can productively suppress the target genes (e.g., TCP genes) of various transcription factors (Guo et al. 2010; Koyama et al. 2007). Five independent transgenic cassava plant lines (named rMeSPL9-SRDX #1 to #5) harboring the MeSPL9-SRDX overexpressing cassette were generated by the use of Agrobacterium-mediated transformation of embryonic calli (Zhang et al. 2000). The basic transcriptional level of rMeSPL9-SRDX in each transgenic line was confirmed using qRT-PCR. #2, #3, and #4 lines exhibited an exceptional upregulation of rMeSPL9-SRDX, approximately 60-fold higher relative to that of WT (Fig. 2b). The positive integration of the 35S::MeSPL9-SRDX T-DNA was confirmed in transgenic lines using Southern blotting. Three lines (#2, #3, and #4) were verified as positive insertion into the genome by EcoRI digestion, while no signal was detected in line #1 or #5 (Fig. 2c). At the same time, we also tried to generate rMeSPL9 overexpression transgenic plants using embryonic calli as receptors for transformation. However, the obtained calli failed to regenerate into plants, which is consistent with previous results that extremely high levels of SPL9 may lead to embryonic lethality (Wang et al. 2008). Therefore, #2 and

Fig. 2 Molecular and phenotypic analysis of rMeSPL9-SRDX transgenic cassava plants. a Schematic presentation of the construction of p35S::rMeSPL9-SRDX and base mutation information of rMeSPL9 resistant to miR156 without changing protein sequence. 35S, CaMV 35S promoter; Ter, NOS terminator; HYG, Hygromycin. b Relative expression of MeSPL9 in WT and transgenic lines. Error bars indicate SDs obtained from three independent experiments. Significance of differences was determined by Student’s t test. ***P < 0.001. c Transgene integration validation in WT and transgenic lines by southern blotting. d Phenotype evaluation of WT and transgenic plants in field. e Shoot tips (left) and petiole (right) of WT and transgenic lines. #, Number
#3, these two lines together with WT were used for subsequent studies. We carefully compared the growth performance of WT and transgenic plants. Generally, expression of rMeSPL9-SRDX had little effect on the development of cassava plant and were not obviously different from WT (Fig. 2d). However, we noticed hyperaccumulation of anthocyanin at the stem–petiole junction and petiole of rMeSPL9-SRDX plants (Fig. 2e). These results are in line with those obtained from the previous reports that SPL9 participates in inhibiting anthocyanin biosynthesis in Arabidopsis (Gou et al. 2011; Wang et al. 2020).

**MeSPL9 negatively mediates drought tolerance at the seedling stage**

Cassava is established to be tolerant under drought conditions (Okogbenin et al. 2013), and it was of interest to assess whether rMeSPL9-SRDX overexpression could influence drought tolerance of transgenic plants. Therefore, 5-week-old WT and rMeSPL9-SRDX plants grown in pots were subjected to drought stress by water depletion. Prior to drought treatment, all plants displayed active growth status (Fig. 3a). After water depletion for 3 weeks, WT plants exhibited obvious dehydration symptoms including severe wilting and dehydrated leaves. In contrast, rMeSPL9-SRDX lines displayed more green leaves and relatively less severe dehydration. After rewatering, the rMeSPL9-SRDX plants exhibited increased drought tolerance with higher survival rates relative to WT (Fig. 3a, b). Detached leaves were subjected to dehydration to monitor the water loss rate. As can be seen in Fig. 3c, the WT leaves lost more water compared to the rMeSPL9-SRDX leaves during the entire experiment. Furthermore, proline content, a key indicator of plant drought adaptation (Dobra et al. 2011; Sperdouli and Moustakas 2012; Szabados and Savoure 2010), was markedly increased in the rMeSPL9-SRDX lines compared to WT (Fig. 3d). Collectively, the above results suggest that MeSPL9 negatively regulates drought stress adaptation in cassava.

![Fig. 3](image-url) Downregulation of MeSPL9 in cassava increases drought tolerance. a Representative phenotypes of WT and rMeSPL9-SRDX plants after drought treatment. Survival rate b, leaf water loss rate c, and proline content d in WT and rMeSPL9-SRDX lines under control and drought conditions. Error bars indicate SDs obtained from three independent experiments. Significance of differences was determined by Student’s t test. *P < 0.05; **P < 0.01; ***P < 0.001
Identification of MeSPL9-regulated genes and related pathways

To elucidate the molecular mechanism underlying the enhanced drought tolerance of rMeSPL9-SRDX lines, we performed RNA-seq to investigate the transcriptomic changes between two genotypes (WT and rMeSPL9-SRDX lines) under two conditions (control and drought). To this end, the whole transcriptome profiles of rMeSPL9-SRDX and WT plants under control (designated #2C, #3C for rMeSPL9-SRDX lines and WTC for wild type) and drought treatment conditions (designated #2D, #3D for rMeSPL9-SRDX lines, and WTD for wild type) were compared. In WT, a total of 1668 DEGs were detected under drought conditions. Among them, 76% of the genes (1263/1668) were upregulated and 24% of the genes (405/1668) were downregulated (Fig. 4a, Table S2). In contrast, 1940 and 2385 drought-responsive DEGs were identified in rMeSPL9-SRDX line #2 and #3, respectively, among which approximately 71% of the genes were upregulated. These data suggested that there were larger shifts in gene expression under drought stress in rMeSPL9-SRDX lines. MeSPL9-regulated DEGs were determined by performing pairwise comparisons between rMeSPL9-SRDX lines and WT (#2C vs. WTC, #3C vs. WTC, #2D vs. WTD and #3D vs. WTD). As a result, 644 and 308 DEGs were detected in the #2 and #3 plants, respectively, under control conditions compared to WT. Similarly, 550 and 345 DEGs were found in these two lines, respectively, during drought treatment relative to WT (Fig. 4a, Table S2). We observed that the number of upregulated DEGs was much greater than that of downregulated DEGs in rMeSPL9-SRDX plants, suggesting that MeSPL9 may inhibit some of the drought-responsive genes.

Further investigation demonstrated that the expression of 15 and 30 genes were promoted and suppressed in rMeSPL9-SRDX lines in response to drought stress, respectively (Fig. 4b, c). Gene ontology (GO) enrichment analysis revealed that the DEGs belonging to ‘response to abiotic/biotic stress’ were overrepresented both in the control- and drought-treated rMeSPL9-SRDX plants (Fig. 4d, e). To further elucidate the roles of the MeSPL9-regulated DEGs, KEGG enrichment analysis was performed. Anthocyanin biosynthesis and proline metabolism were significantly upregulated in rMeSPL9-SRDX lines under control condition. However, the majority of genes related to monoterpeneoid biosynthesis were strongly up-regulated after drought treatment. It is noteworthy that genes involved in alpha-linolenic acid metabolism were notably overrepresented under both control and drought conditions (Fig. 4f, g). The biosynthetic process of hormone JA using alpha-linolenic acid as substrates in chloroplast has been extensively studied (Han 2017; Wasternack and Hause 2013). The above results indicated that the DEGs involved in plant metabolism and JA signaling pathways may play key roles during drought stress.

JA participates in regulation of stomatal closure in rMeSPL9-SRDX plants

Our KEGG analysis revealed that MeSPL9 may repress JA biosynthesis and signaling in cassava plants. Indeed, previous reports have demonstrated that SPLs can attenuate JA responses by stabilizing JAZ proteins (Mao et al. 2017), the repressor of JA signaling pathway. As we know, JA function as an important regulator in plant development and response to abiotic stimuli, including drought, as well as biotic stimuli (Ahmad et al. 2016; Wasternack and Strnad 2016). In this study, we further found that a number of drought-induced DEGs involved in JA biosynthesis, catabolism, and signaling processes were significantly upregulated in rMeSPL9-SRDX plants under both control and drought conditions compared with WT according to the transcriptional data (Fig. 5a, b). To validate the results obtained from the transcriptomic analysis, we analyzed the expression of various JA biosynthesis-related genes by qRT-PCR. The expression of MeLOXs, MeAOS, MeAOC, and MeOPRs in rMeSPL9-SRDX lines was continuously induced before and after drought treatment, which is mostly consistent with the RNA-seq results (Fig. S1A-F). These findings prompt us to evaluate the endogenous JA level in transgenic cassava plants. As shown in Fig. 5c, no significant differences in SA and ABA contents were observed between transgenic and WT plants, while JA content was markedly induced in rMeSPL9-SRDX lines compared to WT, indicating that MeSPL9 may negatively regulate the synthesis of endogenous JA.

Previous reports have shown that JA promotes drought resistance of plants by moderating stomatal closure (Xing et al. 2020). We therefore postulate that JA accumulation may confer enhanced drought resistance in rMeSPL9-SRDX plants. To test this postulation, exogenous JA was applied to wild-type cassava seedlings before drought treatment. As a result, seedlings sprayed exogenous MeJA exhibited higher survival rate after drought treatment compared with the control (Fig. 5d, f), indicating that JA was able to increase drought resistance in cassava. Meanwhile we examined the stomatal apertures of both WT and rMeSPL9-SRDX leaves.
treated with PEG and JA, respectively. As shown in Fig. 5e, smaller stomatal aperture was observed in cells compared to WT under normal conditions, which corresponded to the lower water loss rates as mentioned above. Stomatal aperture assays also revealed that both exogenous JA and PEG treatment can inhibit stomatal opening in WT and transgenic leaves, whereas cells conferred a relative lower stomatal closing capacity than WT and showed hyposensitivity to exogenous JA (Fig. 5e, g). Taken together, these results suggested that the elevated JA
levels, especially in rMeSPL9-SRDX plants, are responsible for stomatal closure in order to prevent water loss, thereby leading to drought resistance.

rMeSPL9-SRDX plants contain higher levels of soluble sugars

To gain additional insight into the mechanism underlying drought resistance conferred by rMeSPL9-SRDX overexpression, we analyzed soluble sugar levels in PEG treated leaves. By comparison, the sugar contents in rMeSPL9-SRDX leaves, such as glucose, fructose, and galactose were significantly higher to the WT under control condition, and were elevated even further as a result of drought treatment (Fig. 6d). This result suggested that accumulation of soluble sugar contents could serve as another indicator for evaluating the drought response in rMeSPL9-SRDX leaves. When combined with the transcriptome data presented above, the genes related to carbon metabolism and plant glycolysis pathway appear to be positively associated with drought response. Subsequently, we observed the expression of genes encoding enolase 1 (ENO1), glucose 6-phosphate/phosphate translocator 2 (GPT2), pyruvate kinase (PK), and vacuolar invertase (INVA) in the starch and sucrose metabolism pathways was remarkably upregulated in rMeSPL9-SRDX plants (Fig. 6e). Glyceraldehyde-3-phosphate dehydrogenases (GAPCs) are ubiquitous proteins that function in the glycolytic pathway and are involved in stress response (Guo et al. 2012; Li et al. 2019b). In rMeSPL9-SRDX plants, the expression level of MeGAPC was also significantly increased. Moreover, we also discovered downregulated genes by overexpressing rMeSPL9-SRDX, such as sucrose-phosphate synthase (SPS), which serves as one of the rate-limiting steps in sucrose synthesis in plants (Fig. 6e). Additionally, the expression of MeENO1, MeGPT2, MePK, and MeGAPC was notably induced under drought stress. However, no particular difference in the expression of MeINVA and MeSPS was observed between control and drought conditions.

Discussion

Although cassava plants are often classified as drought tolerant due to that they are able to grow through the dry seasons in tropical areas, persistent drought stress seriously restricts its growth and production (E1-Sharkawy 2004). Using high-throughput RNA-seq, numerous drought-responsive genes and related regulatory networks have been identified in cassava (Fu et al. 2016; Li et al. 2017a; Utsumi et al. 2012). It is currently proposed that the strategy in cassava response to water stress is ‘avoiding drought’ through enhancing root growth and encouraging leaf abscission to reduce water transpiration (Liao et al. 2016; Okogbenin et al. 2013). In the present study, we reported that MeSPL9 acted as a negative regulator in cassava response to drought stress. Although several studies have confirmed in various species the participation of SPLs in plant response to drought stress (Cui et al. 2015; Feyissa et al. 2019; Visentin et al. 2020), the underlying mechanisms and downstream targets remain largely unclear. Here we show that the transgenic plants overexpressing rMeSPL9-SRDX, a dominant-negative chimeric gene, exhibited a representative drought resistant phenotype. It is suggested that this phenomenon was achieved by the accumulation of protectant metabolites, such as phytohormone JA, anthocyanidin, proline, and soluble sugars, through transcriptional regulation of metabolic pathways.

On top of participating in plant response to abiotic stimuli, miR156-targeted SPLs are largely conserved among various plant species and proposed to play a prominent role in the regulation of plant growth and development, including phase change, root regeneration, and wax synthesis (Li et al. 2019a; Wang et al. 2008; Wu et al. 2009; Ye et al. 2020). Previously, miR156 overexpression enhanced the tolerance to drought stress in plants, but also impaired the overall growth of transgenic plants (Cui et al. 2015; Feyissa et al. 2019). In Arabidopsis, single-knockout mutants of miR156-targeted SPL genes exhibited no significant morphological phenotype changes (Wang et al. 2008). Similarly, in cassava, constitutive expression of rMeSPL9-SRDX can promote drought resistance without altering the growth features of transgenic plants. Thus, characterization of MeSPL9-related genes and pathways in cassava plants are benefit for generating drought-tolerant cultivators.

Under control and drought conditions, rMeSPL9-SRDX lines showed enrichments of proline and anthocyanins. These two metabolites, as important antioxidants, contribute...
Fig. 6 Expression of carbon metabolism-related genes and soluble sugar levels in WT and rMeSPL9-SRDX lines under drought conditions. a-d Contents of glucose (a), fructose (b), galactose (c), and sucrose (d) in WT and rMeSPL9-SRDX lines after drought treatment. e Relative expression levels of MeENO1, MeGPT2, MePK, MeGAPC, MeSPS, and MeINVA in WT and rMeSPL9-SRDX lines under control and drought conditions. Error bars indicate SDs obtained from three independent experiments. Significance of differences was determined by Student’s t test. *P < 0.05; **P < 0.01; ***P < 0.001
significantly to osmotic homeostasis and protection of macromolecules in plants suffering from abiotic stresses (Sperdouli and Moustakas 2012; Szabados and Savoure 2010). Increased anthocyanin or proline production has been demonstrated to significantly enhance resistance to abiotic stimuli in various plant species (Castellarin et al. 2007; Lotkowska et al. 2015; Naing et al. 2017). Indeed, a previous study revealed that SPL9 may negatively regulate anthocyanin production via directly inhibiting the expression of anthocyanin biosynthesis-related genes by destabilization of a MYB-bHLH-WD40 transcriptional activation complex in Arabidopsis (Gou et al. 2011). Therefore, the enhanced accumulation of proline and anthocyanins strongly correlated with the drought stress tolerance of rMeSPL9-SRDX plants.

JA and ABA are the key phytohormones that positively regulate biotic and abiotic stress adaptation. A number of reports have demonstrated that JA accumulation can happen immediately when plants are subjected to abiotic stresses (Riemann et al. 2015). Overexpression of JA biosynthesis genes or exogenous application with JA could significantly enhance water stress tolerance by promoting the antioxidant enzyme activation or regulating the plant growth and tissue water status (Ozturk et al. 2015; Riemann et al. 2015; Wasternack and Strnad 2016). For instance, overexpression of the JA biosynthesis gene LOX and AOS can confer drought stress resistance (De Domenico et al. 2012; Xing et al. 2020). ABA also plays an important role in the plant’s response to drought stress through control of stomatal aperture and water transpiration (Brugiere et al. 2017). In this study, the rMeSPL9-SRDX lines exhibited increased JA content due to higher expression level of key JA biosynthesis-related genes, including MeLOXs, MeAOS and MeAOCs, compared with the WT plants. However, the contents of ABA did not change significantly. It has also been reported that JA could decrease transpiration by the regulation of stomatal closure (Daszkowska-Golec and Szarejko 2013; Suhita et al. 2004). In cassava, we provided evidence that JA treatment could magnify stomatal closure. Comparing with WT, rMeSPL9-SRDX lines exhibited lower water loss rates and smaller stomata aperture. Taken together, these results indicate that rMeSPL9-related stress tolerance is partially JA-dependent in cassava.

Soluble sugars act not only as an energy source, but also as osmolytes to maintain normal transpiration and leaf-water content under drought conditions (Wei et al. 2019). In this regard, the improvement of drought resistance by overexpressing rMeSPL9-SRDX has further encouraged us to evaluate the changes in concentration of soluble sugars in the leaves. Consistent with the previous studies (Pinheiro et al. 2011; Wei et al. 2019; Wingler and Roitsch 2008), while no differences in sucrose content were observed in rMeSPL9-SRDX lines, the contents of glucose, fructose, and galactose significantly increased. In our study, expression of starch-sucrose metabolism-related genes, such as MeENO1, MeGPT2, MePK, and MeGAPC, were higher in rMeSPL9-SRDX than in WT plants during drought conditions. Evidence for GAPC enzymes in plant response to abiotic stresses has come from a number of independent studies. They have been demonstrated to transduce the ROS hydrogen peroxide (H₂O₂) signal in Arabidopsis by interacting with plasma membrane-associated phospholipase D (PLDδ), and characterized as cytoplasmic proteins that have important role in ABA signaling pathway. The loss of GAPCs exhibited decreased stomatal sensitivity to ABA and rendered plants less responsive to water deficits than the wild type (Guo et al. 2012). Therefore, when the MeGAPC activity is raised in transgenic lines, the in planta ROS signaling may be altered, resulting in improved drought tolerance. In plants, ENO1 catalyzes the glycolytic conversion of 2-phosphoglycerate (2-PGA) to phosphoenolpyruvate (PEP), which is an intermediate of glycolysis and delivers ATP and pyruvate with the presence of cytosolic PK (Prabhakar et al. 2010). GPT2 has previously been demonstrated to participate in regulating the metabolite distribution between compartments and to play a crucial role in interpreting environmental signals (Dyson et al. 2015). The enhanced MeENO1, MePK and MeGPT2 activities in rMeSPL9-SRDX plants would produce more soluble carbohydrates, which could be beneficial since they serve as osmoticum under drought stress. Meanwhile, due to the altered activities of MeSPS and MeINVA manifest in the synthesis/hydrolysis of sucrose, the overall levels of glucose and fructose were increased, but without an alteration in sucrose content. INVA has been demonstrated to facilitate water influx and maintain desired osmotic homeostasis by hydrolyzing sucrose into hexoses under abiotic stress conditions (Dahro et al. 2016). Due to this mechanism, it appears that higher levels of glucose and fructose in rMeSPL9-SRDX plants, might contribute to the enhanced drought tolerance.

Taken together, in this study, we isolated and characterized the novel function of MeSPL9 in the regulation of drought adaptation of cassava plants. Our studies demonstrate that MeSPL9 negatively regulates drought tolerance through controlling anthocyanin biosynthesis, JA, proline and soluble sugars accumulation. Further research will be focused on the identification of the molecular network of MeSPL9 in the regulation of stress adaptation.

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Declarations

Conflict of interest The authors declare that there is no conflict of interest.

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