



# Morpho-physiological and molecular evaluation of drought tolerance in cassava (*Manihot esculenta* Crantz)

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## ABSTRACT

Understanding drought tolerance mechanisms of cassava is a pre-requisite to improve the performance of the crop in water-scarce regions. Several hypotheses have been formulated to suggest how cassava can withstand a prolonged period of drought. We performed field trials under drought conditions with a selection of 37 cassava genotypes to identify phenotypic and molecular patterns associated with drought tolerance. Plant morphologies varied significantly between cassava genotypes under drought conditions in Kenya, which indicates a strong genetic basis for phenotypic differences. Drought stress reduced yield by 59%, the number of edible storage roots by 43% and leaf retention by 50% on average. Over three years and in two experimental field sites, the most drought tolerant genotype bulked 7.1 (± 2.1) t/ha yield while the most drought susceptible genotype yielded 3.3 (± 1.4) t/ha under drought conditions. The significant positive correlation of yield under irrigated and non-irrigated conditions suggests that selection of genotypes with high yield performance under well-watered or control conditions should be prioritized to identify genotypes with superior performance under drought stress. The positive correlation between yield and leaf retention provided further evidence that leaf longevity positively contributes to yield in water-deficit conditions. Yield differences could be attributed in part to variation in stomatal conductance ( $g_s$ ) because selected drought tolerant genotypes maintained higher  $g_s$  and delayed stomatal closure as compared to drought susceptible genotypes. Further analysis revealed that genetic or molecular differences for  $g_s$  between drought tolerant and susceptible genotypes could be detected at early stages of water deficit. These differences likely involve both abscisic acid (ABA)-dependent and ABA-independent molecular pathways.

## 1. Introduction

Climatic changes aggravate both biotic and abiotic stresses, which have adverse effects on worldwide agricultural productivity (Raza et al., 2019; Lamaoui et al., 2018; Stevanović et al., 2016). Abiotic stresses constrain crop production and threaten global food security (Lipiec et al., 2013; Fahad et al., 2017). Reduced precipitation and

changes in rainfall patterns are causing frequent onset of droughts around the world (Lobell et al., 2011). Predictions indicate an increase in the frequency, intensity and severity of drought stress in the near future (Nadeem et al., 2019) with yield reductions of 21 and 40% in wheat and maize respectively being attributed to drought stress on a global scale (Daryanto et al., 2016). Therefore, without sufficient interventions to secure agricultural production under changing climatic

**Abbreviations:** ABA, abscisic acid; ANOVA, analysis of variance; ACZ, agro-climatic zone; cDNA, complementary deoxyribonucleic acid; Ct, cycle threshold; DAP, days after planting; DNA, deoxyribonucleic acid; DRGs, drought responsive genes; DS, drought susceptible; DT, drought tolerant; GEFC, gene expression fold change; GOI, gene of interest;  $g_s$ , stomatal conductance; IITA, international institute of tropical agriculture; Lsd, least significant difference; M-MuLV, moloney murine leukemia virus; MAP, months after planting; PP2A, serine-threonine protein phosphatase 2A; RT-qPCR, reverse transcription quantitative polymerase chain reaction; RNA, ribonucleic acid; SMC, soil moisture content; SPSS, statistical package for social scientists; STPs, sampling time points

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and environmental conditions, the negative impact of abiotic stress will be more severe for several crops that are important in large food-vulnerable regions, particularly in Sub-Saharan Africa and Southern Asia (Rosenthal et al., 2012; Rosenthal and Ort, 2012).

One sustainable mitigation measure is breeding and improving stress-tolerant staple crop varieties that can produce higher yields under adverse climatic conditions such as drought. Cassava is an important staple crop for food-insecure populations in Sub-Saharan Africa (Lobell et al., 2008; Rosenthal et al., 2012) and is expected to positively buffer the region under the negative impacts of climatic change (Jarvis et al., 2012). However, cassava productivity can be significantly reduced by insufficient rainfall or low soil fertility (El-Sharkawy, 2004), thus affecting its role as a food security crop. For example, a two-month drought stress during the stages of rapid leaf formation, root initiation and tuberization (1–5 months after planting) can reduce cassava storage root yield by up to 60% (Connor et al., 1981; Alves, 2002). Despite this, cassava is regarded as ‘a drought, war and famine reserve crop’ (Burns et al., 2010) because it can still produce a yield under adverse conditions often considered non-viable for other crops (El-Sharkawy, 2007; Okogbenin et al., 2013).

Traits such as high stomatal sensitivity to limit evapotranspiration, leaf retention and deep rooting capacities have been suggested to contribute to good performance of cassava under water limiting conditions (Alves, 2002; Okogbenin et al., 2013; Lenis et al., 2006; El-Sharkawy, 2007). The physiological characterization of cassava responses to drought has mostly focused on the content of the stress hormone ABA and the associated stomatal conductance (Alves and Setter, 2004a). For example, the sensitivity of cassava stomata to incipient water deficit has been associated with large increases in ABA content (Alves and Setter, 2000). The ABA content of both mature and expanding cassava leaves increases between 3–6 days of water deficit and decreases after re-watering (Alves, 2002; Alves and Setter, 2004a). ABA has also been suggested as a key contributor to the rapid arrest of cassava leaf growth under water stress and quick resumption of leaf expansion after re-watering (Alves and Setter, 2004b). However, the role of ABA regulation in genotypes displaying various degrees of drought tolerance and its potential in targeted breeding of cassava with increased drought tolerance have not been established.

Molecular studies to characterize the cassava response to drought have so far been few. Lokko et al. (2007) identified candidate expressed sequence tags with known roles in drought-response or unique to dehydration-stressed RNA libraries from cassava undergoing drought stress. High-density oligo-microarrays have been used to characterize the transcriptome of cassava *in vitro* plantlets subjected to artificial drought stress (Utsumi et al., 2012). Turyagyenda et al. (2013) identified four differentially regulated cassava genes in two cassava genotypes contrasting for drought resistance in a pot experiment and suggested their importance in oxidative burst mitigation and osmotic adjustment. Recent work suggests that drought tolerance in cassava is associated with the maintenance of a robust developmental programme sustaining storage root growth under water stress. Therefore, the biomass-partitioning ratio at an early stage of storage root development could be a useful indicator for a genotype to favor storage root growth when resources are limited by water stress (Duque, 2012). Consistent with these observations, a positive correlation between partitioning index at 7 months after planting and harvest index for drought-tolerant genotypes has been suggested as the basis for screening cassava germplasm for drought tolerance (Olasanmi, 2010). We performed three-year field trials at two sites in Kenya to assess the performance of 37 cassava genotypes differing for agronomic performance under drought conditions using water-sufficient and water-limiting conditions. Genotypes contrasting for yield performance in the water-limiting regime were subsequently selected for experiments in controlled greenhouse conditions to analyze the underlying physiological and molecular mechanisms associated with drought susceptibility and tolerance.

## 2. Materials and methods

### 2.1. Experimental sites

Field drought trials were conducted between 2010 and 2012 at two sites: Kibwezi (longitude 37°98"E, latitude 2°40"S and elevation of 914 m above sea level and Kiboko (longitude 37° 43"E, latitude 2° 12"S and an altitude of 975 m above sea level), both located within the drought prone Eastern province of Kenya (Shisanya et al., 2011; Mganga et al., 2010a). The two sites are classified under agro-climatic zone five (ACZ-V) within the arid and semi-arid lands of Kenya, which are characterized by soils with low plant nutrient availability, daily mean temperature varying from 15 °C to 35 °C and an average annual rainfall of 450–900 mm which is often poorly distributed and erratic (Sombroek et al., 1982; Jaetzold et al., 2006; Hornetz et al., 2000). Greenhouse experiments were carried out at ETH Zurich Research Station located in Lindau-Eschikon, Switzerland on latitude 47°26"N, longitude, 8°40"E and altitude of 540 m above sea level (Schneider et al., 2011).

### 2.2. Field drought trials

The 37 cassava genotypes assessed in this study were sourced from IITA, Ibadan, Nigeria (Suppl. Table 1), based on previous observations of drought susceptibility and tolerance. Soil characteristics (Suppl. Table 2A) and weather elements (rainfall, relative humidity and temperature) were recorded and analyzed from weather stations located within the sites (Suppl. Figs. 1–3). Between October 2009 and February 2012, (Suppl. Table 2B), three successive multi-seasonal field experiments were carried out in a randomized complete block design (RCBD). Ploughed land was split into four blocks of equal size. Each block was then divided into two plots and each plot further sub-divided into two sub-plots for four replicates. Two treatments, irrigated treatment (IRT) and non-irrigated treatment (NIRT) and cassava genotypes were randomly assigned to the plots and sub-plots respectively. Cassava cuttings of uniform length (~30 cm) was horizontally planted in soil in four rows per sub-plot and four stakes per row (total of 16 plants) at the recommended 1 m spacing between plants and 1 m between the rows (Ng and Ng, 2002). For homogenous plant germination and establishment, all plants were irrigated to field capacity three times per week via an overhead or sprinkler system in Kiboko and for three hours daily through a drip system in Kibwezi.

Irrigation was sustained for three months after which non-irrigated treatment was initiated by withholding total irrigation. To mimic common agronomic practiced by smallholder cassava farmers, no fertilizer (inorganic and organic) was applied during planting or establishment of the trial. Field drought trials were terminated through destructive harvesting at nine (9) months after planting (MAP) (Suppl. Table 2B). Eight plants from each cassava genotype per treatment replicate were selected (from inner rows) for determination of agromorphological traits. Leaf retention was visually scored as percent of the leaf-covered stems to the total plant height, the number of edible storage roots (NESR) was counted from each plant and their fresh storage roots weighed as yield following standard phenotypic approaches (Fukuda et al., 2010; Okogbenin et al., 2013). Yield, leaf retention and NESR data were subjected to analysis of variance (ANOVA) using SPSS under general linear model for multivariate. Fischer's least significant difference (LSD  $\alpha$  = 0.05) was used to separate group means.

### 2.3. Greenhouse assays

Three highest yielding and three least yielding genotypes under NIRT were selected from the field drought trials and subsequently used for greenhouse assays. For greenhouse experiment, plants were first multiplied and grown for 3–4 weeks *in vitro* prior to transfer to soil. The plantlets were subsequently hardened in soil for three weeks under

greenhouse conditions of 26 °C/17 °C (day/night) temperature, 60/50% (day/night) RH, 14 hours light at 35 K-lux intensity and average air ventilation rate of 84.7%. Plantlets of uniform size, growth and vigour were selected and transplanted in 4-litre potted soil composed of 40% sand, 35% clay, 25% silt and 21% organic matter (RICOTER Erdaufbereitung AG, Aarberg, Switzerland). Before planting, soil moisture content (SMC) or 'pot capacity' (PC) was determined as described by Alves and Setter (2004b). Plants were grown and maintained at ~100% PC for 60 days. At 60 days after planting (DAP), four plants from each genotype were subjected to three treatments in a completely randomized design with three replicates. The treatments included water deficit that was attained by withholding total irrigation, control or well-watered plants that were maintained at ~100% pot capacity and re-watering treatment, which was initiated once stomata conductance ( $g_s$ ) could not be measured from plants under water deficit. Daily, between 9.00 – 11.30 am, leaf  $g_s$  were measured from three fully expanded leaves using SC-1 Leaf Porometer (Decagon Devices Inc., Pullman, WA).

## 2.4. Leaf sampling, RNA extraction and cDNA synthesis

Based on declining SMC in the greenhouse experiments, upon WD induction (WDI) (Fig. 1), leaf materials were collected at four sampling time points (STPs). Leaves for STP1 were harvested 3 days after WDI i.e. at ~65% SMC, STP2 leaves collected 5 days after WDI i.e. at ~45% SMC, STP3 leaves taken 9 days after WDI i.e. at ~20% SMC and STP4 leaves harvested after 24-h re-watering (WDR) treatment i.e. at ~80% SMC (Fig. 1). Leaves from WW plants (control) were also harvested at each STP. Three upper fully expanded leaves (10<sup>th</sup>, 11<sup>th</sup> & 12<sup>th</sup> from bottom - Suppl. Fig. 4) from each of the three plants were harvested separately (per plant), pooled and stored at -80 °C for subsequent RNA isolation. Total RNA was extracted using pine tree RNA extraction method (Chang et al., 1993), with modifications adopted from Moreno et al. (2011). RNA concentration and purity was determined through Thermo Scientific™ NanoDrop (ND-1000) and integrity determined via 0.8% agarose gel electrophoresis. It is important to note that RNA extracted from genotype TME-419 was consistently of poor quality or integrity. The RNA was degraded, unsuitable for cDNA synthesis and thus TME-419 was subsequently excluded from gene expression analysis.

Prior to cDNA synthesis, genomic DNA contamination was removed from each isolated RNA via digestion with DNase 1 (Thermo Fisher Scientific). The treatment consisted of 1.0 µg total RNA, 1.0 µl 10X reaction buffer (with MgCl<sub>2</sub>) and 1.0 µl DNase 1 (RNase-free). The mixture was adjusted to final volume of 10.0 µl with nuclease free water.

The reaction was incubated at 37 °C for 30 minutes and terminated with addition of 1.0 µl EDTA, followed by incubation at 65 °C for 10 minutes. The DNase-treated RNA was then used as a template for cDNA synthesis using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The cDNA synthesis reaction contained 1.0 µl random hexamer primer, 4.0 µl 5X reaction buffer, 1.0 µl RiboLock RNase Inhibitor (20 u/µl), 2.0 µl 10 mM dNTP mix and 1.0 µl RevertAid M-MuLV Reverse Transcriptase (RT; 200 u/µl). The 9.0 µl master mix was then added to 11.0 µl DNase-treated RNA and incubated for 5 minutes at 25 °C followed by 42 °C for 60 minutes. The reaction was terminated by incubating at 70 °C for 5 minutes. The cDNA sample was then stored at -80 °C for subsequent RT-qPCR.

## 2.5. Selection of drought responsive genes and reference gene

The nucleotide or protein sequences of selected drought responsive genes (DRGs) associated with ABA-dependent and ABA-independent signaling pathways (Suppl. Table 11) were sourced from genomic databases TAIR (<https://www.arabidopsis.org>, accessed on October 2013) (Lamesch et al., 2012) and NCBI ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov), accessed October 2013). Cassava orthologs of selected genes were identified through phytozome BLAST tool (<http://www.phytozome.jgi.doe.gov>, accessed October 2013) of cassava genome database v4.0 (Prochnik et al. 2012). In cases where the query sequence of the gene of interest (GOI) produced multiple cassava genes during BLAST analysis, the cassava homolog with the lowest Expected (E) value was selected. Transcript sequences of the selected cassava genes were retrieved from Phytozome and used to design gene specific primers (Suppl. Table 12). Reference gene, *Manihot esculenta* serine-threonine protein phosphatase 2A (PP2A) (Czechowski et al., 2005; Moreno et al., 2011) was selected, validated and used to normalize expression of DRGs in the present study. Stability of PP2A expression across all genotypes and under different water regimes was confirmed using BestKeeper v1.0 software (Pfaffl et al., 2004).

## 2.6. Primer design, efficiency and RT-qPCR

Primers were designed using PerlPrimer software (Marshall, 2004), following criteria such as annealing temperature ( $T_m$ ) of 60 ± 1 °C, primer length of 18–25 bases, 40–60% GC content and 60–150 bp amplicon size (Udvardi et al. 2008). Most primers were designed from the 3'-untranslated region (3'-UTR) since it is generally unique than the coding sequence and closer to the reverse transcriptase start site (Udvardi et al., 2008; Taberlet et al., 1991). Specificity of each primer

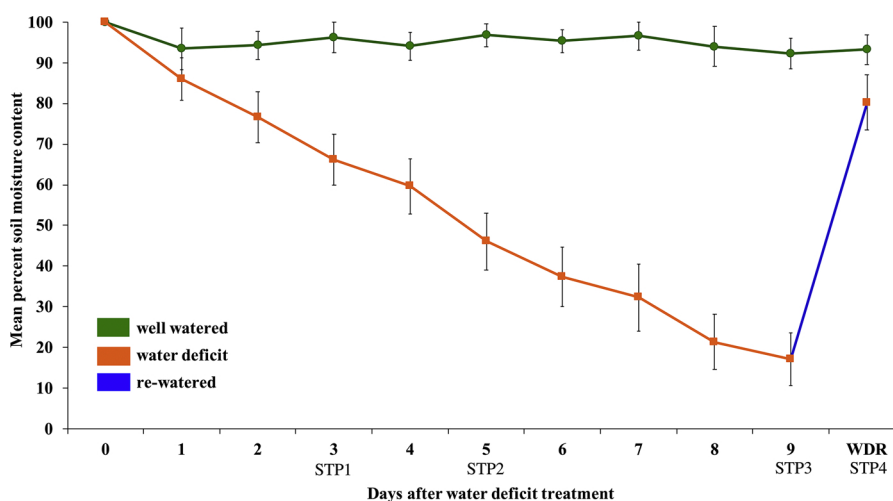


Fig. 1. Leaf sampling time points (STP1, STP2, STP3 and STP4) based on overall mean soil moisture contents (SMC) of well watered, water deficit and re-watered treatment under greenhouse experiments.

pair was confirmed through 1% agarose gel electrophoresis (single product of expected size) and analysis of dissociation curves. Primer efficiencies were derived from amplification plots or calculated using the raw fluorescence data ( $\Delta R_n$ ) that was exported as output file and subsequently imported into the LinRegPCR program (Ramakers et al., 2003; Ruijter et al., 2009). Primer pair efficiencies ranged between 1.90 – 2.02 (95 – 101%) (Suppl. Table 13) and therefore considered as suitable for reliable qPCR analysis (Applied Biosystems, 2008; Bio-Rad Laboratories, 2013). Stability of the PP2A reference gene was confirmed under well watered (WW), water deficit (WD) and re-watered (WDR) conditions using Bestkeeper software (Pfaffl et al., 2004; Sang et al., 2013; Zhang et al., 2019).

RT-qPCR was performed on synthesized cDNA using 7500 Fast Real Time PCR System (Applied Biosystems®, Foster City, CA). Three cDNA samples representing three biological replicates for each genotype per treatment and sampling time point were subjected to RT-qPCR analysis. The 20.0  $\mu$ l PCR volumes consisted of 1.0  $\mu$ l (10 pmoL) primers (forward & reverse), 4.0  $\mu$ l cDNA templates, 4.0  $\mu$ l sterile deionized water (ddH<sub>2</sub>O) and 10.0  $\mu$ l Fast SYBR® Green Master Mix (Thermo Fisher Scientific). The PCR thermal cycles profile applied were adopted from Moreno et al. (2011). The process involved initial cDNA denaturation at 95 °C for 20 seconds; 40 cycles of denaturation at 95 °C for 3 seconds; annealing at 60 °C for 15 seconds and extension at 72 °C for 30 seconds. The default dissociation step consisted of 95 °C for 15 seconds; 60 °C for 1 minute; 95 °C for 15 seconds and 60 °C for 15 seconds. The dissociation curve analysis was carried out at the default setting of the 7500 Fast Real Time PCR System to confirm the specificity of each reaction.

## 2.7. Statistical analysis

### 2.7.1. Data from field trials and greenhouse assays

Field collected data (yield, NESR and leaf retention) were subjected to analysis of variance (ANOVA) using Statistical Package for Social Scientists (SPSS) version 21 (SPSS Inc., 2012). The general linear model (multivariate) was applied with Fischer's least significant difference (LSD  $\alpha$  = 0.05) procedure used for Post Hoc tests between dependent variables (yield, NESR and leaf retention) and fixed factors (location, seasons, treatments and genotypes) (Suppl. Table 3). Additional tests were carried out to analyze interactions between fixed factors for every dependent variable (Suppl. Table 3). Pearson's product moment correlation coefficient ( $r$ ) test was used to analyze the inter-relationships between traits, while the overall percent performance of each trait was computed relative to control treatment. Greenhouse generated leaf stomatal conductance ( $g_s$ ) data was subjected to ANOVA using Sigma-Plot analysis software version 12.2 (San Jose, CA). The differences between groups of means were separated by standard deviations.

### 2.7.2. Data from RT-qPCR

For gene regulation, qPCR cycle threshold (Ct) data was generated using 7500 Fast System SDS software with default settings. The relative gene expression ratio was computed based on primer efficiencies and Ct differences of treated samples versus control treatment following a modified mathematical model described by Pfaffl (2001) (see Eq. 1). Expression ratio (R) of DRGs (Suppl. Table 11) in each cassava genotype per sampling time point (STP) was normalized with reference gene PP2A (Moreno et al., 2011; Reddy et al., 2016). Expression ratio (R) of DRGs (Suppl. Table 11) in each cassava genotype per sampling time point (STP) was normalized with reference gene, PP2A. This was done by dividing each biological sample or replicate per treatment (WD, WDR & WW) with PP2A. Once normalized to PP2A, gene expression fold change (GEFC) was calculated by dividing the ratio from treated samples (WD & WDR) with ratio of WW controls where a ratio above 1.0 was considered up regulation and below 1.0 considered down regulation. Significance of differences between water deficit (WD), re-watered (WDR) and well-watered (WW) or control treatment pairs in gene regulation were tested with a student t-test ( $P \leq 0.05$ ). The GEFC

were then converted into heat maps (Figs. 6 and 7).

$$\text{Expression ratio(R)} = \frac{(E_{\text{goi}})^{\Delta C_t \text{ goi(WW-WD / WDR)}}}{(E_{\text{ref}})^{\Delta C_t \text{ ref(WW-WD / WDR)}}} \quad (1)$$

Where E = primer efficiency; goi = gene of interest or DRGs; ref = reference gene or PP2A;  $\Delta C_t$  = change in Ct; WW = well-watered treatment (control); WD = water deficit treatment; WDR = re-watered treatment

## 3. Results

### 3.1. Identification of cassava genotypes contrasting for drought tolerance under field conditions

During the annual field experiment periods, long season rains (January – May) were punctuated by a 4-month drought (June – September) before the onset of short season rains (September – December) in both locations (Suppl. Figs. 1–3). The mean monthly relative humidity (determined between 9.00 – 11.00 AM) was fairly constant at both sites, with a lower humidity in Kibwezi, while temperatures were comparable and constant (Suppl. Figs. 1–3). Both sites had dominant clay and sandy clay loam soil textures (Suppl. Table 2A). Higher mineral ions or contents (Cl<sup>-</sup>, Na<sup>+</sup>, Ca<sup>2+</sup> and Mg<sup>2+</sup>), low pH (7.51) and low K<sup>+</sup> ions (0.66) were analyzed in soils from Kiboko compared to lower mineral contents (Cl<sup>-</sup>, Na<sup>+</sup>, Ca<sup>2+</sup> and Mg<sup>2+</sup>), higher pH (8.52) and higher K<sup>+</sup> ions (0.82) recorded in soils from Kibwezi (Suppl. Table 2A). Except NESR under location (Ln), ANOVA results showed significant ( $P \leq 0.001$ ) differences in yield, NESR and leaf retention between seasons (Sn), genotypes (Gt) and treatments (Tm) (Suppl. Table 3). The Ln\*Sn\*Tm\*Gt interaction was significant ( $P \leq 0.001$ ) for LER but non-significant ( $P > 0.05$ ) for yield and NESR (Suppl. Table 3).

Analysis of overall mean output showed significantly more yield, NESR and leaf retention in cassava plants under IRT compared to plants under NIRT (Table 1). Overall yield under NIRT were significantly ( $P \leq 0.01$ ) and positively correlated ( $r = 0.591$ ) with yield under IRT (Table 2). Similar positive correlations between yield-NIRT and yield-IRT were also recorded in both experimental sites and years. For instance, the correlation was significant ( $P \leq 0.01$ ) at KBK-S1 ( $r = 0.891$ ) and KBZ-S1 ( $r = 0.699$ ) as well as  $P \leq 0.05$  at KBK-S2 ( $r = 0.415$ ), and KBZ-S2 ( $r = 0.531$ ), with non-significant negative relations ( $P > 0.05$ ;  $r = -0.007$ ) only observed in KBZ-S3 (Table 2). Other correlation analysis under NIRT showed significant ( $P \leq 0.01$ ) and positive correlation between yield and NESR ( $r = 0.516$ ) as well as between yield and LER ( $r = 0.449$ ), while correlations between NESR and LER was non-significant for this condition (Table 2). Under control or IRT, a significant ( $P \leq 0.03$ ) and positive correlation ( $r = 0.859$ ) was observed between yield and NESR while correlations between yield and leaf retention as well as NESR and leaf retention were not significant (Table 2).

All field-screened cassava genotypes were classified either as drought tolerant (DT) or drought susceptible (DS) groups based on yield data (Suppl. Tables 4 and 5). Under NIRT, (with both sites and all seasons considered), the genotypes with significantly higher yield were classified as DT while those with significantly lower yield were categorized as DS (Suppl. Table 6). Based on this criteria, five genotypes (94/0039, 95/0306, 98/0002, I92/0067 and 92/0342) were selected as DT while another five genotypes (PYT, 92/0427, TME-419, I96/1439 and 96/0409) were classified as DS (Suppl. Table 6). Analysis of physiological and molecular response of cassava to drought stress took advantage of those cassava genotypes contrasting for response to drought at the yield level.

Table 3 summarizes the average yield performance of the different genotypes. When yields under both NIRT and IRT were considered, genotypes 94/0039, 95/0306 and 98/0002 bulked significantly higher



**Table 1**

Overall mean field output for Yield, NESR and LER under irrigated and non-irrigated treatments in each location.

	KIBOKO						KIBWEZI					
	YIELD (tons of fresh roots ha <sup>-1</sup> )		NESR (number of fresh roots plant <sup>-1</sup> )		LER (% leaf retained plant <sup>-1</sup> )		YIELD (tons of fresh roots ha <sup>-1</sup> )		NESR (number of fresh roots plant <sup>-1</sup> )		LER (% leaf retained plant <sup>-1</sup> )	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
<b>Season 1</b>												
Irrigated	7.95 <sup>a</sup>	0.11	8.95 <sup>c</sup>	0.18	71.16 <sup>e</sup>	0.86	4.11 <sup>a</sup>	0.07	8.11 <sup>c</sup>	0.13	59.67 <sup>e</sup>	0.60
Non-Irrigated	6.02 <sup>b</sup>	0.09	8.08 <sup>d</sup>	0.19	62.89 <sup>f</sup>	0.89	3.17 <sup>b</sup>	0.06	7.09 <sup>d</sup>	0.14	40.49 <sup>f</sup>	0.62
<b>Season 2</b>												
Irrigated	3.55 <sup>a</sup>	0.06	5.46 <sup>c</sup>	0.11	69.84 <sup>e</sup>	0.531	4.44 <sup>a</sup>	0.07	7.84 <sup>c</sup>	0.13	55.55 <sup>e</sup>	0.61
Non-Irrigated	2.46 <sup>b</sup>	0.07	4.67 <sup>d</sup>	0.12	50.93 <sup>f</sup>	0.542	1.80 <sup>b</sup>	0.08	4.63 <sup>d</sup>	0.15	27.93 <sup>f</sup>	0.68
<b>Season 3</b>												
Irrigated	-	-	-	-	-	-	3.92 <sup>a</sup>	0.07	7.10 <sup>c</sup>	0.13	71.02 <sup>e</sup>	0.62
Non-Irrigated	-	-	-	-	-	-	1.60 <sup>b</sup>	0.09	4.06 <sup>d</sup>	0.17	53.32 <sup>f</sup>	0.81

NESR = number of edible storage roots; LER = leaf retention; SEM = standard error of mean; means with different letters in each column (for each trait) are significantly different at  $P \leq 0.001$ .

root yield (in both conditions) in all growing periods at each site while similar performances with one exception were recorded in genotypes I92/0067, 92/0342 and 96/2132 (Table 3). The NESR was significantly higher for most DT genotypes as compared to DS genotypes under NIRT, whereas under IRT NESR differences were not significant between the two cassava genotype groups (Suppl. Tables 7 & 8). Genotypic differences for leaf retention (Suppl. Table 3) were also significant but this varied with location. For instance, under NIRT in Kiboko (S1 & S2) the least leaf retention (~31%) was recorded in a DS genotype compared to the least leaf retention (~45%) of a DT genotype (Suppl. Table 9). Under the same condition (NIRT) in Kibwezi, the least leaf retention (~19%) and (~23%) were observed in a DS and a DT genotype (Suppl. Table 10), respectively.

### 3.2. Stomatal conductance in selected cassava genotypes under controlled conditions

Cassava genotypes contrasting for yield performance in NIRT field conditions were subsequently assessed in controlled greenhouse conditions. Significantly, higher stomatal conductance ( $g_s$ ) were measured consistently in WW cassava plants compared to plants the WD condition (Fig. 2). Similar comparisons were made between WW and WDR plants. Genotypic variations for  $g_s$  were also observed especially starting at 6

days after WD treatment (Fig. 3). For example, DT genotypes (98/0002, 94/0039 & 95/0306) exhibited significantly higher  $g_s$  compared to DS genotypes (I96/1439, 92/0427 & TME-419) with significantly lower  $g_s$  between day 7, 8 and 9 (Fig. 3). These implied that upon WD treatment, DT genotypes showed less decline in  $g_s$  (maintained higher  $g_s$ ) compared to their DS counterparts with rapid  $g_s$  decline. The genotypes also showed differences for  $g_s$  after re-watering (WDR) that increased soil moisture to 80% (Fig. 1). For example, all DS genotypes re-gained significantly higher  $g_s$  compared to DT genotypes (Fig. 4). Under well watered or control treatment,  $g_s$  variation between DT and DS genotypes were not significant (Suppl. Fig. 5).

### 3.3. Expression patterns of drought-responsive genes in selected cassava genotypes

Candidate genes with potential functions in drought response previously characterized in *Arabidopsis thaliana* and in other crops were identified in cassava and their expression tested in cassava genotypes contrasting for their response to drought in the present study. The DRGs (Suppl. Table 11) were categorized into either ABA-dependent (ABA-D) or ABA-independent (ABA-I) pathways (Fig. 5) based on Arabidopsis classification. It should be noted that this classification remains to be validated in cassava. After WDI, the expression patterns of ABA-D

**Table 2**

Correlations between yield, leaf retention and number of storage roots non-irrigated and irrigated conditions in different experimental seasons and sites.

	Overall_YLD-IRT	KBK-S1_YLD-IRT	KBK-S2_YLD-IRT	KBZ-S1_YLD-IRT	KBZ-S2_YLD-IRT	KBZ-S3_YLD-IRT
Overall_YLD-NIRT	0.591**					
KBK-S1_YLD-NIRT		0.891**				
KBK-S2_YLD-NIRT			0.415**			
KBZ-S1_YLD-NIRT				0.699**		
KBZ-S2_YLD-NIRT					0.531*	
KBZ-S3_YLD-NIRT						-0.007 <sup>ns</sup>
	NSR_NIRT	NSR_IRT	LR_NIRT	LR_IRT	LR_NIRT	LR_IRT
YLD_NIRT	0.516**					
YLD_IRT		0.859*				
YLD_NIRT			0.449**			
YLD_IRT				0.289 <sup>ns</sup>		
NSR_NIRT					-0.058 <sup>ns</sup>	
NSR_IRT						-0.200 <sup>ns</sup>

IRT = irrigated treatment; NIRT = non-irrigated treatment; YLD = yield; NSR = number of storage roots; LR = leaf retention; \*\*correlation is significant at  $P \leq 0.01$ ; \*correlation is significant at  $P \leq 0.05$ ; ns = correlations is non-significant ( $P > 0.05$ ); KBK = Kiboko; KBZ = Kibwezi; S1, S2 & S3 = season 1, season 2 and season 3

**Table 3**

Average yield (tons of fresh roots ha<sup>-1</sup>) of selected drought tolerant and drought susceptible cassava genotypes in non-irrigated and irrigated conditions in field trials in Kenya.

Treatment	Non-Irrigated treatment					Irrigated (control) treatment				
Site	Kiboko		Kibwezi			Kiboko		Kibwezi		
Genotype	Season 1	Season 2	Season 1	Season 2	Season 3	Season 1	Season 2	Season 1	Season 2	Season 3
<i>Drought tolerant genotypes</i>										
94/0039	8.31	3.47	4.39	2.87	2.25	10.61	3.83	4.93	6.35	4.79
95/0306	7.44	3.13	3.82	2.93	2.17	8.94	4.34	4.83	4.79	3.71
98/0002	7.59	3.09	3.28	2.62	2.44	8.50	3.59	4.39	5.10	2.79
192/0067	7.77	3.36	3.31	2.31	2.19	11.02	3.55	4.00	4.19	3.73
92/0342	5.63	3.77	2.74	2.27	1.31	8.27	4.74	3.99	4.01	5.41
96/2132	7.09	-	3.88	1.45	1.47	9.86	-	4.18	4.64	3.57
<i>Drought susceptible genotypes</i>										
PYT	4.61	1.97	1.67	0.78	0.82	6.50	2.50	3.36	3.68	4.32
92/0427	4.25	1.97	2.66	1.35	0.96	6.30	2.38	5.09	3.81	4.24
TME-419	5.64	2.05	2.72	1.15	1.04	8.16	3.25	4.13	4.63	4.55
196/1439	6.49	1.84	2.36	1.50	0.86	7.42	3.24	2.79	3.13	3.32
96/0409	3.66	2.80	2.82	1.33	1.07	5.74	3.18	4.35	3.93	2.97

(Fig. 6) and ABA-I genes (Fig. 7) were significantly changed and differed between cassava drought-tolerant and susceptible genotypes. As expected, an increasing number of ABA-D genes were up-regulated with decreasing SMC. For example, more genes were up-regulated at ~20% SMC (STP3) compared to those up-regulated at ~65% SMC (STP1) (Fig. 7). Further, fewer ABA-D genes were up-regulated at higher soil moisture contents such as 80% SMC (STP4). Gene expressions also varied with genotype. For example, at STP1, more ABA-D genes were up-regulated in DS genotypes and in 196/1439 in particular compared to either down-regulation or non-significant expression changes of these genes in DT genotypes and 95/0306 in particular (Fig. 6).

ABA-D genes with contrasting expression patterns between DT and DS cassava genotypes included *NCED3*, *RD29A/B*, *SLAC1* and *SNAC1*. These genes were up-regulated in DT genotypes and down-regulated in DS genotypes at STP1 (65% SMC) (Fig. 6). Nearly all ABA-D genes were up-regulated in both DT and DS cassava genotypes at 45 and 20% SMC (Fig. 6). The only exceptions were *PYR1* that was down-regulated in both DT and DS genotype at 45% SMC, *OST1* and *DSTP* that were both down-regulated in DT and up-regulated in DS genotypes at 45% SMC, as well as *PLDa1* and *PYR1* that were up-regulated in DT and down-regulated in DS genotypes at 20% SMC (Fig. 6). After re-watering at STP4 (80% SMC), four genes, *ABI1*, *RD20*, *PLDa1* and *MYB44* were up-regulated in DT and down-regulated in DS genotypes, while *NFYA5*, *SCaBP5* and *SNAC1* were up-regulated in DS genotypes (Fig. 6). Nearly

all ABA-I genes were up-regulated upon WD induction at 65%, 45% and 20% SMC (Fig. 7). The only exceptions were *ATAF1* that was not up or down regulated at STP3 (20% SMC) in any genotype as well as *DREB2A/B* and *RD29A/B*, which were down-regulated in DT genotypes and up-regulated in DS genotypes at STP1 or 65% SMC (Fig. 7). Upon re-watering at 80% SMC (STP4), all the five ABA-I genes (*ATAF1*, *ERD10*, *DREB1A/B*, *DREB2A/B* and *RD29A/B*) except *ERD10* were down-regulated in the DS cassava genotype 92/0427 (Fig. 7). Expression patterns of these genes in the remaining genotypes were different at STP4 (Fig. 7).

#### 4. DISCUSSION

##### 4.1. Effects of water deficit on cassava morphology and yield

The typical bi-modal rainfall pattern of the region (Maingi et al., 2001; Kamau et al., 2010; Mganga et al., 2010b) and the dominant clay and sandy clay loam soil textures in both locations with low organic content and fertility that are common in semi-arid areas (Hornetz et al., 2000) made the two selected sites suitable for our field experiments (Suppl. Figs. 1–3). Although the mineral contents of soil profiles in both locations were relatively low (Suppl. Table 2A), no mineral fertilizer or organic manure was applied. This was to mimic as much as possible some of the common agronomic practices observed in cassava farmers

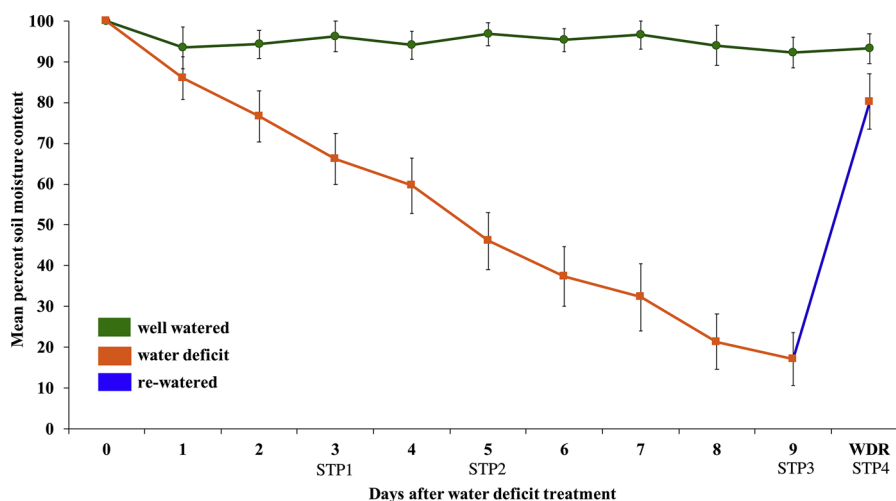


Fig. 2. Mean stomatal conductance ( $g_s$ ) under well-watered (control), water deficit and re-watered treatment at different sampling time points (STP1, STP2, STP3 and STP4) under greenhouse experiment.

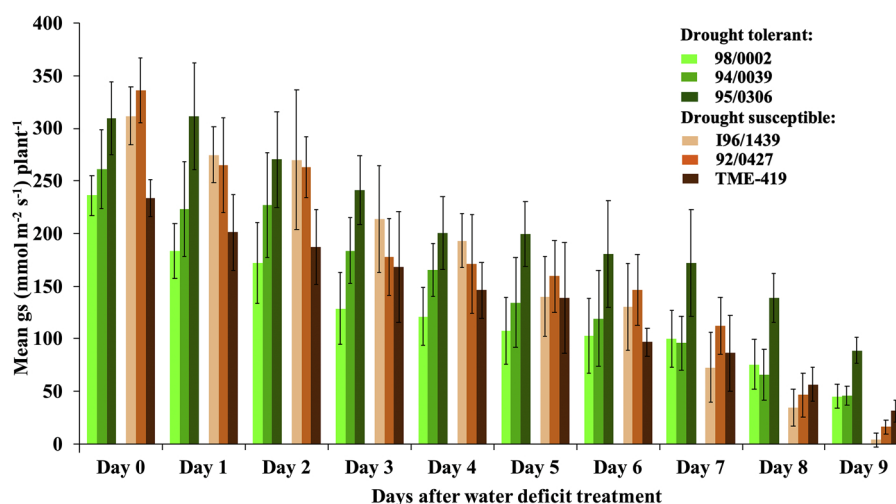


Fig. 3. Mean stomatal conductance ( $g_s$ ) variations between drought tolerant (DT) and drought susceptible (DS) cassava genotypes subjected to nine-day water deficit treatment under greenhouse experiments. Error bars = standard deviations

in both locations. Indeed smallholder farmers in Africa use little or no fertilizer at all when cultivating cassava (Fermont et al., 2009; Nweke, 1994; Kelly, 2006; Biratu et al., 2018). The significant variation among the cassava genotypes between conditions as well as genotype and treatment interactions ( $Gt \times Tm$ ) as well as locations and seasons ( $Ln \times Sn$ ) for yield, NESR and LER indicated strong genetic variability for drought tolerance (Suppl. Table 3). Although genotypic variability suggests a potential for selection of drought tolerant cassava genotypes (Nduwumuremyi et al., 2017), the significant  $Ln \times Gt \times Tm$  interactions present a challenge in identifying or selecting superior genotypes (Tumuhimbise et al., 2014). The significant  $Ln \times Gt \times Tm$  interaction highlight the importance of conducting multi-location field trials to identify the most stable genotypes generally and specifically adapted to semi-arid environments (Nduwumuremyi et al., 2017).

The overall impact of NIRT relative to IRT confirmed the negative effect of water deficit on cassava growth and yield (Tables 1–3), which was more severe in Kibwezi than in Kiboko, perhaps as the result of irrigation methods or rainfall, rates of evapotranspiration, or moisture retention capacities of the soils, but comparable to previous reports (Connor et al., 1981; Alves, 2002; Aina et al., 2007a). Performance of traits within and between locations and seasons also varied. For example, overall mean output of yield, NESR and LER under both treatments was higher in season one compared to season two in Kiboko field

trials (Table 1). Differences in the timing of rainfall onset and duration of rainfall probably contributed to seasonal trait variation. For instance, season one trial was initiated at the onset of the short rainy season (September 2009) and terminated in June 2010 after the long seasonal rains (Suppl. Table 2B; Suppl. Figs. 1 & 2), compared to season two trial that was established during drier month of July 2010 and terminated in April 2011 (Suppl. Table 2B; Suppl. Figs. 2 & 3). Additionally, during trial in season one, there was rainfall for more than two months before harvest (Suppl. Fig. 1) compared to trial in season two that had longer dry period (Suppl. Fig. 2). Thus, the number of months with rainfall in season two was two months fewer than in season 1. This might have contributed to the higher performance among traits in season one compared to season two (Table 1). While studying the impact of water stress on fresh tuber yield and dry matter contents of cassava under field conditions, Bakayoko et al. (2009) equally reported seasonal yield variation based on planting and harvesting time points. Cassava plants exposed to drought during establishment stage (Santisopasri et al., 2001; Pardales and Esquibel, 1996) and immediately before root harvest (Bakayoko et al., 2009) exhibits reduced productivity.

The generally significant and positive correlation between yield under NIRT and IRT indicates that cassava genotypes with high yield under NIRT are also likely to perform better under IRT conditions (Table 2). Therefore, our results suggest that initial screening of highest

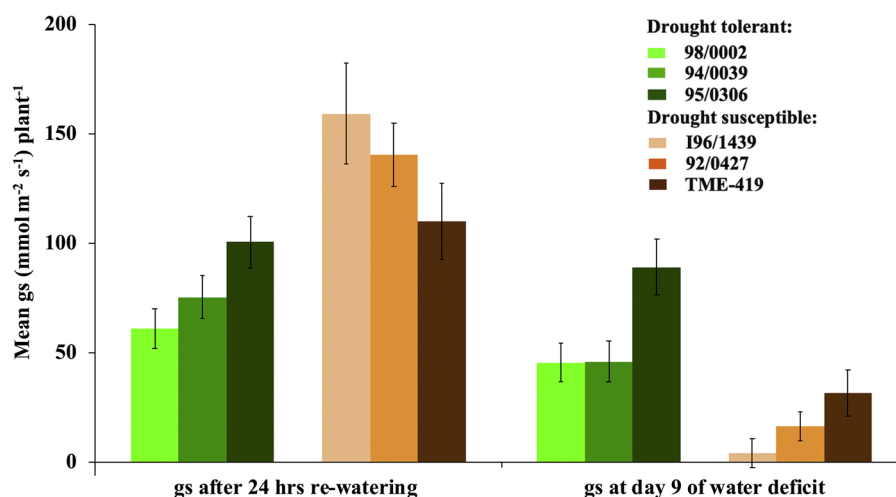
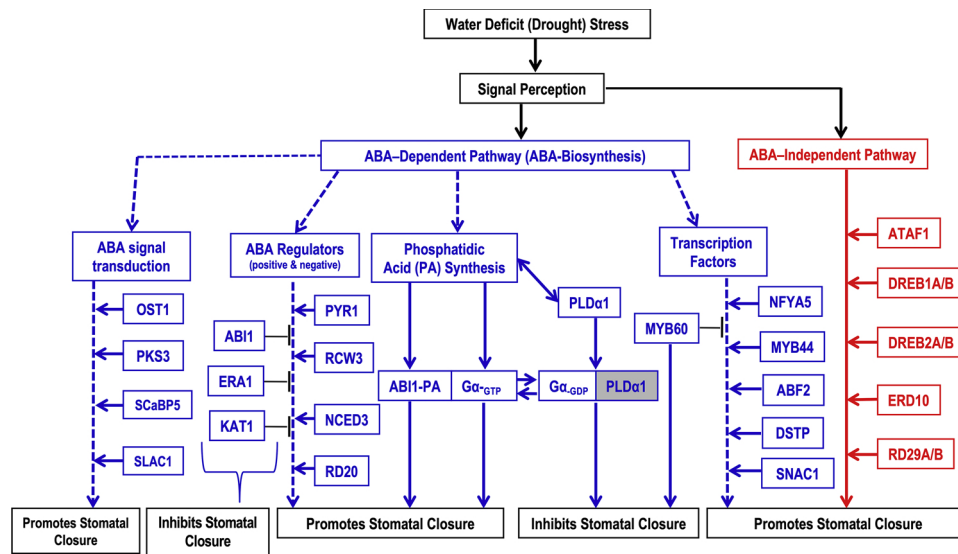


Fig. 4. Mean stomatal conductance ( $g_s$ ) variations between drought tolerant (DT) and drought susceptible (DS) cassava genotypes (at day 9 of water deficit and after 24 hours of re-watered treatments) under greenhouse experiments. Error bars = standard deviations



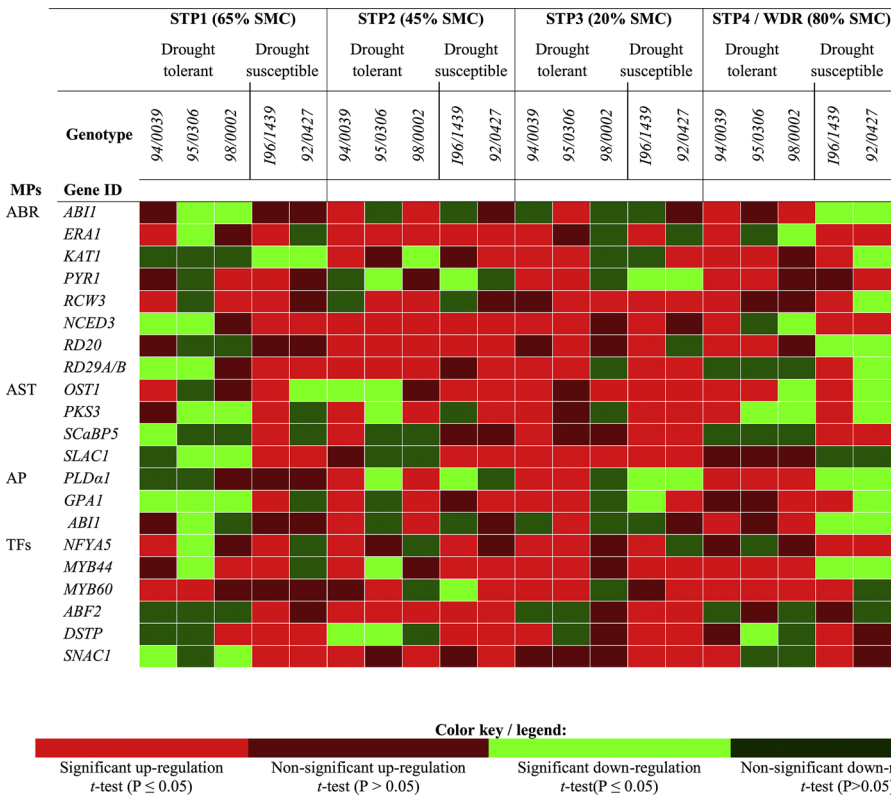
**Fig. 5.** A model adopted and modified from [Shinozaki and Yamaguchi-Shinozaki \(2007\)](#) representing selected Drought Responsive Genes (DRGs) profiled in cassava and categorized into either ABA-D or ABA-I molecular pathways. ABA-D = ABA-Dependent; ABA-I = ABA-Independent

yielding cassava genotypes under well-watered (control) conditions is a reasonable strategy for subsequent screening under water deficient conditions to select genotypes for high yield under drought conditions. The positive correlation between yield and leaf retention under NIRT suggests a positive effect of leaf retention on high cassava yield under drought stress ([Table 2](#)). Similarly, [Lenis et al. \(2006\)](#) observed a positive correlation between leaf retention and fresh root production. Prolonging leaf longevity could aid in producing cultivars with improved yield and root quality ([Fregene and Puonti-Kaerlas, 2002](#)).

The positive and significant correlations between yield and NESR ([Table 2](#)), which is an indicator of cassava sink strength ([Pellet and El-Sharkawy, 1994](#)), suggests that in addition to storage root weight,

NESR can also be included in a selection index designed to improve cassava production. Thus, NESR should be increased to obtain a higher cassava yield because a lower storage root sink capacity reduces the canopy photosynthetic rate and increases leaf starch ([Gray, 2000](#), [De Souza and Long, 2018](#); [Stitt, 1991](#)). Similar correlations between yield and NESR have been previously reported in cassava ([Adjepong-Danquah et al., 2016](#); [Tumuhimbise et al., 2014](#)). The non-significant correlation between NESR and leaf retention under NIRT that we found differs from previously reported LER capacity of cassava during periodic drought that positively correlated with root quality or number of commercially viable storage roots ([Fregene and Puonti-Kaerlas, 2002](#)).

Although cassava is generally considered to be drought-tolerant



**Fig. 6.** Heat map showing differential regulation patterns of ABA-dependent genes (orthologous to ABA-dependent genes in Arabidopsis) between drought-tolerant (DT) and drought-susceptible (DS) cassava genotypes at sampling time point (STP) 1 (65% SMC), STP2 (45% SMC), STP3 (20% SMC), and STP4 (WDR / 80% SMC). STP = sampling time point; MPs = molecular pathways; ABA Regulators; AST =

ABA Signal Transduction; PAP = Phosphatidic Acid Pathway; TFs = Transcription Factors.



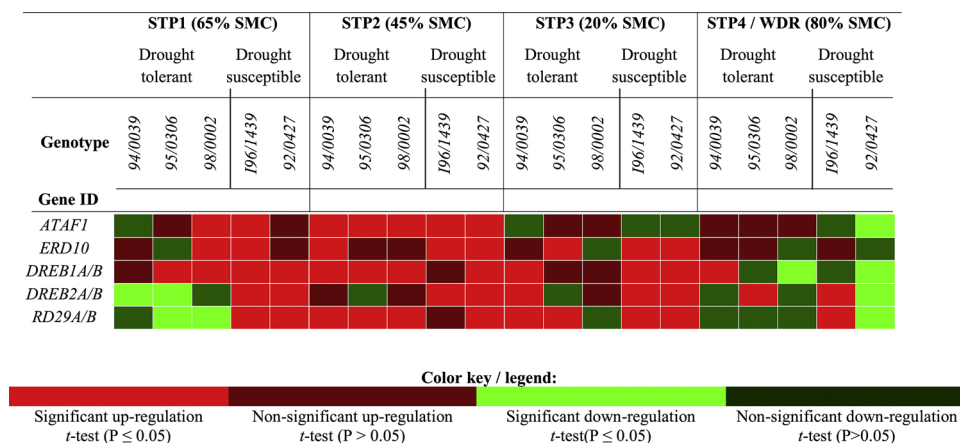


Fig. 7. Heat map showing differential regulation patterns of ABA-independent genes (orthologous to ABA-dependent genes in Arabidopsis) between drought-tolerant (DT) and drought-susceptible (DS) cassava genotypes at STP1 (65% SMC), STP2 (45% SMC), STP3 (20% SMC), and STP4 (WDR / 80% SMC). SMC = soil moisture contents; STP1, STP2, STP3 & STP4 = sampling time points 1, 2, 3 and 4.

(Alves, 2002; Bergantin et al., 2004), our results provide the range of yield reduction that can be expected from exposure to sub-optimal water regimes (Tables 2 and 3). Water availability is among the most significant genotype-dependent abiotic constraint for cassava (Oliveira et al., 2015). Based on storage root yield under NIRT we identified the best performing or drought-tolerant (DT) and least-performing or drought-susceptible (DS) cassava genotypes (Table 3). Interestingly, most DT genotypes also bulked higher yield under IRT compared to DS genotypes, suggesting that better-performing genotypes selected under water deficit can also be expected to sustain higher performance in areas or during periods with sufficient rainfall. The DT genotypes 94/0039, 98/0002, 95/0306, 192/0067, 92/0342 and 96/2132 were particularly significant at the top of 15 highest yielding genotypes under both NIRT and IRT (Table 3). These genotypes showed good yields irrespective of conditions, years or locations and could be incorporated into breeding programs for drought tolerance. They could produce reasonable yields with unpredictable precipitation and temperature patterns associated with climate change. As previously reported, the wide variation within the cassava germplasm for tolerance to prolonged drought presents the possibility to breed and select for stable and relative high yields under favorable and adverse conditions (El-Sharkawy and Cock, 1987).

Variations in other morphological traits such as NESR and leaf retention can also be useful markers. For instance, under NIRT, we found significantly higher NESR for most DT genotypes compared to DS genotypes (Supp. Tables 7 & 8), suggesting a higher root sink strength in drought-tolerant genotypes. The number of storage roots harvested per plant may also be an indicator of root sink strength, which is of value in cassava breeding (El-Sharkawy, 2004; Pellet and El-Sharkawy, 1994). However, we note that genotypic differences for NESR were not significant under IRT, indicating that DT genotypes maintain higher root sink strength under NIRT conditions. Variation in leaf retention was not significant between DT and DS genotypes under IRT, but the higher leaf retention in DT genotypes under NIRT field conditions (Supp. Tables 9 & 10) suggest that these genotypes also maintain a higher source capacity. It may thus be preferable to breed and select for better leaf retention when developing varieties adapted to dry areas (Okogbenin et al., 2013).

#### 4.2. Drought tolerance is associated with differential stomatal conductance in response to water deficit

The reduction of stomatal conductance ( $g_s$ ) in plants subjected to WD is often associated with the decrease of soil moisture content. Similarly, Shan et al (2018) recorded a substantial  $g_s$  decline in cassava plants under drought stress conditions compared to well-watered plants. Cassava maintains a high  $g_s$  and internal  $CO_2$  concentration under optimal water conditions, but rapidly closes stomata in response

to even a small decrease in soil water potential (El-Sharkawy and Cock, 1984; Alves and Setter, 2000). Importantly, we found no consistent differences for  $g_s$  between DT and DS genotypes as both groups of genotypes showed a continuous reduction of  $g_s$  as SMC decreased (Fig. 3). However, between 7 – 9 days after water deficit induction, the reduction of  $g_s$  became more pronounced in DS genotypes (shown by lower  $g_s$ ) compared to DT genotypes with higher  $g_s$  (Fig. 3). Cassava plants rapidly recover from drought stress after a rainfall by producing new leaves with even higher  $g_s$  (El-Sharkawy, 2006, 2007). Relatively similar phenomenon was also observed in greenhouse experiments, in which selected genotypes and particularly DS genotypes showed a rapid and significant increase in  $g_s$  24 hours after re-watering, but in fully expanded leaves (Fig. 4). Collectively, our results indicate that sensing of water deficit is similar in DT and DS genotypes but eventually leads to significant differential reduction of  $g_s$  over a 9-day period (Fig. 3). The selected DT genotypes appear to be less responsive to water deficit conditions as suggested by the delayed increase in stomata aperture upon re-watering as compared to DS genotypes. Relatively similar variation in stomatal leaf conductance has been observed and thus  $g_s$  seems to be useful parameter in pre-selecting sources of germplasm conferring adaptation to prolonged dry periods (Iglesias et al., 1995). The improved yield performance of DT genotypes under NIRT field conditions can therefore be partially explained by their capacity to prolong stomatal opening, enabling them to photosynthesize for a longer period. The faster opening and closing of stomata has a greater rate of energy consumption per unit leaf area than slower opening and closing (Raven, 2014), which could further accentuate the yield difference between DT and DS genotypes.

Despite this, results in the present study cannot authoritatively associate variation in stomatal conductance with plant yield, as these variables were measured in plants cultivated in different systems (greenhouse and field) and stress conditions (short – 9 day water deficit; or long-term stress – 9 months). Additional factors such as rooting depth response may be one of the differences in genotypes that can explain adaptations to water stress, likely interacting with stomatal conductance responses and thus yield differences. Although we did not measure root depth in the current study, previous research showed that during water scarcity, cassava fibrous roots can extend for more than 2 meters into deeper and wetter soil, from where the plant can extract between 20 – 40% of its total water uptake (El-Sharkawy et al., 1992). Also, cassava can maintain adventitious root elongation in drought conditions, which results in a relatively broad horizontal spread of the root system that can recover quickly from drought by lateral root branching and that may be related to good cassava growth and yield performance (Subere et al., 2003). Cassava's access to deep-water layers (Okogbenin et al., 2013) enables the crop endure long periods of drought stress and perhaps extended stomatal conductance and photosynthesis for better yield performance as observed in DT genotypes.

Cassava is capable of partially retaining their photosynthetic capacity under prolonged water shortage (Okogbenin et al., 2013) for sustained production.

#### 4.3. Molecular characterization of stomatal conductance in cassava under water deficit

Our results show that stomatal closure was associated with a decrease in  $g_s$  under water-deficit conditions while stomatal opening was linked to sustained or high  $g_s$  under control or well-watered conditions in all genotypes (Fig. 2). Similar stomatal closure, opening and re-opening in cassava has been reported under field conditions (Alves and Setter, 2000; El-Sharkawy, 2006, 2007). The accumulation of ABA in cassava leaves is correlated with  $g_s$  and/or transpiration rates and rapid stomatal closure under drought stress (Alves and Setter, 2000). ABA is involved in the regulation of stomata opening and closing to regulate water loss (Mishra et al., 2006). In response to drought, plants synthesize ABA, which triggers closing of stomata to reduce water loss (Schroeder et al., 2001). Studies in model plant species have shown that drought stress signaling is mediated by ABA-dependent (ABA-D) and ABA-independent (ABA-I) pathways to activate several drought-inducible genes (Roychoudhury et al., 2013; Shinozaki and Yamaguchi-Shinozaki, 2007; Chinnusamy et al., 2004). To date, these two pathways have not been well characterized in cassava in the context of water deficit conditions. Our results show that ABA-D and ABA-I genes in the cassava genotypes differing in drought tolerance likely mediate signaling of water deficit conditions as well. Previous experiments that mimicked drought stress using PEG-mediated dehydration in cassava also found changes in both ABA-D and ABA-I regulatory networks and genes (Fu et al., 2016; Li et al., 2017; Li et al., 2017b), similar to the activation of ABA-D and ABA-I pathway genes in other plants (Tuteja, 2007; Shinozaki and Yamaguchi-Shinozaki, 1997, 2007).

The expression changes of ABA-D and ABA-I genes we observed at 65% SMC suggest a varied molecular response to water scarcity in cassava genotypes, similar to the large natural variation in the expression of stress-related genes in Arabidopsis subjected to soil WD in greenhouse experiments (Rymaszewski et al. 2017). It was also reported that some genes respond to water stress very rapidly whereas others show slow response after drought (Shinozaki and Yamaguchi-Shinozaki, 1997). Most of the ABA-D and ABA-I genes were up-regulated in both DT and DS cassava genotypes at 45% and 20% soil moisture, suggesting a gradual response to water deficit that is consistent with the corresponding significant reduction in stomatal conductance. It is however cautionary to note that the molecular evaluation in the current study refers to the early response to drought stress, as plants were subjected to a 9-day water deficit treatment.

#### 4.4. Regulation of genes involved in ABA-dependent pathway

We found that expression of ABA-D genes varied with levels of water deficit and between DT and DS cassava genotypes. The number of up-regulated ABA-D genes generally increased with decreasing SMC in both DT and DS cassava but more strongly in DS genotypes and concomitant with their rapid decrease of  $g_s$  compared to the gradual reduction of  $g_s$  in DT cassava (Fig. 6). The reduction of  $g_s$  induced by stomatal closure has been linked to ABA accumulation in cassava leaves in water deficit conditions (Alves and Setter, 2000). The larger number of genes down-regulated after re-watering, especially in DS cassava genotypes, was also correlated with the faster recovery of stomatal conductance in these genotypes. This is consistent with the role of ABA in the signal transduction pathway that connects decreases in relative humidity or moisture to  $g_s$  reduction (Xie et al., 2006).

The contrasting regulation of ABA-D genes between DS and DT cassava at 65% SMC (*NCED3*, *RD29A/B*, *SCaBP5*, *PKS3*, *SLAC1*, *GPA1*, *SNAC1*), 45% SMC (*OST1*, *DSTP*, *ABI1*, *RCW3*), 20% SMC (*PYR1*, *PLDa1*) as well as 80% SMC after re-watering (*ABI1*, *RD20*, *PLDa1*,

*MYB44*, *SCaBP5*, *NFYA5*) (Fig. 6) indicates availability of soil moisture as a key parameter for responses of the genotypes to water deficit. These genes can serve as useful markers for early, moderate and late inducers of stomatal responses to drought stress in cassava or recovery from water deficit conditions. Thus, the genetic analysis of drought stress responses in cassava should involve varying time-courses of drought stress induction, similar to Arabidopsis transcriptome studies of controlled moderate and sub-lethal water deficit conditions (Harb et al., 2010).

The regulation of the six ABA-D (*NCED3*, *SCaBP5*, *PKS3*, *SLAC1*, *GPA* and *SNAC1*) and two ABA-I (*RD29A/B* and *DREB2A/B*) marker genes that were up-regulated in DS and down-regulated in DT cassava genotypes at early stages of WD (at 65% SMC; Figs. 6 and 7), could be associated with the decrease in stomatal conductance ( $g_s$ ) as an early avoidance response to drought stress (Harb et al., 2010). Their roles in stomatal closure and drought tolerance have also been reported in other crops or plants. For example, higher ABA synthesis, rapid stomatal closure and drought tolerance were correlated with increased expression of *NCED3* and *RD29A/B* in petunia, tobacco and cassava (Estrada-Melo et al., 2015; Kasuga et al., 2004; Utsumi et al., 2012), *SCaBP5* and *PKS3* in Arabidopsis (Guo et al., 2002), *SLAC1* in rice and Arabidopsis (Kusumi et al., 2012; Imai et al., 2015), *GPA1* in Arabidopsis (Wang et al., 2001; Li et al., 2009), *SNAC1* in cotton and rice (You et al., 2013; Liu et al., 2014) and *DREB2A/B* and *RD29A/B* in canola (Yang et al., 2010). Genes that are markers for moderate and late reduction of  $g_s$  are correlated with the acclimation of plants to long-term drought (Harb et al., 2010) and are often used as proxies for photosynthesis performance and production in this condition. Of these genes, *OST1* and *DSTP* were up-regulated in DS and down-regulated in DT cassava genotypes while *ABI1*, *RCW3*, *PYR1* and *PLDa1* were up-regulated in DT genotypes and either down-regulated or not significantly regulated in DS genotypes. Thus, these genes are useful markers for drought responses in cassava at lower soil moisture levels.

As critical positive regulators of ABA signal transduction (Belin et al., 2006), *OST1* is involved in limiting water loss in Arabidopsis leaves through regulation of stomatal closure (Mustilli et al., 2002; Yoshida et al., 2006) while *DSTP* regulates drought tolerance in rice via stomatal aperture control (Huang et al., 2009). Since *ABI1* negatively regulates ABA signaling (Gosti et al., 1999; Merlot et al., 2001), its increased expression in DT cassava would be consistent with the sustained stomatal opening and gradual  $g_s$  reduction in WD conditions. The up-regulation of the aquaporin gene *RCW3* in DT cassava could have a similar effect as the over-expression of *RCW3* in rice, which enhanced drought tolerance (Lian et al., 2004). *PYR1* positively regulates ABA-mediated stomatal closure (Klingler et al., 2010; Okamoto et al. 2013) and *PYR1* quadruple (*pyr1pyl1pyl2pyl4*) Arabidopsis mutant plants elicited strong insensitivities in ABA-induced stomatal closure and ABA-inhibition of stomatal opening (Nishimura et al., 2010). *PLDa1* mediates ABA regulation of stomatal movements (Hong et al., 2008). Under drought stress, increased expression of *PLDa1* resulted in rapid stomatal closure and decreased transpirational water loss in tobacco (Hong et al., 2008) and decreased water loss and improved seed production in *Brassica napus* (Lu et al., 2013). As summarized in these literature reviews, the roles of these genes could perhaps be also correlated with the current contrasting expression patterns observed between DT and DS cassava genotypes under WD and further linked to the differential stomatal conductance response.

The differential expression of drought-responsive genes after recovery from water deficit is correlated with differences in  $g_s$  between DT and DS cassava genotypes. For instance, the up-regulation of *RD20*, *PLDa1* and *MYB44* in some DT genotypes could indicate restricted stomatal re-opening and thus slower  $g_s$  recovery in DT genotypes, while their down-regulation in DS genotypes would allow faster stomatal re-opening and  $g_s$  recovery. Arabidopsis *rd20* mutants have higher transpiration rates that are correlated with enhanced stomatal opening and a reduced tolerance to drought stress compared to WT (Aubert et al.,

2010). Transgenic Arabidopsis plants over-expressing AtMYB44 show more rapid ABA-induced stomatal closure, a reduced rate of water loss and enhanced tolerance to drought compared to wild type and *atmyb44* mutant plants (Jung et al., 2008).

The increased expression of *SCaBP5* and *NFYA5* in DS cassava genotypes implies a slower rate of stomatal re-opening or a slower  $g_s$  recovery after re-watering, which would contradict the observed faster  $g_s$  recovery amongst DS genotypes. In Arabidopsis, *NFYA5* is strongly induced by drought stress in an ABA-dependent manner (Li et al., 2008). Similarly, under water deficit conditions (65, 45 and 20% SMC), *NFYA5* was consistently up-regulated in both DT and DS cassava genotypes (Fig. 6). However, up regulation of *NFYA5* upon re-watering in both DS genotypes (Fig. 5a) implied lower  $g_s$  in these genotypes, an observation that does not concur with the higher  $g_s$  the DS genotypes exhibited compared to their DT counterparts (Fig. 4). This contradiction cannot be explained in the current study. Previously, drought insensitive *nfya5* mutant plants showed enhanced water loss compared to transgenic lines over-expressing *NFYA5*, which had reduced water loss and tolerance to drought compared to wild type (Li et al., 2008).

#### 4.5. Regulation of genes involved in ABA-independent pathway

Some transcription factors (TFs) respond to dehydration but not to ABA and are referred to as ABA-independent dehydration-responsive TFs (Yang et al., 2010). The general up-regulation of the five ABA-I TF genes under WD in all cassava genotypes would be consistent with the synthesis of protective proteins and osmolytes such as dehydrins and proline, which is activated by these TFs (Budak et al., 2013; Vaseva et al., 2012; Movahedi et al., 2012). Similar WD-induced up-regulation of ABA-I genes has been reported in other plants, including *ATAF1* (Lu et al., 2007), *ERD10* (Kovacs et al., 2008), *DREB1A/B* or *CBF* (Shinozaki and Yamaguchi-Shinozaki, 2000), *DREB2A/B* (Sakuma et al., 2006) and *RD29A/B* (Jia et al., 2012) (Fig. 5). Over-expression of *DREB1B* enhanced drought tolerance in transgenic potatoes (Movahedi et al., 2012) while rice plants transformed with *DREB1A* were significantly dehydration tolerant (Datta et al., 2012).

Up-regulation of *DREB2A/B* and *RD29A/B* genes in DS cassava genotypes at 65% SMC (Fig. 7) was consistent with the increased stomatal closure or faster rates of  $g_s$  decline in these genotypes, a similar response previously observed in canola (Yang et al., 2010). The down-regulation of *DREB2A/B* and *RD29A/B* in DT cassava genotypes at 65% SMC (Fig. 7) can perhaps explain the reduced  $g_s$  rates and increased stomatal opening in these genotypes. *RD29A/B* genes are commonly used as markers to monitor stress response pathways in plants and have the potential to confer abiotic stress resistance in crop species grown in arid and semi-arid regions (Jia et al., 2012; Seki et al., 2003; Msanne et al., 2011). Similarly, over-expression of *DREB2A* improved drought tolerance in pea (Jovanović et al., 2013) and Arabidopsis (Sakuma et al., 2006). ABA-I genes clearly have a role in cassava WG regulation as well because *ATAF1*, *DREB1A/B*, *DREB2A/B* and *RD29A/B* were rapidly down-regulated after re-watering in the DS cassava genotype 92/0427, consistent with the rapid recovery of  $g_s$  in this genotype (Fig. 7).

In conclusion, we identified a panel of drought-tolerant cassava genotypes that will be useful for breeding to expand cassava production in the arid and semi-arid areas of Africa. The significant correlations we found between yield in IRT and NIRT conditions suggest that initial selection of new varieties for yield could even be performed under normal water conditions because they likely will also perform well in water-scarce environments. Drought-tolerant cassava varieties will be essential for maintaining yield stability, which is vital for sustaining food security especially in arid and semi-arid regions of the developing world where smallholder farmers will be particularly affected by climate change.

## Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## CRediT authorship contribution statement

**Charles Orek:** Conceptualization, Data curation, Formal analysis, Methodology, Writing - original draft, Writing - review & editing. **Wilhelm Gruissem:** Resources, Writing - review & editing. **Morag Ferguson:** Project administration, Supervision, Writing - review & editing. **Hervé Vanderschuren:** Funding acquisition, Resources, Methodology, Project administration, Supervision, Writing - review & editing.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.fcr.2020.107861>.

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