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Survey for the Expression Levels of Drought Tolerant Genes in Cassava Varieties in Tanzania

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ABSTRACT: *Cassava (Manihot esculenta Crantz) is an important root crop to resource-poor farmers in Sub-Sahara Africa, where the production is hampered by drought stress constraints. Given the difficulties associated with cassava breeding, a molecular understanding of drought tolerance in cassava will help in the identification of markers for use in marker-assisted selection and genes for transgenic improvement of drought tolerance. This study was to improve efficiency in breeding for drought tolerance cassava through molecular techniques by determining the expression levels for each drought tolerant genes in eight selected cassava varieties grown in screen-house environment.From the four genes (ALDH7B4, ZFP252, MSD and RD28) that have previously been biologically validated as conferring or being associated with drought tolerance in other plant species; ALDH7B4 gene was confirmed as being exclusively up-regulated in all varieties except in variety IS 30474. Results showed further that the four genes were exclusive up-regulated in Kiroba compared with other cassava varieties. Based on ALDH7B4 gene, it was hypothesized that the basis of the tolerance at the cellular level in those varieties is through mitigation of the osmotic and oxidative adjustment. The ALDH7B4 gene can now be tested in the context of cassava breeding, as possible quantitative trait loci and engineering drought tolerance in transgenic cassava or used for introgression into other improved cassava germplasms for climate change mitigation.*

Keywords: *Cassava, Drought, Gene, Tolerance, Up-regulated.*

I. Introduction

Cassava (*Manihot esculenta* Crantz)serves as major food and cash crop for smallholder farmers and family in Africa. The crop is the 3rd most important source of calories in the tropics after rice and maize (El-Sharkawy, 2006; FAO, 2010). Cassava is a major part of the diet for nearly a billion people in approximately 105 countries mostly in Sub-Saharan Africa, Asia, the pacific and South America (FAO, 2008). Over 50 % of the global cassava production occurs in Africa (FAOSTAT, 2009; Alabi *et al*., 2011). Globally, about 70 % storage roots from cassava are consumed as human food (El-Sharkawy, 2004). The greatest per capita consumption (800g per person) recorded in Sub-Saharan Africa where it is the main energy source for over 40 % of the population (Scott *et al.*, 2000; Nhassico*et al*., 2008). A typical composition of cassava root is 70 % moisture, 24 % starch, 2 % fiber, 1 % protein and 3 % other substances including minerals (Westby, 2002; Tonukari, 2004). Cassava is an important staple crop in more than half of Tanzania and a subsistence crop, especially in the semi-arid areas (Kulembeka, 2010).

There are major challenges facing cassava production in Sub- Saharan Africa; ranging from abiotic to biotic (IITA, 2007; Reynolds and Tuberosa, 2008; Kulembeka, 2010). Abiotic stresses account for more than 50 % of potential yield losses in major crops worldwide (Rosegrant and Cline, 2003; Peters *et al*., 2004). Drought is one of the major factors that have been reported to hamper cassava productivity among smallholder farmers in the face of climate change (MAFSC, 2009). It is currently one of the major abiotic stress, which limits the production of crops and vital food for human survive (Lokko *et al.*, 2007).Future increase and intensity of drought due to climatic changes especially in most agriculturally productive zones around the world is anticipated (Reynolds and Ortiz, 2010; Mir *et al*., 2012). Cassava brown streak and Cassava mosaic disease are some of the biotic constraints as well as the low yielding potential of some of the local varieties (Mkamilo, 2005).

Food insecurity is one of this millennium's serious and most shared problems throughout the world and specifically in developing countries. Many factors, including climate change and particularly drought constitute

the basis of Food insecurity in the world (Passioura, 2007). Increased effect of drought stress incapacitates farmers' ability to cultivate crops and produce high yields (Sheffield and Wood, 2008). It is under these changing climatic conditions that the future food production will need to be doubled to feed the human population expected to plateau at nine billion by 2050 (Cassman*et al.,* 2003; Godfray*et al.,* 2010; Tilman *et al.,* 2011). This poses a serious challenge to farmers, crop breeders and the larger scientific community, especially in most food insecure regions of the world such as Sub-Saharan Africa (Rosenthal *et al.,* 2012). The impact of climatic change is expected to be very high and it double (Lobell *et al.,* 2008; Rosenthal *et al.,* 2012).Cassava is one of the widely produced and consumed crop in Africa (Lokko *et al.,* 2007). In Tanzania according to FAOSTAT, (2009) cassava is the six most essential diets and one of the most important food crops. Cassava is important in sustaining food security and improve livelihood for most of the small-scale farmers. To circumvent a paramount problem such as food insecurity, there is a need of using molecular tools to identify and characterize the genes that confer drought tolerant traits in cassava crops to plan how best these can be used in other drought vulnerable crops and thus alleviate food insecurity in African populations. Given the inherent challenges with cassava breeding, an understanding of the molecular basis of cassava drought responses and tolerance can help greatly in the development of appropriate varieties (Valliyodan and Nguyen, 2006; El-Sharkawy, 2007).

1.1 Constraint with cassava production

There are major challenges facing cassava production in Sub- Saharan Africa; ranging from abiotic to biotic (IITA, 2007; Reynolds and Tuberosa, 2008; Kulembeka, 2010). Abiotic stresses account for more than 50 % of potential yield losses in major crops worldwide (Rosegrant and Cline, 2003; Peters *et al*., 2004). Drought is one of the major factors that have been reported to hamper cassava productivity among smallholder farmers in the face of climate change (MAFSC, 2009). However extreme environmental fluctuations are known to lead to significant yield reductions in the crop (El-Sharkawy, 2007). These factors include unsuitable soil types with low nutrient levels, low or high temperatures and prolonged drought sometimes caused by insufficient rainfall.

Cassava production in East Africa is constrained by both abiotic and biotic factors, which are aggravated by sub-optimal management practices (Bull *et al.,* 2011). Cassava mosaic disease (CMD) and Cassava brown streak disease (CBSD) are major viral diseases constraining cassava production (Winter *et al*., 2010). Tanzania has recorded crop losses of up to 74 % due to CBSD, but in severely affected areas, leading to 100 % damage in susceptible varieties (Legg *et al.,* 2011). Cassava brown streak disease can cause significant reduction in both quality and quantity of cassava in all coastal areas of Tanzania, Kenya and Mozambique and in the lakeshore areas of Malawi (Legg *et al*., 2011). Another major challenge for smallholder farmers in Tanzania, is the lack of facilities and storage which causes the cassava products to spoil (Coulson and Diyamett, 2012). Insignificant investment has been made to research, breed and improve its production when compared to major cereal crops. The crop has received little attention from government policy makers, researchers and research institutes further inhibiting the crop's production potential that should meet its growing demand (FAO, 2008).

1.2 The economic importance of cassava

Cassava is one of the major diets for nearly a billion people in sub-Saharan Africa, Asia, the pacific and Latin America (FAO, 2008). It is reported that over 50 % of the global cassava production occurs in Africa (El-Sharkawy, 2006; FAOSTAT, 2009; Alabi *et al.,* 2011). Cassava production is expected to reach 290.8 million metric tons per year by 2020 (Scott *et al.,* 2000). In 2007, Africa was the world's largest producer with 118 million tons out of a global production of 22 832 million tons (FAOSTAT, 2008). In the developing world, cassava is regarded as a "drought war and famine" crop (Burns *et al.,* 2010). This is because the crop can grow in low fertility soils, is easily propagated, requires little cultivation, and can tolerate sporadic and seasonally extended drought episodes (De Tafur*et al.,* 1997; El-Sharkawy, 2002; Hillocks, 2002). The highly perishable tuberous roots of cassava can be left in the soil and retrieved only when needed for up to 3-4 years after maturity (Ceballos *et al.,* 2004; El-Sharkawy, 2004; Lebot, 2009; Okogbenin*et al.,* 2013). Globally, about 70 % storage roots from cassava are consumed as human food (El-Sharkawy, 2004) particularly in sub-Saharan Africa where it is the main energy source for over 40 % of the population (Scott *et al.,* 2000; Nhassico*et al.,* 2008). Cassava can provide some form of food security during periods of climatic or agricultural instability and social unrest (Burns *et al.,* 2010; Koledoye*et al.,* 2012). More than 90 % of cassava produced in Sub-Saharan Africa is used for fresh consumption and processed foods and the remaining used for animal feed and other industrial uses such as starch (Sanni*et al.,* 2009; Okogbenin*et al.,* 2013). Cassava is used as a raw material for starch production, papermaking, as a lubricant in oil wells and in the textile industry as substrate to producedextrinsand glues production (Cock, 1985).

1.3 Nutritional Values of Cassava

Cassava is the third most essential sources of calories in the tropical regions after rice and maize (El-Sharkawy, 2006; FAO, 2010). Cassava roots typically are composed of 70 % moisture, 24 % starch, 2 % fiber, 1 % protein and 3 % includes mineral substances (Westby, 2002; Tonukari, 2004). The carbohydrate content in root ranges from 80 % to 90 % on a dry matter basis (Montagnac*et al.,* 2009). Cassava roots are commonly

processed into flour or products such as tapioca, fufu, farinha or gari, and can also be eaten fried or as boiled chips (Balagopalan, 2002). Cassava has high content of dietary fibre, magnesium, sodium, riboflavin, nicotinic acid, and citrate (Bradbury and Holloway, 1988). The iron and vitamin A levels are low (Westby, 2002), however some varieties with yellow roots contain significant amount of βeta-carotene (Ferreira *et al.,* 2008; Akinwale*et al.,* 2010; Carvalho *et al.,* 2012). The leaves contain more proteins, minerals, and vitamins than the tubers (Westby, 2002; Montagnac*et al.,* 2009). Cassava leaves are also consumed fresh or cooked (Achidi*et al.,* 2005; Lebot, 2009). The leaves and tubers can be used as animal feed (Balagopalan, 2002).

II. Molecular characterization of response to drought stress in cassavas

The scientific world turns to carry out an evaluation of drought effects on genetic parameters in cassava and value of breeding cassava. Those parameters included yield of storage roots, the mineral content in leaves and dry matter content of cassava tubers. Those genetics parameters within this study may be useful in the future for cassava breeding programs (Ochieng' Orek, 2014). The modern genomic tools are used in identifying the key genetic traits associated with yield-limiting factors such as drought stress in cassava (Ochieng' Orek, 2014). Cassava tissue and genotypes are valuable tools for the development of microarrays, to study genetic diversity, gene discovery and expression profiling. Those molecular instruments can create a clear understanding of molecular issues for drought tolerant crops (Zeng *et al.,* 2006*;* Xu *et al.,* 2008*).* Sequence analysis is one of the molecular evaluations used to determine cassava in response to drought; while twenty thousand full-length cDNA clones that revealed significant levels of lineage with specific expansion of genes were directly related to stress responses. This is a valuable tool that can be used by the breeding community for the improvement of cassava varieties (Cellier *et al.,* 1998*;* Lokko *et al.,* 2007*;* Sakurai *et al.,* 2007*;* Ochieng' Orek, 2014).

A microarray analysis conducted in three cassava genotypes and identified 168 up-regulated genes and 69 down-regulated genes (Utsumi *et al.,* 2012). The understanding of drought tolerant traits in cassava can help with the identification of molecular markers that will be used in transgenic genes improvement (Turyagyenda *et al.,* 2013). The study provided molecular insights into drought tolerance trait in cassava. There was a studied on fifty-three cassava genotypes in Uganda and it indicated that MH96/0686 was tolerant to drought and leaf retention under water stress when compared to other cassava varieties (Turyagyenda *et al.,* 2013). The finding of this experiment showed that expression of Zinc finger protein (ZFP252) gene in rice increased free proline along with soluble sugars the amount by elevating the expression of stress defense genes. Tolerance to salinity was enhanced in the rice and in addition, drought stress genes were enhanced (Xu *et al.,* 2008).

2.1 Drought Responsive genes

Given the inherent challenges with cassava breeding, an understanding of the molecular basis of cassava drought responses and tolerance can help greatly in the development of appropriate varieties (Valliyodan and Nguyen 2006; El-Sharkawy, 2007).Conventional breeding has been hindered by cassava's high heterozygosity, genotype by environment (G × E) interaction, long life cycle (Ceballos *et al.* 2004) and limited seed production, while molecular breeding is hindered by limited information on genomic regions and genes associated with drought tolerance in cassava. Efforts to improve cassava's water use efficiency through conventional breeding have been limited in many parts of the world, including much of Sub Sahara Africa. Breeding programs in Latin America have successfully identified germplasms with increased levels of drought tolerance, with 2–3 times the yield of typical cassava genotypes in semi-arid conditions (El-Sharkawy, 2007). A range of cassava drought-tolerance levels has also been characterized in West Africa (Okogbenin*et al.,* 2003). Efforts are now under way in eastern Africa to begin breeding for drought tolerant cassava. A range of cassava drought-tolerance levels has also been characterized in West Africa (Okogbenin*et al.,* 2003). Efforts are now under way in eastern Africa to begin breeding for drought tolerant cassava.

Zinc finger protein (*ZFP252)* maintains cell membrane integrity and promotes proline synthesis *(*Sanchez et al., 1998; Xu *et al.,* 2008*).* One or more "Zinc Finger" is possessed by the zinc finger protein gene, which bonds most of Zinc Ions by its residues, Histidine (His) and Cysteine (Cys). In abiotic and biotic stresses in rice plant, zinc finger protein respond significantly by regulating the plant molecular mechanism to those conditions (Li *et al.,* 2013). A cys-2/his-2-type (C2H2) zinc finger protein is a transcription factor that regulates gene expression by binding DNA in promoter regions of genes (Hardy, 2010). The C2H2 transcription factor has been associated with drought stress response in many plant species such as *Cicer arietinum*, *Petunia hybrida*, *Oryza sativa*,and *Glycine max* (Huang *et al.,* 2009). Overexpression of *ZFP245* enhanced activity of reactive oxygen species scavenging enzymes and elevated free proline levels in rice, thus increasing drought tolerance (Huang *et al*., 2009).

Aldehyde Dehydrogenase 7B4 (*ALDH7B4*) is a gene involved in the function as an antioxidant/reactive oxygen species (ROS)/scavenging by reducing the levels of lipid peroxidation (Kotchoni*et al.,* 2006; Ochieng' Orek, 2014*).* Reactive Oxygen species inhibit photosynthesis and cause cellular damage to plant during salt or drought stresses (Missihoun, 2010). A model plant *Arabidopsis thaliana* was used and Aldehyde dehydrogenase

gene was considered as 'aldehyde scavengers'; its main function is to eliminate toxic aldehydes in plant by causing oxidative stresses (Hou and Bartels, 2014).

The responsiveness to Desiccation (*RD28*) gene is known to have early response to dehydration in plants. It had been known for its early involvement in transporting essential molecules across the leaves and stem in plants during osmotic stress (Silva *et al.,* 2012*)*. Early response to dehydration and drought stress in the plant is enhanced by the responsiveness to Desiccation gene (Daniels *et al.*, 1994; Obidiegwu *et al.,* 2015). Abscisic acid also activates transcription factors that involved in expression of downstream-stress responsive genes such as Responsive to Dehydration (RD) and early Responsive to Dehydration (Pardo, 2010). The promoter region of *RD29A/COR78/LTI78* contains both an abscisic responsive elements and dehydration responsive element binding proteins/C-repeat binding factor, which functions in Abscisic acid-dependent and Abscisic acid-22 independent gene expression respectively in response to drought stress (Seki *et al*., 2003).

Manganese Superoxide Dismutase (*MSD*) involves with playing a role in oxidative stress tolerance in plants. During drought stress plant can be detoxified by *MSD* (Alscher *et al.*, 2002). When plants are affected by drought stress condition, Manganese Superoxide Dismutase provides an inflammation defense subsequently causing cellular homeostasis (Li and Zhou, 2011). The *MSD* gene scavenges for reactive oxygen species (Fryer *et al*., 2002), *ZFP252* maintains cell membrane integrity and promotes proline synthesis (Xu *et al*., 2008). The *ALDH7B4* reduces the levels of lipid peroxidation (Kotchoni*et al*., 2006) and RD28, a turgor-responsive, plasma membrane aquaporin found in plasma membranes of plant tissues and enhances drought tolerance through abscisic acid-independent pathway (Kotchoni*et al*., 2006).

III. Materials and methods

Eight cassava varieties (Table 1) were collected from Kibaha, Dodoma and Mtwara during September to October 2015. Cassava cuttings for each were packaged in a paper envelope for the purpose of safely transporting them at Mikocheni Agriculture Research Institute (MARI); and they were washed with distilled water to disinfect them. The cassava cuttings were planted in pots filled with soil (4 kg) each in the screen house at (MARI). These known improved cassava varieties were selected for this study.

Table 1: List of Cassava varieties included in this study.

Cassava varieties	Pedigree	Collection sites
Kizimbani	Kirobahalfsib	Mtwara
KBH 2006/374	Kibahahalfsib	Kibaha
KBH 2002/135	Kibahahalfsib	Kibaha
UKG 92/053	Kibahahalfsib	Dodoma
IS 30474	Kibahahalfsib	Dodoma
Kiroba	Amanihybrid	Kibaha
KBH 95/517	Kibahahalfsib	Kibaha
KBH 97/212	Kibahahalfsib	Kibaha

Table 2: Sequence of primers used for RT-qPCR reaction.

Gene symbol	Primers ID	Primer sequence (5′–3 ′)	Length (bases)	Amplicons size (bases)
ZFP252	ZFP1F	CTCTATTCTCAGCGCACATTCC	22	245
	ZFP1R	AGCATAACGAGGCAGAGAGC	20	
MSD	MSD1F	ATGAATGCAGAAGGTGCTGCA	21	269
	MSD1R	GAAGGGCATTCT TTGGCATAC	21	
RD28	RD282F	TGCACTGCTGGTATC TCAGG	20	237
	RD282R	GATCTCAGCTCCCAATCCAG	20	
ALDH7B4	ALDH1F	GGATGGAATGCATGCATTGCACTG	24	263
	ALDH1R	CTGATTCACTGTTTGTTGCACCATC	25	

Source: Turyagyenda*et al.* (2013).

Table 3: Reference gene (*βeta –Actin*) sequences used for RT-qPCR

Gene symbol	Primers ID	Primer sequence (5′–3 ′)	Length (bases)
BETA-ACTIN	ACTIN1F	TGCAGACCGTATGAGCAAG	19
	ACTIN1R	CACCCTTGGAAATCCACATC	20

Source: (Guo *et al.,* 2009; Yang *et al*., 2011).

3.1 Experimental design and treatment

The experiment was set up in a randomized complete block design (RCBD) with three biological replicates. Three cassava cuttings (20 cm in length) for each variety were planted vertically in 4 kg of sterilized soil, in 5 liters plastic buckets. Treatment levels consisted of control (well-watered) and water deficits under Screen house environment at the temperatures ranging from 40 – 45 $^{\circ}$C during day, with humidity typically at 50 to 65 %. Both the control and treatment samples for all plants were watered with 500 ml of water every 2 days until 60 days after planting. After 60 days, plants in the stress treatment were gradually subjected to drought stress condition for additional 30 days. During the 30 days of stress, control samples received (500 ml) of water after every two days and water stress treatment samples received 250 ml of water after every four days. Irrigation was stopped 90 days after planting (DAP).

3.2 Sampling and Laboratory Analysis

Three leaves of each cassava varieties (upper, middle, and lower leaves) were sampled from both the control and treatment from the three biological replicates in the screen house after stressing them. Samples of cassava leaves were harvested one variety at a time, compressed into an envelope, well labeled.

Ribonucleic acid (RNA) was extracted from 0.15-2.0 gram of fresh cassava leaf (that had been frozen at -80 °C) samples using a modified Chang *et al.* (1993) Cetyltrimethyl ammonium bromide (CTAB)-based protocol. A Modification was made to the Chang *et al*. (1993) method to reduce the time and cost of extraction without reducing quality and yield of the RNA extracted from leaves of cassava plants. In the modified protocol, all centrifugation steps were carried out at 4 °C. The CTAB extraction buffer was prepared using 2 % CTAB, 100 mM Tris hydrochloric acid, 20 mM Ethylene diaminetetracetic acid (EDTA), 1.4 M Sodium Chloride (NaCl), 5 % βeta-mercapto ethanol and 2 % Polyvinyl Pyrrolidone (PVP). A fresh leaf of cassava sample, weighing 0.15 – 0.2 gram was grinding with 700 µl of extraction buffer. Other steps did not change except that RNA precipitation was carried out using 2 volumes of absolute ethanol instead of Lithium Chloride (LiCl) precipitation (Appendix 1). It was incubated at -20 °C, overnight to completely precipitate nucleic acid from the leaves of the cassava.

The RNA quantity and quality of each of the samples was measured by Cecil CE3021 spectrophotometer (Cecil Instruments, Cambridge, UK) at the absorbance wavelengths ratios of 260 nm and 280 nm (A_{260}/A_{280}). A ratio ranging between 1.88 and 2.2 was considered an acceptable quality; and furthermore, investigation on the quality of RNA was conducted by an agarose gel electrophoresis.

Master mix was prepared for cDNA synthesis using deoxynucleoside triphosphates (dNTPs), DEPC treated water and oligodT (18) to anneal to poly-A tails of the massager ribonucleic acid (mRNA) and reverse transcriptase to convert the mRNA to cDNA by reverse transcription, following manufacturer's instructions (Bioneer Corporation, Daejeon, South Korea). Two control reactions were added for each sample throughout the processed. Each of the control reactions had no RNA templates. The control reactions were to assess the quality of reagents, and the absent of contaminations.The synthesized cDNAs quantity and quality for each sample were determined using a Cecil CE3021 spectrophotometer (Cecil Instruments, Cambridge, UK). High quality cDNA was obtained at the absorbance wavelength ratio of A_{260}/A_{280}. The resulting cDNAs were standardized by diluting to a final working concentration of 50 ng/µl.

3.3 Genes expression analysis

The quantitative RT-PCR for the four genes (*ZFP252*, *ALDH7B4*, *MSD* and *RD28)* was performed on a standard real time PCR System (Agilent Technologies Stratagene with Mx3000P Software version 4.10) using SYRB Green JumpStart TaqReadyMix (Sigma, USA). These reactions were run on three biological replicates for each variety with the primers in Table 2. For each of the biological replicate, there were duplicate reactions run with the total volume of 20 µl per each reaction. Each reaction consisted of 2 µl of cDNA, 1 µl (10 pmol) each of the forward (F) and reverse (R) gene specific primers, 10 µl of 2 x SYBR Green I ready mix, 0.02 µl of passive reference dye and 5.98 µl of deionized water. There were two reactions of negative control containing only reagents with no cDNA templates. Thermal Profile conditions; initial denaturation at 94 $^{\circ}$C for 2 minutes for cycle 1 and denaturation at 94 $^{\circ}$C for 30 minutes, annealing at 55 $^{\circ}$C for 1 minutes and extension at 72 $^{\circ}$C for 30 seconds for 40 cycles. The dissociation curve was carried out on default setting to confirm the specificity of each reaction. The amplification efficiencies of the targets (genes of interest) and the endogenous control (reference gene) were determined by performing RT-qPCR on 50, 25, 10, 1 and 0.5 ng of cDNA dilutions for all experimental samples. In addition, the coefficient of determination (R^2) and standard deviations for three biological replicates were determined to calibrate pipetting accuracy and the reproducibility, respectively.

The relative gene expression for the four genes (*ZFP252*, *ALDH7B4*, *MSD* and *RD28)*, were obtained by comparing each of the gene with βeta-*Actin gene* (house-keeping gene). The ΔΔ Ct method for relative gene quantification was used to make the various comparisons from the RT-qPCR threshold (Ct) data, specially using the Relative Expression Software Tool (REST) version 2009, computed based on the analytical model by Pfaffl*et al.* (2002). The reactions for the RT-qPCR were normalized using the cassava βeta-*Actin gene* with the primers listed in Table 3. The βeta-*Actin gene* was used as reference gene for all comparison in the selected

varieties (Guo *et al.*, 2009; Yang *et al*., 2011). Expression data from control plants were used as standard calibrator or baseline for comparisons with the treatments for each variety; and Student paired sample *t-test* was applied to determine whether an up-or down regulation of a gene was significant (P<0.05). The expression in the control plant was taken as unity (one). A gene is significantly up-regulated or down-regulated when its expression in a treatment is higher than or lower than that of the baseline respectively, and when the *t-test statistics* is lower than 0.05 % (at 95 % significance level). Expression of more than one is up-regulation and expression less than one is down-regulation. The *t-test statistics* showed whether the up-regulation or down-regulation is significant or non-significant (NS).

IV. Results

All the genes of interest *(ZFP252, ALDH7B4, MSD* and *RD28*genes*)* and the reference gene (*βeta-Actin*) amplified with threshold cycles (Ct) ranging from 25 to 29. The amplification efficiencies of the reference and target genes ranged between 98.24 to 101.39 %, which is considered acceptable. The linearity for coefficient of determination (R^2) indicated the accuracy of pipetting and the threshold cycles (Ct). Standard deviations of all biological replicates were less than 0.105, which validated an acceptable reproducibility. Negative control reactions (no cDNA templates) assessed the quality of reagents, primers dimers and absent of contaminations. No amplification was detected in the negative controls.

The relative gene expression for each of the four genes (*ZFP252, ALDH7B4, MSD* and *RD28* gene) compared with *beta-actin gene* between the controls (well-watered plants) and the treatment (stressed plants) within each of the eight varieties by using the ΔΔ Ct method for quantification by Pfaffl*et al.* (2002). Results showed efficiencies of targets and reference genes were approximately equal. Results revealed that all of the four genes were significantly (P<0.05) up-regulated in Kiroba as shown in Table 4. The study further revealed that *ALDH7B4* gene was significantly (P<0.05) up-regulated in all varieties except in IS30474. The results also showed that KBH 94/774 and KBH 97/212 had the highest number of down-regulated genes in expressions. Whereas IS30747 had the highest number of non-significant gene expression as shown in Table 4: and Figure. 1.

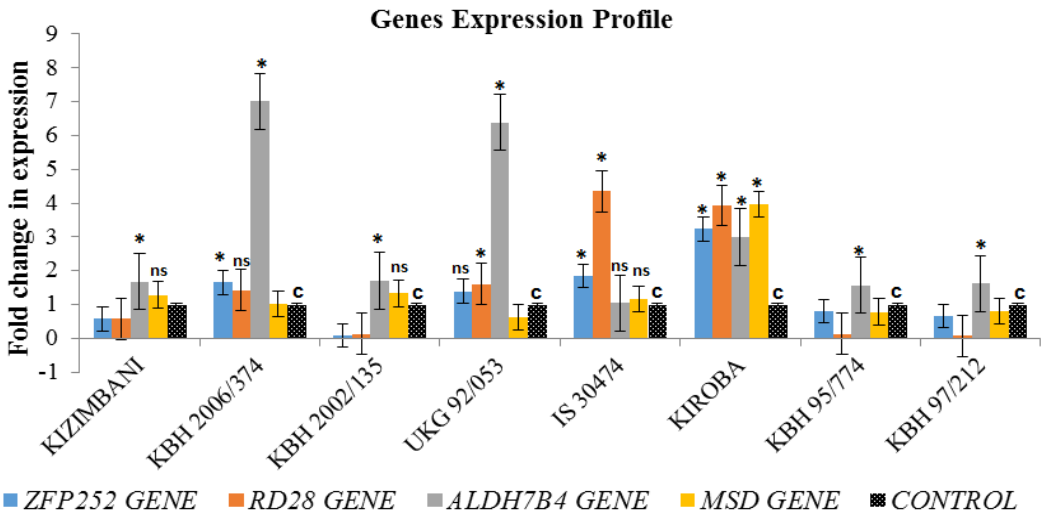

Fig: The fold change in expression for *ZFP2525, RD28, ALDH7B4* and *MSD* genes in eight varieties relative to the reference gene (*βeta-Actin)*. The fold change in expression for *ZFP2525, RD28, ALDH7B4* and *MSD* genes in eight cassava varieties relative to the reference *βeta-Actin* gene. Paired Sample *t-tests* significance: * = P<0.05, as up-regulated. The control (C) is the standard baseline/calibrator at unity (one), while ns = non-significant.

Table 4: Fold change in expression of genes in treatment (water stress) against control (Well

Varieties	Gene	Expression	SE	95 % CI	Probability (P = 0.05)	Results
Kizimbani	*ZFP252*	0.571	0.171 – 0.646	0.019 - 0.661	0.001	Down-regulated
	RD28	0.571	0.183 – 0.711	0.320 – 0.577	0.009	Down-regulated
	ALDH7B4	1.670	1.618 – 3.031	1.579 – 8.281	0.001	Up-regulated
	MSD	1.283	0.431 – 1.682	0.321 – 2.446	0.084	NS
KBH 2006/374	*ZFP252*	1.652	1.781 – 2.561	1.620 – 5.973	0.003	Up-regulated
	RD28	1.421	0.718 – 2.196	0.133 – 4.407	0.055	NS
	ALDH7B4	7.012	2.869 – 5.828	2.408 – 11.979	0.000	Up-regulated
	MSD	1.023	0.718 – 2.196	0.455 – 3.115	0.212	NS
KBH 2002/135	*ZFP252*	0.091	0.097 – 0.681	0.045 – 0.869	0.004	Down-regulated
	RD28	0.132	0.121 – 0.510	0.069 – 0.838	0.000	Down-regulated
	ALDH7B4	1.694	1.249 – 3.456	1.169 – 9.838	0.000	Up-regulated
	MSD	1.323	0.541 – 2.141	0.447 – 3.899	0.093	NS
UKG 92/053	*ZFP252*	1.392	0.214 – 0.998	0.149 – 1.024	0.099	NS
	RD28	1.601	1.341 – 5.182	1.226 – 9.174	0.004	Up-regulated
	ALDH7B4	6.383	1.601 – 4.127	1.409 – 8.641	0.003	Up-regulated
	MSD	0.612	0.731 – 0.873	0.018 – 0.689	0.000	Down-regulated
IS 30474	*ZFP252*	1.851	1.912 – 3.191	1.802 – 7.087	0.011	Up-regulated
	RD28	4.355	1.994 – 4.144	1.937 – 11.309	0.000	Up-regulated
	ALDH7B4	1.046	0.651 – 3.416	0.553 – 3.926	0.390	NS
	MSD	1.171	0.245 – 1.891	0.146 – 2.267	0.064	NS
Kiroba	*ZFP252*	3.233	1.301 – 4.564	1.230 – 10.976	0.003	Up-regulated
	RD28	3.927	1.716 – 4.011	1.681 – 9.496	0.002	Up-regulated
	ALDH7B4	2.991	1.513 – 3.511	1.491 – 8.789	0.001	Up-regulated
	MSD	3.972	1.991 – 4.881	1.955 – 11.058	0.000	Up-regulated
KBH 95/774	*ZFP252*	0.794	0.711 – 0.881	0.083 – 0.951	0.012	Down-regulated
	RD28	0.126	0.412 – 0.813	0.099 – 0.954	0.001	Down-regulated
	ALDH7B4	1.563	1.341 – 3.491	1.297 – 9.813	0.009	Up-regulated
	MSD	0.779	0.114 – 0.712	0.046 – 0.921	0.006	Down-regulated
KBH 97/212	*ZFP252*	0.658	0.315 – 0.331	0.085 – 0.412	0.006	Down-regulated
	RD28	0.067	0.182 – 0.561	0.097 – 0.643	0.000	Down-regulated
	ALDH7B4	1.615	1.456 – 4.516	1.373 – 10.887	0.017	Up-regulated
	MSD	0.806	0.841 – 0.612	0.422 – 0.717	0.043	Down-regulated

water) varieties

CI = Confidence interval at 95 %; Expression = fold change in the expression of a gene in water stress relative to control and treatment andNS = non-significant.

V. Discussion

This study was conducted in a screen house environment at Mikocheni Agriculture Research Institute, Dar es Salaam. The aim of this study was to improve efficiency in breeding for drought tolerance cassava through molecular techniques; by further determining the fold change in expression for each drought tolerant gene in eight selected improved cassava varieties (Kizimbani, KBH 2006/374, KBH 2002/135, UKG 92/053, IS 30474, Kiroba, KBH 95/517, and KBH 97/212) in Tanzania. This is the first study of its kind, to our knowledge, conducted on the eight varieties of cassava, which additional aimed at investigating molecular characteristics of gene expression levels. This study revealed and confirmed the presence of the four genes (*ZFP252*, *ALDH7B4*, *MSD* and *RD28*). They have been associated with drought adaptation, or the tolerance in improved cassava genotype (MH96/0686) from Uganda (Turyagyenda *et al.*, 2013).

Aldehyde dehydrogenase is encoded from the gene *ALDH7B4*, which was up-regulated specifically in MH96/0686 by 2.815-fold. It plausibly this gene may hence be involved in the enhancement process of drought tolerance in cassava under drought stress (Turyagyenda *et al.*,2013). In the findings of this study, the gene was up-regulated with fold change of 1.670-fold in Kizimbani, 7.012-fold in KBH 2006/374, 6.383-fold in UKG 92/053, 2.991-fold in Kiroba, 1.563-fold in KBH 95/774 and 1.615-fold in KBH 97/212 (Table 8). This gene was over-expressed in KBH 2006/374 and UKG 92/053. The findings of this study are in strong agreement with the studies by Kotchoni*et al.* (2006), who observed that transgenic *Arabidopsis thaliana* plants with increased amounts of *ALDH7B4* were more tolerant to dehydration and salt stress than wild-type plants. They reported further that over-expression of the *ALDH7B4* gene in transgenic plants under drought and salt stress reduced the level of lipid peroxidation, signifying that the gene confers both oxidative and osmotic stress tolerance in *Arabidopsis thaliana* through reactive oxygen species (ROS) scavenging and reducing lipid peroxidation. Additional result revealed that the gene can be induced by pathogens and might therefore be a multi-stress-responsive gene (Zimmermann *et al.*, 2004). The fold change by 2.815 over-expression of this gene in drought tolerant cassava indicated the gene may take part in the enhancement of drought tolerance in cassava, probably by reducing lipid peroxidation through ROS scavenging (Kotchoni*et al.*, 2006).

The gene *ZFP252* that translates a zinc finger protein has been conveyed during water stress to confer drought tolerance in plants by maintaining cell membrane integrity. It was revealed that the relative electrolyte leakage, an indicator of membrane injury, was lower under drought stress in *Oryza sativa ZFP252*-transformed rice plants than in non-transformed *Oryza sativa ZFP252* knock-out plants (Morsy*et al.*, 2005; Xu *et al.*, 2008). The findings suggest that *ZFP252* protects plants from stress by retaining cell membrane integrity. The higher soluble sugars and free proline contents were in transformed *Oryza sativa* plants than non-transgenic *Oryza sativa* plants (Xu *et al.*, 2008). Results suggest that enhanced stress tolerance under salt and drought stresses of *ZFP252*-transgenic plants might partially be through activation of proline synthesis and proline transference pathways by *Oryza sativa ZFP252*. Drought tolerance through osmotic adjustment was due to higher proline levels (Sanchez *et al.*, 1998; Xu *et al.* 2008). In this study, *ZFP252* gene was also exclusively up-regulated in KBH 2006/374 by 1.652-fold, 1.851-fold in IS 30474 and 3.233-fold in Kiroba. It is therefore very suggestive that this gene is among few that enhance drought tolerance in cassava and specifically in KBH 2006/374, IS 30474 and Kiroba, through increasing the free osmo-protectant proline and soluble sugars as observed in earlier studies (Sanchez *et al.*, 1998).

The gene *MSD* translates into manganese superoxide dismutase (MnSOD) enzyme that plays a role in oxidative stress tolerance in plants. Over-expression of superoxide dismutase (SOD) in transgenic plants increases oxidative stress tolerance (Basu*et al*., 2001; Wang *et al.*, 2005). In this study, findings agreed with the relative expression in Kiroba by 3.972-fold. It shows a level that can believably confer increased oxidative stress and drought tolerance in cassava. Studies by Sen Gupta *et al.* (1993) showed that a 3-fold increase in total pea copper or manganese superoxide dismutase activity in transgenic tobacco resulted in an increase significantly in resistance to membrane impairment. A 1.5 to 2.5-fold increase in total (SOD) enzymes activity was reported by Basu*et al.* (2001) in transgenic *Brassica napus* plants transformed with wheat MnSOD increased oxidative stress resistance as compared with wild-type controls. Wang *et al.* (2005) reported that a 1.4-fold increase in total Superoxide dismutase enzymes activity in the MnSOD transgenic rice plants was enough to increase oxidative stress resistance and drought tolerance when the gene was fused with a chloroplast transit peptide sequence to target the manganese superoxide dismutase to the chloroplast. The 3.148-fold increase of expression was observed in drought tolerance genotype MH96/0686 (Turyagyenda *et al.*,2013). Superoxide dismutase enzymes are involved in scavenging

reactive oxygen species that are produced in plants during water stress (McKersie*et al.*, 1996; Fryer *et al.*, 2002). It is therefore hypothesized that this gene confers drought tolerance through ROS scavenging in cassava.

The *RD28* gene encodes the responsiveness to Desiccation. The expression of t *RD28* gene was increased1.511-fold by water stress, being exclusively up-regulated in the drought tolerant genotype, suggesting that it plays a role in enhancement of drought tolerance in cassava. In this study, findings in expression for *RD28* gene were 4.355-fold in Kiroba, 3.927-fold in KBH 95/774 and 1.601 in UKG 92/053. Daniels *et al.* (1994) finding showed that *RD28* gene is a turgor-responsive, mercury-resistant plasma membrane aquaporin found in plasma membranes of all plant tissues except seeds. Earlier studies by Yamaguchi-Shinozaki *et al.* (1992) revealed that *RD28* gene enhances drought tolerance through an abscisic acid-independent pathway. It transports small molecules across cell membranes by protecting desiccated cells; and finding in this study believed that it enhances the cells' desiccation tolerance in drought tolerant cassava through osmotic adjustment.

The Transcription factors (TFs) interact with cis-elements in the promoter region of several stresses related genes and thus up or down-regulating the expression of many downstream genes resulting into impacting abiotic stress tolerance (Agarwal and Jha 2010). In *Arabidopsis thaliana* genome about 1500 TFs are described which are involved in expression of stress responsive genes (Riechmann*et al.*, 2000). The dehydration responsive element binding proteins/C-repeat binding factor (DREBs/CBF) are important transcription factors that induce a set of abiotic stress-related genes, thus impacting stress tolerance to plants. They play an important role in Abscisic acid (ABA)-independent pathway that activates stress response genes (Stockinger *et al.*, 1997; Liu *et al.*, 1998; Udvardi*et al.*, 2007). These proteins specifically bind to and activate the expression of genes in the promoter of the drought response gene *RD28* (Yamaguchi-Shinozaki and Shinozaki, 1993). It is probably possible that transcription factor DREBs/CBF might not bind to the promoters of gene *RD28* during the abscisic acid-independent pathway mechanism thus resulted to down-regulation of the gene in three varieties (Kizimbani, KBH 2002/135, and KBH 95/774).

VI. Conclusion

Successful amplification of *ZFP252, ALDH7B4, MSD* and *RD28* fragments by primers in this study suggests the present of these genes in the selected cassava varieties.The present study revealed at the molecular level an exclusively up-regulation of *ALDH7B4* gene with statistical significance in all varieties except IS 30474. Based on the gene known function in other species, it is likely that the tolerance to drought stress at the cellular level in these varieties consist of reduction of osmotic adjustment and oxidative stress through reactive oxygen species (ROS) scavenging and reduction of lipid peroxidation; and *ALDH7B4* gene influenced these varieties tolerance to drought. In addition, findings showed Kiroba as the only variety with all four genes exclusively up-regulated. Physiologically, the eight varieties exhibited indications of tolerance to drought stress during the entire experiment. The *ALDH7B4* gene can now be tested in the context of cassava breeding, as possible quantitative trait loci and engineering for drought tolerance in transgenic. Kiroba can also be considered for test in cassava breeding programs to engineering drought tolerance trait due to the up-regulation of the four genes. The significance of this research finding is to generate new molecular data to bridge the information gap and provide more tools for breeders to use for introgression into other improved cassava germplasms for climate change mitigation. In addition, poverty alleviation and sustainable food security are benefiting factors from this study.

Based on the findings of the present study, it is recommended that other studies should be conducted.
To further understand the mediating signaling pathways in response to abiotic stresses, it will be essential to identify and characterize the downstream and upstream molecules of *ZFP252, ALDH7B4, MSD* and *RD28* by microarray, yeast hybrid system and so on. To research on Kiroba concerning its mineral contents in leaves and tuber qualities; this analysis is warranted to determine whether the up-regulation of these four genes (*ZFP252, ALDH7B4, MSD* and *RD28)* influence or affect any nutritional qualities during abiotic stresses. To further design field-based trials, re-evaluating experiments and compare findings with screen-house environment conditions; and provide information back to plant breeders and farmers in the field as the best realistic research design to generate sustainable results.

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