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GENE EXPRESSION VALIDATION FOR STORAGE ROOTS DEVELOPMENT IN CASSAVA (*Manihot esculenta*)

MASTER OF SCIENCE IN PLANT BREEDING

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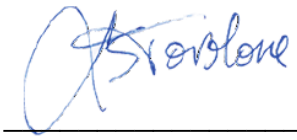
Livia Stavolone		October, 2018
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ABSTRACT

Cassava storage roots are used for food security and are increasingly important as an industrial commodity in developing economies. However, the biology of root formation in cassava is poorly understood. The main aim of this research is identification of specific genes that are differentially expressed during root bulking in cassava at different time points and increasing the knowledge of storage roots bulking to facilitate the early time point selection for root bulking trait.

To elucidate the mechanisms involved with storage root bulking in cassava, a transcriptomic study of cassava storage roots and fibrous roots formation was undertaken along with phenotypic information for the genotype TMEB 419 and indicated differential gene expression for storage roots and fibrous roots. Apart from TMEB 419 multiple genotypes has been used for confirmation study.

Therefore, in this thesis and for the first time, the expression levels of some genes selected from transcriptomic data were studied by using quantitative real time PCR (RT-qPCR) to confirm their involvement in root bulking development in the genotype TMEB419 at several time points. Furthermore, these same genes were tested in three different genotypes (1090576, TMEB 14 and Kaleso) to verify if their expression levels were similar to the observed levels in the genotype TMEB 419.

RT-qPCR analysis indicated interesting results from multiple genotypes to TMEB419 RNA Seq results. In particular, the genotype 1090576 showed a positive correlation with the reference genotype TMEB419 and most of the genes behave similarly. Confirmation with TMEB 419 has revealed many genes expression pattern with the RNA Seq information except at first time point that has disagreeing results.

To finally prove the hypothesis that SR are forming independently from FR, the expression level of the identified key Differentially Expressed Genes (DEGs) only in SR, should be confirmed in each root emerging from planting stems in the first month after planting

Keywords: Gene expression, Storage root formation, Real time PCR, yield and RNA Seq.

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DEDICATION

This study is wholeheartedly dedicated to my beloved parents my wife and my children (Glory and Grayson), who have been my source of inspiration and gave us strength when we thought of giving up, who continually provide their moral, spiritual, emotional, support.

I also dedicate this thesis to my brothers, sisters, relatives, mentors, friends, and classmates who shared their words of advice and encouragement to finish this study.

STATEMENT OF THE AUTHOR

By my signature below, I declare that this thesis is my work. I have followed all ethical principles of scholarship in the preparation, data collection, data analysis, and completion of this thesis. I have given all scholarly matter recognition through accurate citations and references. I affirm that I have cited and referenced all sources used in this document. I have made every effort to avoid plagiarism.

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BIOGRAPHICAL SKECTH

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1.0 INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a perennial shrub, commonly known as tapioca, mandioca, manioc and yuca in different parts of the world. It belongs to the family Euphorbiaceous, sub family Crotonoidea and tribe Manihotae. It is belonging to the genus *Manihot* that is reported to have about 100 species of which *Manihot esculenta* is the only commercially cultivated species (Alves, 2002; Jennings and Iglesias, 2002). It is mainly cultivated for its starchy roots. Cassava provides more than half of the calories consumed by more than 800 million people in Sub- Saharan Africa (SSA), Latin America and Asia. Cassava has moved from being a subsistence crop to a fully commercial crop due to its income generating capacity and enormous potential for industry, animal feed and human consumption.

Cassava is native to North - Eastern Brazil, although its wild relatives can be found in Northern South America, Central America and Mexico. Molecular evidences by using Single Nucleotide Polymorphisms (SNPs) and Simple Sequence Repeat (SSR) markers showed that cassava was domesticated in the Southern Amazon region, in Brazil about 9000 years ago and was likely domesticated from wild species of the *Manihot* genus i.e. *Manihot esculenta* and *M. flabellifolia* (Olsen and Schaal, 2001; Olsen, 2004).

Major cassava producing countries in the world are Nigeria, Thailand, Indonesia and Brazil. It is mainly cultivated for local consumption and predominantly grown by marginal, low-input farmers as an emergency crop. It is a rural crop tolerant to drought and acidic soils with good performance on degraded soils where other crops fail. Generally, it can be produced on a range of edaphic and climatic conditions (Kawano *et al.*, 1998).

High yield potential of cassava makes it a suitable option over other grain staples where population pressure and crop failure are major challenges (Al-Hassan, 1993; Nweke, 1996). Cassava is one of the most important staple foods in the human diet in the tropics and ranked fourth after rice, sugar cane and maize as the most important source of calories in the human diet. A typical composition of cassava root is 70% moisture, 24% starch, 2% fiber, 1% protein and 3% other substances including minerals (Alfredo *et al.*, 2000; Westby, 2002 ; Tonukari, 2004).

Cassava storage roots have high starch content and can form the major source of various intermediate products including flour, starch and dextrin for food, feed, confectionery, pharmaceutical, adhesives, explosives and other industrial uses. Apart from its importance as animal feed and industrial raw material, cassava has emerged as an important biofuel resource (Egesi *et al*., 2007). In addition, the cassava storage roots can be left in the ground for several years before harvesting, this provide security against famine

World population is estimated to increase from 7.3 billion to 8.5 billion by 2030 (United Nations, 2015) but the agricultural productivity is increasing in an inadequate rate. The major challenge is climate change, particularly increasingly high temperatures and low amount of rainfall.

Due to drought tolerance of cassava and its high caloric content, more effort on research have been put in this crop in Sub-Sahara Africa. As C3 plant, cassava has an unusually high rate of photosynthetic carbon assimilation (43μ mol $CO_2/m^2/s$) as well as a high temperature optimum (45°C) for photosynthesis (Hunt *et al*., 1977; Edwards *et al*., 1990. In addition, cassava has been reported to have one of the highest rates of CO_2 assimilations into sucrose of any plant measured (Hunt *et al*., 1977; 1993). This massive accumulation of starch into cassava roots and the physiological adaptation, motivated researchers to investigate on biosynthetic pathways and enzymatic proteins functions and how they are regulated. For these reasons, it could be hypothesized that cassava was an excellent candidate for enhancing carbohydrate allocation to sink tissues.

Unlike many of the world's major crop plants, cassava is not principally acquiescent to genetic improvement through normal sexual crosses. Many varieties flower rarely and seed production is habitually low. In the field, cassava is typically propagated clonally by stem cuttings. This propagation strategy is ideal for molecular approaches to crop improvement as gene segregation through outcrossing is limited. In 1996, the first stable genetic transformation of cassava was reported using Agrobacterium and micro particle-mediated delivery of DNA to plants. Additional reports of the genetic transformation of cassava have followed (Sarria *et al*., 1993; Zhang *et al*., 2000 a, b); however, only recently have transgenic plants been generated with enhanced agronomic traits.

Cassava has one of the highest rates of CO_2 fixation and sucrose synthesis for any C_3 plant, but rarely reaches its yield potentials in the field. Therefore, there exists a strong hypothesis

that starch production in cassava storage roots can be increased substantially by increasing the sink strength for carbohydrate (Uzoma *et al*., 2006).

The efforts to improve tuberous root yields made by breeding programs throughout the world have prioritized drought tolerance, cyanogenic content, low soil fertility conditions and resistance to a wide range of diseases (El-Sharkawy, 2004). Concurrently, efforts have also been put into improving cassava nutritional quality (Montagnac *et al*., 2009; Gonzalez., 2011; Sayre *et al*., 2011; Ceballos *et al*., 2013) and agronomic practices

On the other hand, Development of storage root organs in cassava is poorly understood. It consists in the production of large amounts of secondary xylem parenchyma in which starch is synthesized and stored. Storage roots formation is hypothesized to occur when a subset of fibrous roots receives unknown signals to undergo secondary thickening. However, whether cassava storage roots develop as such or derive from fibrous roots modification has not been proved and the molecular bases of cassava root bulking are not yet known.

Previous studies, conducted on cassava plants grown in pots, have described gene expression profile for starch biosynthesis but did not contribute yet the involvement of specific key genes. A recent transcriptome analysis of roots and leaves of cassava genotype TMEB419 cultivated in the IITA-Ibadan fields in normal growth conditions has highlighted the existence of genes specifically (up- or down-) regulated in storage roots during root development. Furthermore, detailed morphological monitoring and functional RNA Seq analysis showed that fibrous and storage roots behave differently from the earliest stages of growth. In fact, potential genes responsible for storage root development in cassava genotype TMEB419 have been identified. Therefore, in this study, we will verify the involvement of these genes in root bulking development by using quantitative real time PCR (RT-qPCR) to test the regulation of their expression levels at several time points during storage root development, and in different genotypes.

Due to revolution in molecular biology techniques, reverse transcription quantitative PCR (RT-qPCR) is considered today as the gold standard for accurate, sensitive and fast measurement of gene expression. With this study we hope to unlock the genetic potential for starch mobility in cassava at specific time.

The general aim of this study was to determine the genes responsible for storage root bulking at different growth stages in cassava crop plant. The specific objectives were to study gene expression profile for storage root formation in cassava, and identify the involvement of specific key genes responsible for storage root bulking at different time points.

2.0 LITERATURE REVIEW

2.1 Genetic diversity of Cassava

Genetic diversity in plants is explained by various evolutionary processes, which include hybridization, mutations, migration and polyploidy (Colombo *et al*., 2000). In cassava, it is believed that the wide range of genetic diversity was generated through centuries of farmer selection (Jennings and Iglesias, 2002). Nassar (2007), however, indicates that the wide genetic diversity in cassava is a result of natural hybridization between the wild *Manihot* spp. and cultivated cassava, as well as controlled interspecific hybrids between *M. esculenta* and several wild *Manihot* spp or through apomixis. Jennings (1963) suggested that a high genetic diversity of cassava genotypes resulted from introduction of cassava genotypes by immigrants, followed by natural hybridization in the fields. Fregene *et al*. (2000) explained that the genetic diversity for example in East African cassava is structured according to the adaptation to biotic and abiotic stresses, agronomic practices, and post-harvest use. (Asante *et al*.,2003) on the other hand stated that as much as the genetic diversity in *Manihot* spp. is high, diversity within a given geographical region may be low, and is associated with the exchange of planting materials between farmers and selection for desired traits.

2.2 Cassava production and trade statistics in the world

Cassava is set to resume its status as one of the fastest expanding food crops, with its production rebounding from year to year. The significant reduction in international trade so far in 2016 has exposed the high vulnerability of cassava non-food sectors to developments in markets in which cassava competes, especially maize (FAO STAT 2016).

The crop's tolerance to erratic weather conditions, however, spared cassava from substantial output reductions, encouraging governments to put cassava expansion high on their agendas, especially in food insecure regions. Production prospects are also positive in countries with dietary diversification programs or those aspiring to limit the imports of staples, particularly wheat and rice. The volume of world trade in cassava in 2016 is expected to slump to a six -year low. International flows of cassava, primarily confined to East and South east Asia, are hugely contingent on industrial and feed demand, particularly from China, the world's leading cassava importer, and on the competitiveness of supplies in Thailand the world's leading exporter. international petition for cassava has fallen, emphasized by the relative narrowness of cassava trade.

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2.1.4 Spatial and temporal distribution of starch

Currently much research has been done to unlock the genetic nature of cassava storage root but yet remain uncertain. The molecular approach seems to be highly promising, (Priscila *et al.*,2013). studied on spatial and temporal distribution of starch in cassava and observed a concentration of starch which may be related to the movement of organic solutes in the xylem vessels and also revealed that there is up-regulated ribosome related genes which go together with high ratio of up-regulated ubiquitin, protease genes in cassava roots. Starch formation occurs simultaneously with starch degradations at early stages of roots development but at root maturity starch degradation decreases particularly due to decrease in UDP-glucose dehydrogenase activity. Starch synthesis, translocation, and accumulation are also associated probably with signaling pathways and constitutive expression of stress-responsive genes may be due to the adaptation of cassava and evolution

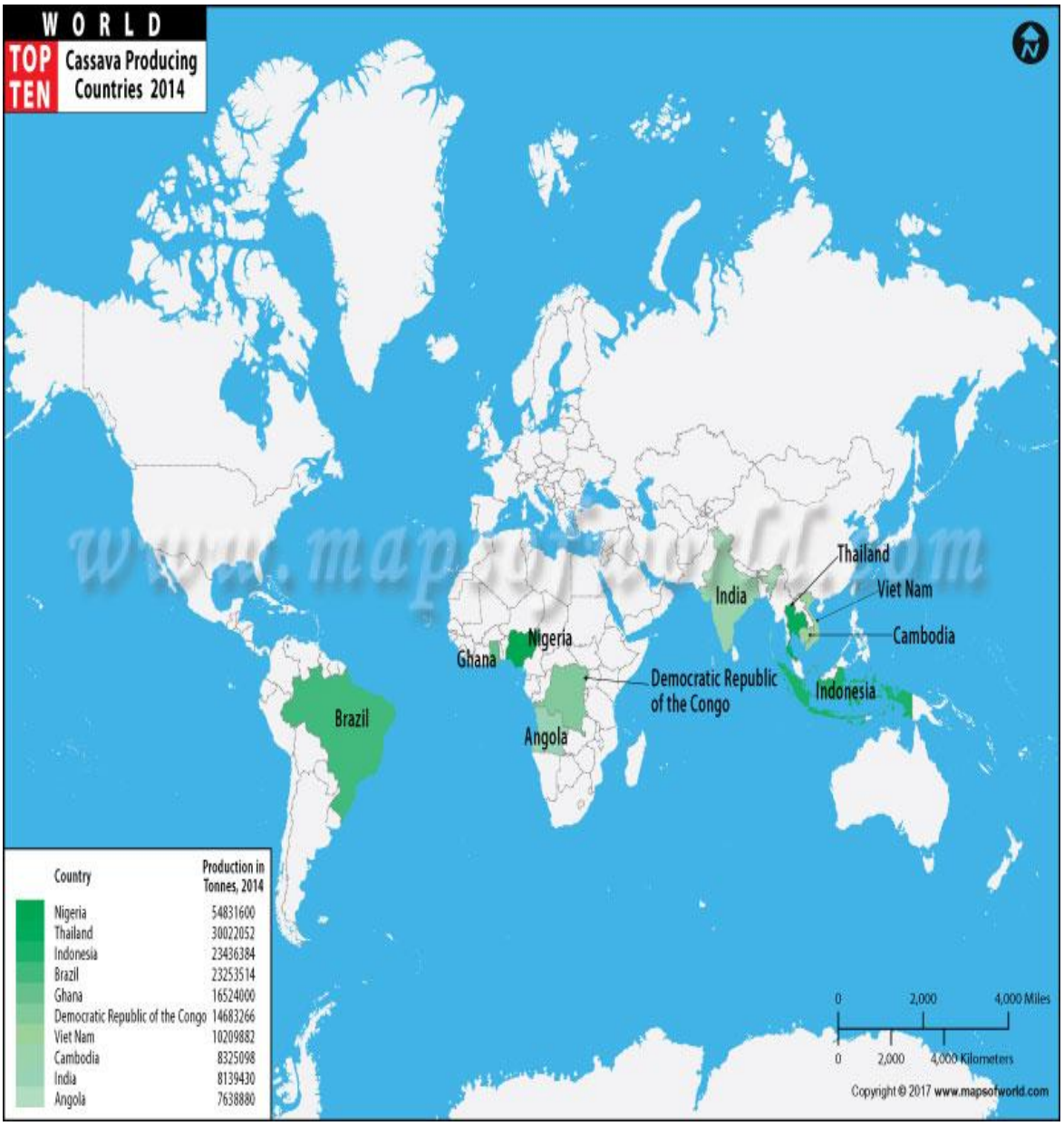

Figure 2.1: Map of the best ten world cassava producing countries, Source: FAOSTAT 2014.

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2.1.5 Genome-Wide expression patterns during tuberization of cassava

Yang *et al.,* (2011) evaluated genome-wide expression patterns during tuberization of cassava and identified differentially expressed transcripts in fibrous root, developing storage root and matured storage roots. 25 significantly changed pathways were identified and glycolysis was the most evident one. Rate-limiting enzymes were identified from each individual pathway, for example, enolase, L-lactate dehydrogenase and aldehyde dehydrogenase for glycolysis, and ADP-glucose pyrophosphorylase, starch branching enzyme and glucan phosphorylase for sucrose and starch metabolism. This study revealed dynamism in some of transcripts.

2.1.6 Initiation of storage root development

In 2015, Chaweewan *et al.,* reported that basal roots were initiated from the cambium while the nodal derived roots are developed from tissues deeper within the stem, at the boundary of the xylem and pith. Further development established the storage organ in which secondary xylem parenchyma, tracheids and vessels were produced from the cambium. Also they observed nodal roots to be a precursor of the storage organs after they received unknown signal. This study has not revealed the key genes responsible for observed structural root development behaviour

2.1.7 Change of fibrous root to storage root.

In cassava the roots are adventitious and arise from the basal cut surface of the stake and occasionally from the buds under the soil. These roots develop to make a fibrous root system. Only a few fibrous roots 3-10 start to bulk and become storage roots. Most of the other fibrous roots remain thin and continue to function in water and nutrient absorption. Once a fibrous root becomes a storage roots, its ability to absorb water and nutrients decrease considerably. The storage roots result from secondary growth of the fibrous roots; thus the soil is penetrated after 45 days by thin roots, and their enlargement begins only after that penetration has occurred.

Anatomically, the cassava root is not a tuberous root, but a true root, which cannot be used for vegetative propagation. The mature cassava storage root has three distinct tissues: bark (periderm), peel (or cortex) and parenchyma. The parenchyma, which is the edible portion

of the fresh root, comprises approximately 85% of total roots weight, consisting of xylem vessels radially distributed in a matrix of starch containing cells.

The peel layer, which is comprised of sclerenchyma, cortical parenchyma and phloem, constitutes 11–20% of root weight. The periderm (3% of total root weight) is a thin layer made of a few cells thick and, as growth progresses; the outermost portions usually slough off. Root size and shape depend on cultivar and environmental conditions; variability in root size within a cultivar is greater than that found in other root crops.

2.1.8 Storage root bulking process

Storage root bulking involves secondary growth by genesis of a circular primary vascular cambium as well as several anomalous circular cambia in the sub-apical region of roots (Doku, 1969; Indira and Sinha, 1970; Hunt *et al.,* 1977; Izumi *et al*., 1999; El-Sharkawy, 2003). At the onset of secondary thickening, primary vascular cambium initials are first laid down within the parenchymatous zone between the protoxylem and protophloem and are connected to form a continuous and irregular cylinder through division of the single layered pericycle (Hunt *et al*., 1977; Ravi *et al.,* 2009). This is accompanied by the formation of a cork cambium in the outer layers of the pericycle. Subsequent vascular cambial activity leads to centripetal production of thin-walled storage parenchyma, secondary vascular tissues and a regular cylinder of vascular cambium. Differentiation of vascular cambium is accompanied by the origin of anomalous circular cambia in the central pith around central metaxylem cells as well as around each of the discrete protoxylem elements. Anomalous circular secondary cambia also originate around secondary xylem elements derived from the vascular cambium (Izumi *et al*., 1999; Ravi *et al*., 2009). Interstitial cambial strips unassociated with vascular tissues also develop within the secondary parenchyma and contribute to storage roots growth. Active cell division in these cambia results in the formation of thin walled, starch storing parenchyma cells causing thickening of storage roots and lignifications. The xylem parenchyma cells store the bulk of starch grains (Doku, 1969; Hunt *et al*., 1977). Starch deposition in the first produced parenchyma cells occurs 25 days after planting (Indira *et al.*,1970). The time of onset of both the secondary thickening and of starch deposition can either be delayed by excising buds, or accelerated by applying sucrose or glucose (Indira *et al*., 1970), implying that the sugar alone can initiate storage root differentiation.

A number of studies have differed on the time when thickened roots appear in cassava during its growth and development (Doku, 1969; Izumi *et al.*, 1999; Okogbenin *et al*, 2002). For

instance, Izumi *et al*., (1999) showed that root bulking begins about 3 months after planting (MAP) but maintained that rapid starch deposition does not occur before 6 months after planting. Based on sequential harvesting experiments in various Ghanaian cassava cultivars, Doku (1969) reported that root bulking of most genotypes began during the second month and produced reasonable fresh storage root yields by 6 months after planting. (Wholey *et al.,* 1974), however, in their trials designed to investigate differences in onset of root bulking and rate of bulking found that thickened roots were present after 2 months after planting, and that root bulking increased with time but, the rate of bulking differed between cultivars.

They also found that after three months, the number of thickened roots per plant remained fairly constant for all cultivars except for one in which the thickened root number increased with time. Based on fresh storage root mass accumulated by different genotypes at different times, Wholey and Cock (1974) concluded that earliness was related to early onset of bulking, rapid bulking, or a combination of both factors. Similar findings on accumulation of different amounts of FSRM at different harvest times by different cultivars have been reported elsewhere indicating existence of early bulking genotypes (Kamau, 2006; Amenorpe *et al*., 2007; Mtunda, 2009; Okogbenin *et al*., 2013).

2.1.9 Change in storage root bulking

The rate of cassava storage root bulking fluctuates over a long period due to changes in the agro-climatic conditions (Ekanayake *et al.*, 1998). Unlike in cereal grains, the cassava storage root can undergo periods of arrested growth during unfavorable conditions and then continue growing once conditions improve. High yielding cultivars have a high bulking rate over a long period, whereas cultivars with intermediate and low storage root yield have a low bulking rate for short duration or low bulking rate for longer duration (Hershey, 2011, Okogbenin *et al*., 2013). Bitai and Lian (1978) found that early maturing, short duration sweet potato cultivars exhibit fast initiation growth of storage roots whereby their yields reach a maximum within a short growing period. They indicated that the bulking rate of tubers of early maturing cultivars declines or even pauses in the early period of growth, whereas for the late maturing cultivars, the bulking rate increases at the middle and later growth period. Suja *et al*. (2009) indicated that high and low yielding cassava cultivars differ in their bulking rate and the period at which they exhibit the maximum bulking rate. They revealed that short duration cultivars exhibit maximum bulking rate during their early growing stage.

Morphological changes and starch accumulation during cassava root development is significant importance for starch production (Wang *et al*,. 2016), they are the consumed part of the cultivated plant. The cassava roots began to develop into storage roots in approximately 2 months, and the starch granules clearly appeared at approximately 3 months under a light microscope. The highest starch content was detected in the tuberous roots after planting for 7 months. The fresh weight and dry matter of the starchy roots steadily increases with time after two months, the starch content slightly decreased with continued tuberous root development (Wang *et al,* .2016).

2.2 Storage root number and Storage root yield

Storage root number (SRN) in cassava is determined during the first 3 MAP Ekanayake *et al*. (1997). Wholey and Cock (1974) also indicated that SRN is generally determined early in the growth cycle although some cultivars appear to continue producing new storage roots up to 7 MAP. The number of thickened roots ranges are 5- 20 per plant. Fewer storage roots are formed in drier environments. The number of fibrous roots which form storage roots depends on several factors such as: genotype, assimilate supply, shading, photoperiod and temperature (Ekanayake *et al.,* 1997). Enyi (1972) showed that the number of shoots per plant may affect the SRN, plants with more than one shoot producing more roots than plants with only one.

Yield in plants refers to the mass of produce harvested from a single plant or the quantity of produce harvested per unit of land area. In cassava, it is often defined in terms of marketable storage root yield, although leaves, stems or even seeds could potentially be additional economic products (Hershey, 2012). Several attempts have been made to describe the ideal cassava plant type for maximum yields and, according to Cock (1975), the highest yielding cassava plants would have a single erect stem, late branching, short thick internodes, and long leaf retention capacity.

However, he recognized environmental limitations of this ideal plant and suggested that in fertile soils harvest index (HI) was important, and that under low soil fertility HI is irrelevant. Hunt *et al.*, (1977) on the other hand suggested that the most prominent trait associated with yield is leaf longevity. Several other indicators of high yielding cassava were suggested including late branching (Cock *et al*., 1979); large individual leaves or profuse branching (Cock *et al*., 1979); narrow-lobed, vertical positioned leaves (Ramanujam, 1985); optimal

leaf area index, large and numerous leaves and high HI (Byrne, 1984). Lahai and Ekanayake (2009) suggested that dry mass production and partitioning are important determinants of storage root yield in cassava and that they could be important selection criteria in breeding for enhanced yield. Byrne (1984) indicated that in selection for high yielding cassava, the obvious approach would be to select for the ideal plant type and that this should be based on late branching, large leaf size, and the number of roots on a mass-screening scale, but not for long leaf life.

2.2.1 The starch biosynthetic reactions

Starch consists of the two glucose polymers amylopectin and amylose, which together form insoluble, semi-crystalline starch granules. Amylopectin accounts for 75–90 % of wild-type starches, has a degree of polymerization (DP) of 105 and a branching level of 4–5 % (i.e., 4–5 % of its linkages are a-1,6-branch points). Amylopectin makes up the structural framework and underlies the semi-crystalline nature of starch. Amylose is considerably smaller and only lightly branched. It is believed to fill spaces in the semi-crystalline matrix formed by amylopectin, probably rendering the starch granule denser. The starch synthesis in plants, occurs by utilizing ADP-glucose as the glucosyl donor for the elongation of α-1,4-glucosidic chains also photosynthetic bacteria synthesise glycogen by following a similar pathway. The first committed step in these pathways is the synthesis of ADP-glucose in a reaction catalyzed by allosteric ADP-glucose pyrophosphorylase (ADPGlc PPase) as carbon intermediate assimilatory pathway (Miguel *et al*., 2013).

ATP + Glc 1-P ⟷ ADPGlc + PPi

ATP = Adenosine triphosphate
ADPGlc = Adenosine Diphosphate Glucose
PPi = Pyrophosphate

This reaction was first described to occur in soybean extracts by Espada (1962). Thereafter, the enzyme was also found in many plant tissues and in bacterial extracts (Preiss *et al*., 1998a; Preiss 1999). ADPGlc PPase is the first committed step in the route leading to glycogen and starch synthesis in bacteria and plants, respectively. The ADPGlc PPase reaction takes place in the presence of a divalent metal ion, $Mg^{2+,}$ and it is freely reversible

in vitro. The hydrolysis of PPi by inorganic pyrophosphatase and the use of the sugar-nucleotide for polysaccharide synthesis cause the ADPGlc synthetic reaction to be essentially irreversible in vivo (Iglesias and Preiss 1992).

In addition, in each tissue of a plant differences in starch granule size and shape are observed within a range time. The diameter of the starch granule changes during the development of the reserve tissue. There are also some fine features, characteristic of each species, for example, the 'growth rings', spaced 4–7μm apart, and the fibrillary organization seen in potato starch, which allows one to identify the botanical source of the starch by its microscopic examination (Morrison and Karkalas 1990; Hizukuri 1995; Preiss and Sivak1998).

The biosynthetic steps required for starch synthesis are relatively simple, involving three committed enzymes: ADP glucose pyrophosphorylase (ADPGPPase; EC 2.7.7.23), starch synthase (SS; EC 2.4.1.21), and starch branching enzyme (SBE; EC 2.4.1.28). Several pathways of starch biosynthesis have been proposed.

These enzymes have been based on *in vitro* studies of isolated enzymes and increasingly on molecular analysis of the genes which gives rise to these proteins. It is generally held that starch is synthesised from sucrose. Most cytosolic sucrose is converted to hexose phosphates which are then transported to the amyloplast via a hexose translocator. The glucose-6-phosphate so formed is then converted into glucose-1-phosphate by the enzyme phosphor glucomutase. The key step in starch biosynthesis in plants takes place inside the amyloplast where the enzyme adenosine diphosphate glucose pyrophosphorylase (AGPase; EC2.7.7.23) catalyses the synthesis of ADP-glucose from ATP and glucose-1-phosphate (Espada, 1962; Preiss, 1982). In the process pyrophosphate is produced. This is removed by inorganic alkaline phosphate thereby driving the reaction in the direction of ADP-glucose synthesis. ADP-glucose is the primed glucose molecule which functions as the glucosyldonor for glucan synthesis by various starch synthases. The starch synthases (SS; EC 2.4.1.21), mainly granule bound starch synthase (GBSS), catalyze the conversion of ADP-glucose into amylose through (1– 4) linkage of ADP glucose to a pre-existing glucan chain. *In vitro* SS are able to utilize both amylose and amylopectin as substrates. How the initial primers for glucan chain formation *in vivo* are produced remains un clear. However, in bacteria, which synthesize glycogen through a process similar to starch biosynthesis in plants, and in maize, likely priming proteins termed glycogenin and amylogenin respectively, have been identified

(Hengge-Aronis and Fisher, 1992; Singh et al., 1995).Amylopectin is thought to be formed mainly due to the action of Branching Enzyme (BE, EC 2.4.1.18) and Soluble Starch Synthase (SSS). BE introduces branch points in the amylopectin molecules by hydrolysis of the alpha (1, 4) glucan chains at 15–20 units from the nonreducing end. It then catalyses the formation of an alpha (1, 6) cross linkage between the reducing end of the cleaved chain and another glucose residue (Smith and Martin, 1993).

Cassava GBSS exhibits high amino acid sequence identity with potato (GBSS (74%) and also with GBSS from other plant species (60–72%). The mature protein has a predicted molecular mass of 58.6 kilodalton (KD). The enzyme was shown to be highly expressed in cassava tubers which are consistent with its prominent role in amylose synthesis therein (Salehuzzaman *et al*., 1993).

2.2.2 Soil and climatic requirement for cassava production

A soil which is light textured soil was classified as moderately suitable due to its medium level of soil chemical characteristics in comparison with the nutrient requirement for cassava production, though; this soil possesses a limitation, which was low fertility, especially macronutrients (N, P and K) which are close to the critical level.

This however, does not preclude its use for sustainable production of cassava, since the soil fertility and nutrient level can be greatly improved with the use of inorganic and organic fertilizers (Ande *et al.,* 2008). Sulphate fertilizers should be avoided so as not to raise the level of soil acidity.

Cassava is often grown almost exclusively in the hot lowland tropics and it is cultivated under a broad range of edaphic and climatic conditions between 30 ºN and 30 ºS latitude and in regions from sea level to 230 m altitude. In the most extreme latitudes where cassava is grown light frosts occasionally occur. Fronts may defoliate the plant, but when warmer weather returns, the plant sprouts from the base and grows normally. In the areas that have large seasonal variations in temperature, cassava is not grown if the annual mean temperature is less than about 20ºC. Cassava is found wherever the climatic conditions are favourable (Alaux *et al.,* 1990)

It is often grown in areas considered marginal for other crops, for example low soil fertility with a pH range of 4.0-8.0, annual rainfall from more than 600 mm in the sub humid and

humid tropics and it is grown all the year round giving the best yields when the growth cycle is synchronized with the seasonal cycle.

The crop plant does not thrive well in hydromorphic soils but is well adapted to low fertility soils that predominate in the tropics, and it is therefore usually grown on highly weathered and leached soils of the orders Oxisols, Ultisols, and Alfisols (Cock 1985). It also does well in a variety of soils, namely, muddy, alluvial or ferralitic, provided there is good aeratin and drainage (Alaux and Fauquet, 1990). When grown in low fertility soils, the total growth of cassava suffers less than that of most of crops. Similarly, for root yield, which are what farmers are interested in, cassava is an efficient producer in low fertility soils. It is produced under low input and output production, particularly when grown as food crop. Most areas under cassava production are considered marginal for other crops (Alves, 2002). Howeler (1981), complied literature on the critical level of soil parameters for cassava. For example, the critical level of soil pH is from 4.0 to 8.0. It has been suggested that high level of nitrogen may increase leaf production at the expense of roots and in one experiment phosphorus and potassium applied before planting depressed survival.

Cassava is sensitive to shading and consequently roots yields are reduced when shaded (Okoli and Wilson, 1986). They reported that less than six shade regimes (0, 20, 40, 50, 60, and 70 % of full sunlight) cassava yield was reduced by 43, 56, 59, 69, and 80% compared to the control treatment of 0% shading. In Africa and the Americas, cassava is commonly intercropped with grains and legumes (Alves, 2002). Cock, (1985) estimated that at least one third of cassava grown worldwide is intercropped thus minimizing the risk of crop failure and obtaining production at different times during the year. This practice makes the best use of available land and labor resources and provides the family with a balanced diet. These attribute places cassava in an important position in traditional tropical cropping system, particularly to the small scale and subsistence farmers. In this system, cassava is usually found intercropped with a variety of other crops, with long or short cycles and food or cash crops (Alves, 2002).

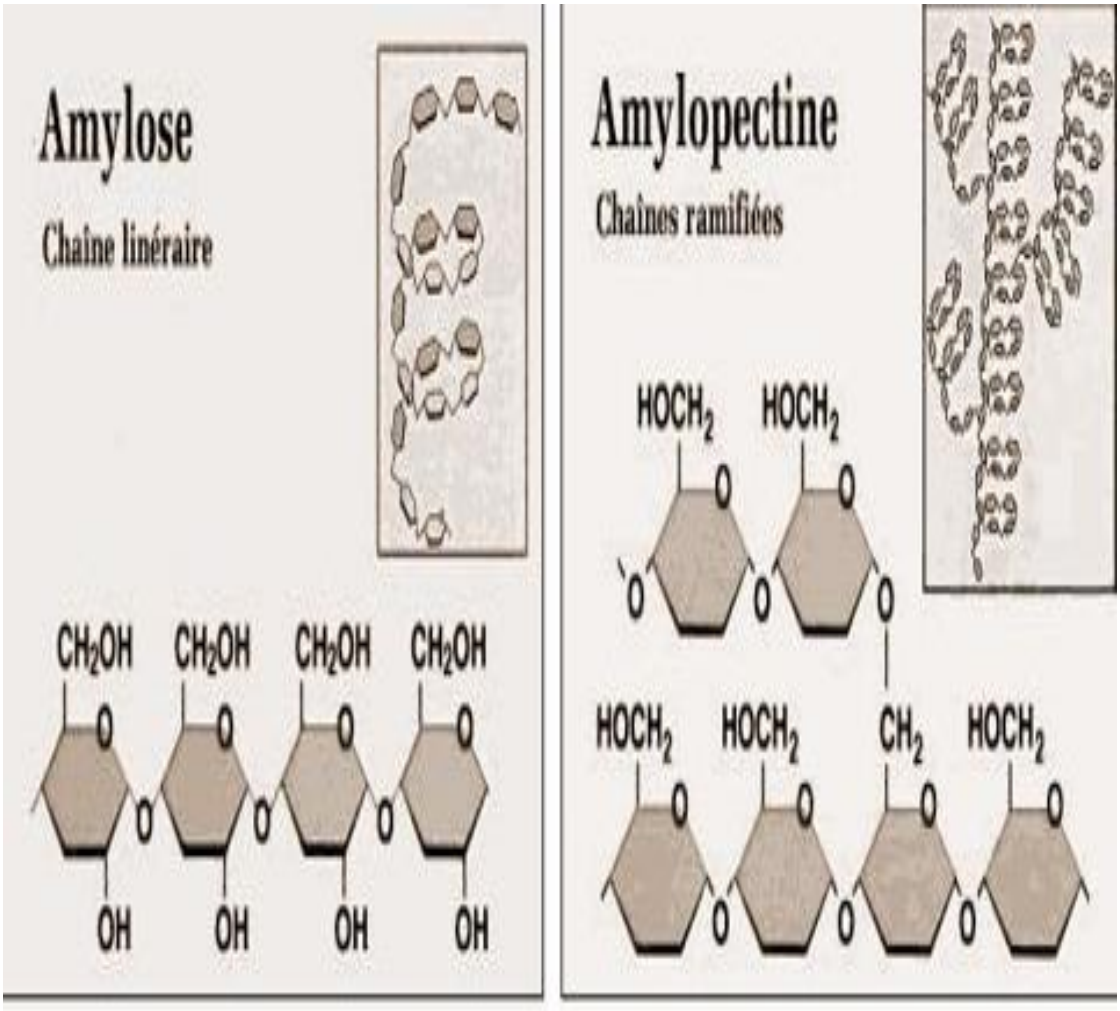

Figure 2.2: The structure of amylose and amylopectin. Amylose is straight chain while amylopectin is branched structure. It is also assumed that amylose fills the gap in between amylopectin structure. Barbara et al., 2016

Figure 2.*2.1*: Formation of starch in plant cells: adjacent amylopectin chains form double helices which pack in crystalline lamellae. (Barbara et al., 2016).

2.2.3 Analysis of gene expression in storage roots of cassava.

A transcriptome analysis of gene expression during the process of storage root development is critical to elucidate it regulation. Several tools are currently available to facilitate functional genomics analysis in cassava such as a genetic map, cDNA libraries, and expressed sequences tags (EST) library (Anderson *et al.,* 2004; Lokko *et al.,* 2007; Raji *et al.,* 2009; Sojikul *et al.,* 2010; Mitprasat *et al*., 2011; Yang *et al.,* 2011). Moreover, several cassava genome sequence are currently available to access for cassava genome analysis in cassava data base.

An effective genetic transformation system has been developed for characterizing target genes including siRNA technology to facilitate gene knockdowns and for over-expression of desired genes in cassava (Taylor *et al.*, 2012; Zainuddin *et al*., 2012).

2.2.4 Quantitative Real Time PCR

Quantitative PCR (qPCR) is a technique used to measure the quantity of a PCR product (usually in a real-time PCR procedure). It quantitatively measures starting amounts of DNA, cDNA or RNA. This technique can also determine whether a DNA sequence is present in a sample and the number of its copies in the sample. Another advantage of Real-Time PCR is rapidity of the assay, since it is not necessary to perform electrophoresis or other procedure after the DNA amplification reaction (Sykes *et al,*.1992).

2.2.5 Transcriptome analysis using microarray

Microarray technology is a high throughput genome-wide analysis method (Hardiman, 2004; Nguyen and Williams, 2006; Wang *et al.*, 2008). Several microarray platforms have been developed to study genetic and cellular processes. The similarities and differences among these platforms depend on large data set manipulation and the complexity of their experimental target expression (Hardiman, 2004). Time-series microarrays provide information as multiple expression profiles at each time point for continuous cellular processes. This technique has been routinely applied to identify expression patterns, detect differentially expressed genes, and construct gene networks (Bar-Joseph, 2004; Hardiman, 2004; Opgen-Rhein and Strimmer, 2007; Nguyen and Williams, 2006; Wang *et al.*, 2008). However, the limited sampling problems in the time-series microarrays cause increased

potential for misleading analysis (Churchill, 2002; Jørstad *et al.*, 2007). Wang *et al.* (2008) developed short time-series microarrays to enhance the accuracy of data-series analysis, with limited sampling and address simplification-based approaches for integration of multi-source information.

Lokko *et al.* (2007) characterized an 18,166 EST dataset for cassava for drought responsive genes and demonstrated that these ESTs can be useful for developing microarrays and gene-derived molecular markers. Carvalho (2010) has also performed a cDNA-microarray platform in collaboration with USDA Fargo to demonstrate genomic analysis in cassava biodiversity and showed the transcriptomic diversity between wild and domesticated cassava.

3.0 MATERIALS AND METHODS

3.1.1 Planting Materials

Selected cassava genotype TMEB419 available at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, was evaluated for storage root development. The genotype was selected based on contrasting yielding capacity among others and preference by farmers in Nigeria. This study is the step forward from a previous transcriptomic analysis of genotype for sink leaves, source leaves, storage roots and fibrous roots conducted at three different time points during the development of storage roots.

Apart from TMEB 419 ,13 genotypes with contrasting traits from high yielding to low yielding capacity were also planted to confirm the expression level of gene responsible for storage root and fibrous roots. The other 13 genotypes planted were KALESO, TMEB14, I102103, I051599, I051601, I030006A, O8100045, I090454, I090576, I071313, MM990302, TMEB419 and IBA30572 which were selected out of 134 genotypes evaluated in IITA. The traits taken into consideration during selection process were root size, dry matter content, root number, and root weight and sprouting where the clones with the contrasting traits were considered *(*Table: 4.1**).**

3.1.2 Study location

The study was conducted in the IITA field with altitude of 230 meters above sea level lying between 7^{o} 29’11.99’’N latitude and 3^{o}54’2.88’’E longitude, mean maximum and minimum temperature is 35 ^{o}C and 25 ^{o}C respectively almost throughout the year. Relative humidity of the place is 74.55 %, mean annual rainfall 1250 mm and soil type is ferric luvisol. All the molecular analyses were conducted in the Bioscience laboratory, IITA.

3.1.3 Field trial

Trial was laid out in a Randomized Complete Block Design (RCBD) with three replications. Healthy stem cuttings, each 25 cm in length were horizontally planted in a flat seedbed at a spacing of 1 x 1 m giving a population density of 10 000 plants ha^{-1}. Each plot measured 14 x 14 m comprising 13 rows of 13 plants each. The first and last rows and the first and last plant within each row of each plot were considered as border plants.

Table 3.2: Traits considered for genotype selection

SN	Genotype	Traits
1	KALESO	High Dry matter (DM), low root size (RS)
		Low root number (RN) and Low shoot weight
2	TMEB14	High Dry matter
		High root size
		High root weight
3	I102103	Low dry matter
		High root size
4	I051599	Low dry matter
		High root size
5	I051601	Low dry matter
		Good vigor
6	O8100045	Low dry matter
		High root number
		High root weight
7	I090454	Low dry matter
		High root number
		High root weight
8	I090576	High dry matter, high root number
		Low root size and
		High root weight
9	I071313	Low dry matter
		High root number
10	MM990302	Low dry matter
		Low root size
		Low shoot weight
11	TMEB419	Reference genotype and high yielding
		High root size, preferred by farmers
12	IBA30572	Poor sprouting
13	I030006A	Good sprouting, Low root weight
		Low shoot weight

The plots and blocks were separated by 1.5 m and 1.5 m alleys, to reduce inter-plot and inter-block plant competition, respectively. The trials were conducted without supplemental irrigation and weeded regularly as weed growth observed. Plants were earthed as there was a heavy rainfall that caused erosion.

3.1.4 Score for cassava mosaic disease severity and incidence (CMD)

The disease severity and incidence were scored at 54 DAP. Disease severity was estimated on a scale of 1 -5 where 1 = no disease and 5= 100% disease (Spence, 1994). Disease incidence was estimated through count of number of diseased and healthy plants per replication and the percent incidence was calculated as here below.

$$\text{Disease incidence (\%)} = \frac{\text{Number of infected plants}}{\text{Total numbers of plants observed}} \times 100$$

3.1.5 Sample Collection

Individual plant genotype tissues from storage roots (SR) and fibrous roots (FR) was collected at different time points. TMEB 419 samples were collected at 35, 44, and 60 days after planting (DAP) and every two months until end of trial i.e. twelve MAP. Multiple genotypes samples were also collected at 35, 44, and 60 DAP respectively. Three biological replicates and three technical replicates were collected for each genotype.

In the field, samples collected were chopped into pieces and wrapped in aluminium foil paper and snap freezed in liquid nitrogen to block cell activities and RNases (to protect RNA molecules from degrading). Samples were stored at - 80ºC until used.

3.1.7 Statistical analysis

Gene expression levels was analyzed by Roche LightCycler480 machine and data obtained were subjected to Statistical Analysis System (SAS) software package version 9.0 and means separated using Duncan Multiple Range Test.

3.1.8 Total nucleic acid extraction from cassava roots

Total nucleic acid was extracted from both fibrous and storage root tissues using the CTAB (Cetyl Trimethyl Ammonium Bromide) extraction protocol as described by Dellaporta *et al.* (1983) with some modifications to allow high-throughput extraction of plant tissues. Approximately 500 mg samples were ground using mortar and pestle in liquid nitrogen and transferred into a properly labeled 1.5ml pre-cooled RNase/DNase free tube. A pre-hated 1000 ul CTAB extraction buffer (2% CTAB, 2% PVP-40, 20 mM Tris–HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA) was added to each sample and vortexed to mix the samples properly with the CTAB solution. The total nucleic acid extraction buffer is used to maintain stable pH environment (cationic) during extraction and to lyses the cell wall and cell membrane to separate polysaccharides and polyphenols. Samples were incubated in water bath at 65ºC for 15 minutes, vortexed at 5-minute interval and were centrifuged at 15000 rpm at 4ºC for 5 minutes.

The aqueous top layer containing the nucleic acid was transferred to 2 ml micro centrifuge tube without transferring the plant debris and 1 ml of chloroform: isoamyl alcohol at ratio of 24:1 (kept at +4° C) was added and centrifuged for 10 minutes at 15000 rpm. The chloroform helps to dissolve the lipids and proteins in the samples and separate them from nucleic acid, while isoamyl alcohol helps to reduce the foaming of chloroform, aids in the separation, and maintains the stability of the layer of the centrifuged, deproteinized solution. The supernatant (upper layer) was transferred into a new 1.5 ml tube, 0.6 volume of cold isopropanol was added into the supernatant to precipitate the nucleic acid, mixed gently by inversion for about 2 minutes and centrifuged at 15000 rpm for 20 minutes. The precipitated pellet was washed with 100 ul of cold 70% ethanol and centrifuged for 5 minutes, the alcohol was decanted and pellet air dried in the laboratory fume hood until the ethanol evaporates completely (about 15-20 minutes). Dry pellet was re-suspended in 100 ul of RNase/DNase free water, TNA quality and quantity were confirmed by denaturing agarose gel electrophoresis and spectrophotometer respectively and stored in -80º C freezers.

3.1.9 Total Ribonucleic acid isolation

Total ribonucleic acid isolation was done and cleaning up using the RNA clean and concentrator kit (Zymo Research). Total nucleic acid (TNA) with a starting mass 10µg was treated with DNase to get rid of Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA) was isolated.

DNase I into DNA digestion buffer was added to the TNA and incubated for 15 minutes at room temperature, 100µl of RNA binding buffer was added to the mixture and mixed thoroughly. One volume of 100% ethanol (150µl ethanol to 150µl mixture) was added and the sample was transferred into a Zymospin IC column in a collection tube, centrifuged and the flow through was discarded. RNA preparation buffer of 400ul was added to the column and centrifuged, flow through was discarded, and 700µl of RNA wash buffer was added to the column and centrifuged for 1 minute at 15000 rpm, 400µl of RNA wash buffer was added again and centrifuged for 2 minutes to ensure complete removal of the wash buffer.

The column was carefully transferred into an RNase free tube, 20 ul of DNase/RNase free water will be added directly to the column matrix, incubated at room temperature for 2 minutes and then centrifuged for 2 minutes at 10000 rpm. The eluted total RNA was stored at -80ºC .

3.2.1 RNA Quantification using the Spectrophotometer

Accurate quantification and determination of the purity of the extracted RNA was done by using Nanodrop spectrophotometer (ThermoFisher Scientific Inc., Denver). Nucleic acid application module was selected in the Nanodrop software. Upper and lower pedestal measurement was cleaned with lint-free tissue paper and 70% ethanol. RNase/DNase free water of 2 µl was added as blank sample into the measurement pedestal and then measured. 2 ul of each RNA samples was dropped into the measurement pedestal and measured. Upper and lower measurement pedestals were wiped dry after each measurement to avoid mixing up of samples or cross contamination. The RNA quantity displayed and the purity of RNA indicated by the 260/280 and 260/230 absorbance ratios was recorded.

Agarose gel electrophoresis was used to examine the quality of the extracted TNA and RNA. It was used for separating molecules based on the rate of movement while under the influence of an electric field. During the electrophoresis the following materials was used: Agarose, 10 X TBE Buffer, 6X Sample Loading Buffer, electrophoresis chamber, power supply, gel casting tray and combs, RNA stain, staining tray, gloves and pipette and tips. The gel was allowed to run for 30 minutes under 100V. The TBE buffer was prepared as follows: 4.84 g Tris base, 1.14 ml glacial acetic acid, 2 ml 0.5M EDTA (pH 8.0) by bringing the total volume up to 1L with DEPC-treated water and 0.5 micrograms of RNA was loaded with equal amount of loading dye. The RNA was normalized prior to cDNA synthesis (Figures 4 and 5).

3.2.3 Primer Design

Primers used for this study was designed with the support of the IDT's Primer Quest software, freely available at http://eu.idtdna.com/Scitools/Applications/Primerquest/, Multiple Sequence alignment by CLUSTTALW www.genome.jp/tools-bin/clustalw, National Centre for Biotechnology information (NCBI) https://www.ncbi.nlm.nih. Nine Primers was designed, one for storage root equal, two for storage root down, five for storage root up and one for fibrous root up. Primers were purchased from Inqaba company.

For maximum RT-PCR reaction some primer parameters were taken into consideration, parameters such as the melting temperature of the primers Tm (60° C), the length of the

amplicon (120), the percentage and the distribution of C and of G at the 3 'end of each were taken into consideration (45-50) and primer size of 20 bp optimum.

Tm = 4(G+C) + 2(A+T)

Where
G = Guanine
T = Thiamine
A = Adenine
C = cytosine

Primer concentrations were optimized before selecting the conditions of amplification. Relative quantitative real time-PCRs were performed using Roche machine. The cycling profile consisted of 95° C for 20s, 40 cycles of 3s at 95° C and 30s at 60° C, one cycle of 10s at 95° C, as recommended by the manufacturer, using 2X Fast SYBR Green PCR Master Mix (Applied Bio systems), 400 nM forward and reverse primers, 50 ng of sample (cDNA) and nuclease-free water in a total volume of 12.5 µL.

Each cDNA sample was amplified in duplicate for each primer pair on a 96-well optical plate. Immediately after the final PCR cycle, a melting curve analysis was performed to verify primer specificity. The PCR efficiency for each primer pair and amplicon was derived from the slope of the regression line fitted to a subset of baseline-corrected data points in the log-linear phase using LighCycler480 software (Roche machine). The regression line was obtained by interpolating values from triplicates of five serial log10 dilutions of input DNA amount and the relative Ct values. Relative quantities of cDNA accumulation were calculated as RQ values using the comparative cycle threshold (Ct) (2-DDCt) method corrected for PCR efficiencies

Data acquisition and analysis were handled by the Roche software that automatically calculates the Ct values and the parameters of the standard curves.

3.2.3 Real Time PCR detection Chemistry

Real-time PCR is a reaction that allows real-time detection of the amount of amplicon that has formed at each reaction cycle. The detection was performed through the SYBR Green, a generic fluorophore able to intercalate in the DNA double stranded molecule. Compared

to the PCR-end-point, PCR-real-time has the advantage of checking whether, at each reaction cycle, the duplication of all the DNA fragments present has been obtained.

Relative quantification determines the changes in steady state mRNA levels of a gene across multiple samples and expresses it relative to the levels of an internal control RNA. The reference gene is a housekeeping gene and can be amplified in the same tube in a multiplex assay or can be amplified in a separate tube. Therefore, relative quantification does not require standards with known concentrations and the reference can be any transcript. Relative quantification is based on the expression levels of a target gene versus one or more reference gene(s) and in many experiments it is adequate for investigating physiological changes in gene expression levels.To calculate the expression of a target gene in relation to an adequate reference gene various mathematical models was established. Calculations are based on the comparison of the distinct cycle determined by various methods, e.g. crossing points (CP) and cycle threshold values (Ct) at a constant level of fluorescence; or CP acquisition according to established mathematic algorithm.

This parameter, defined as amplification efficiency, as 100% when each DNA fragment has been correctly duplicated at each reaction cycle and for all reaction cycles. However, the results of reactions with an efficiency between 90 and 110% are considered reliable. The variation in efficiency between cycle and cycle depends on a series of factors including: concentration and purity of cDNA, concentration of primers, presence of inhibitors, magnesium chloride concentration. In conditions of efficiency within the limits indicated above, the estimation of the amount of DNA which, as is known, can be determined on the basis of the reaction cycle at which the intensity of the fluorescent signal exceeds a certain threshold value is also reliable and can be detected by the machine. The threshold cycle is commonly referred to as Ct (for Cycle threshold). Since real-time PCR performs the quantification during the exponential amplification phase of the reaction, in which the number of amplicons produced is linearly proportional to the initial amount of cDNA molecules, the obtained Ct value will be as high as the number of reaction cycles necessary to reach the quantity of DNA molecules whose fluorescence exceeds the set threshold.

Real-time PCR uses an increase in the intensity of a fluorescent signal generated by an intercalating dye or from the breakdown of a dye-labeled probe during amplification of a target sequence to detect nucleic acids either for their presence or absence or for their

amount. The PCR cycle number where fluorescent signal becomes discernable above background noise is the CT value. Between two samples, a decrease in a CT value (ΔCT) of one cycle represents a doubling of the amount of target. This relationship can be expressed as

The fold increase in the amount of amplified product = 2−ΔCT

Standard deviation, a measure of the degree of variability in a data set, was calculated to determine to what amount the CT values of replicate real-time PCR reactions vary from the mean. It is given by the equation

$$S^2 \equiv \frac{\sum(x-\tilde{x})^2}{n-1})$$

S = Standard deviation

The RNA quantification using real-time PCR was performed by generating a standard curve from a dilution series of an independently quantified supply of reference RNA, plotting their CT values versus the log of the RNA concentration, and determining the amount of RNA contained in the unknown sample from the plot's regression line.
Real-time PCR and the generation of fluorescent signal proceeded at a rate defined by the equation

Rf = Ri(1+E) n

where
Rf = equivalent to the final fluorescence signal of the reporter dye,
Ri = equivalent to the reporter dye's initial fluores-cent signal,
E = the amplification efficiency, and n is the number of cycles.
Amplification efficiency which was determined from a standard curve using the following formula:
Exponential

Amplification=10 (−1/slope)

Efficiency= (10(−1/slope) −1) ×100

Where the slope in this equation was that of the standard curve's regression line. A 100% efficient reaction was having a slope of −3.32. During exponential amplification, a 100% efficient reaction was double every cycle and produced a 10-fold increase in PCR product every 3.32 cycles.

In mathematical terms, that is,

$\log_2 10 = 3.3219$

A slope with a more negative value will represent a PCR reaction having efficiency less than 100% and in need of optimization. A slope having a more positive value can indicate some problem with the reaction.

The standard curves were prepared 4 serial dilutions 1: 5 from a cDNA sample at a concentration of 50 ng / µl. For each dilution the Ct values of both the housekeeping and the gene under examination are calculated, using specific primers for each of them. The standard curve for each gene was obtained by relating the Ct value to the cDNA concentration for each dilution. The amplification efficiencies of the reactions are comparable if the standard curves are parallel with a slope value (angular coefficient) of -3.3 ± 10%. If the lines are not parallel it means that, for the genes considered, the Ct values vary differently as the concentration of the sample in the reaction varies. This indicates that ΔCt is constant, which means that the two reactions behave similarly to changes in the initial concentration of the target sequence. If efficiencies are not similar, the ΔΔCt method cannot be used.

Table 3.2: Primers designed for the confirmation studies from RNA Seq.

Name	Sequence
MeSRE1-qPCR_F	TGGTGAAGGTTGTGGAGTTT
MeSRE1-qPCR_R	AGTACTTCATTAGCAGCATCAGT
MeSRD1-qPCR_F	TGGGTCTTGACAGAGGTAGAGA
MeSRD1-qPCR_R	ATGAGGATACATGGCACACAGA
MeSRD2-qPCR_F	TAGGAGTCTATCCCTGCAACT
MeSRD2-qPCR_R	AGCTCATGAGGAGGTAGAGAA
MeSRU1-qPCR_F	CGTTGGACCTTAGAATATGAGAAGA
MeSRU1-qPCR_R	CATTTCACGAGATGAGCAGAAAC
MeSRU2-qPCR_F	CCACACAAAGCAACGATCTTC
MeSRU2-qPCR_R	GTAAGCTCTTCGCGGTACTT
MeSRU3-qPCR_F	GCTCCGAGCTGCTTATGAA
MeSRU3-qPCR_R	CTTAGCTTGACAGTCCCTCAC
MeSRU4-qPCR_F	CAGCAGAGACCGTTGTTGA
MeSRU4-qPCR_R	CTGGAGTTTCCGATGCTGAT
MeSRU5-qPCR_F	CTGGTTCCTCCTGAGATGTTT
MeSRU5-qPCR_R	GAGGTGGTAGAGGTTGAAGAAG
MeFRU1-qPCR_F	TGCAGACTTTGTGTTCGACTA
MeFRU1-qPCR_R	GTAGAAGGATCCTGAATGGGAAA
MeActin_F	TCCGTGACATGAAGGAGAAG
MeActin-_R	CTGACCATCAGGAAGCTCATA

4.0 RESULTS AND DISCUSSION

4.1 Data analysis of gene expression level for storage and fibrous roots formation

An RNA sequencing study was conducted on cassava genotype TMEB 419 to investigate the genes involved in the development of storage roots. Root tissue was collected in a series of time point, and time point one (30 days after planting, time point two (42 days) and time point seven (60 days) after planting were sequenced. In fact, Morphological observations show that at already at first time point roots appeared of two different type, which were defined potential storage roots and potential fibrous roots. Some of these roots were tagged and their development monitored. Consistently, those expected to bulk indeed became storage roots while those expected to be fibrous did not bulk.

Results of transcriptomic studies showed that some gene are differentially expressed in fibrous roots and storage roots. Among the genes differentially expressed in storage roots, some are increasingly highly expressed over time, others are equally constantly expressed or downregulated through all time points while other are up regulated in fibrous root.

This study was conducted to confirm the differential expression of some genes and thus their involvement in storage roots and fibrous roots formation. Among the genes found to be differentially expressed through transcriptomic studies on cassava genotype TMEB 419 the following ones have been selected for this confirmation study: GDSL-like lipase, UDP-glucosyl transferase, Light-dependent short hypocotyls, Pt2L4 glutamic acid-rich protein, P54 glutamic acid-rich protein, and Unknown seed protein like 1 (USPL1).

Firstly, the expression of the selected gene was confirmed in an independent experiment on cassava roots from the TMEB419. To further investigate whether the differential expression of these genes in storage and fibrous roots could be extended to other cassava genotypes the gene expression level study was conducted on a group of different cassava genotypes at 60 days after planting, the genotypes to be analysed were selected based on phenotypic characteristics such as root size, root number, dry matter, root weight and sprouting.

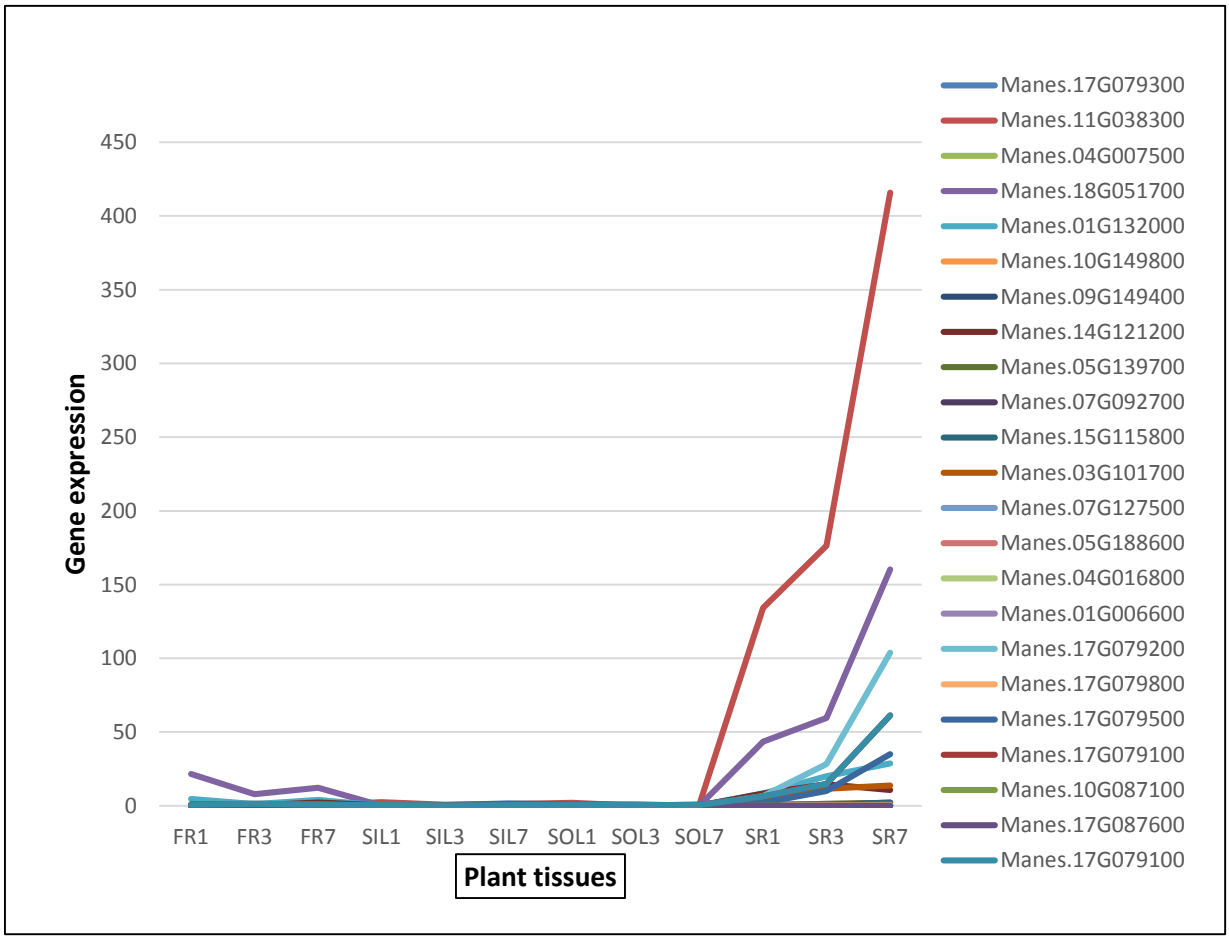

Figure 4.1.1: The gene expression levels for fibrous roots storage roots sink and source leaves at time point 1,3 and 7 consecutively from the RNA Seq.

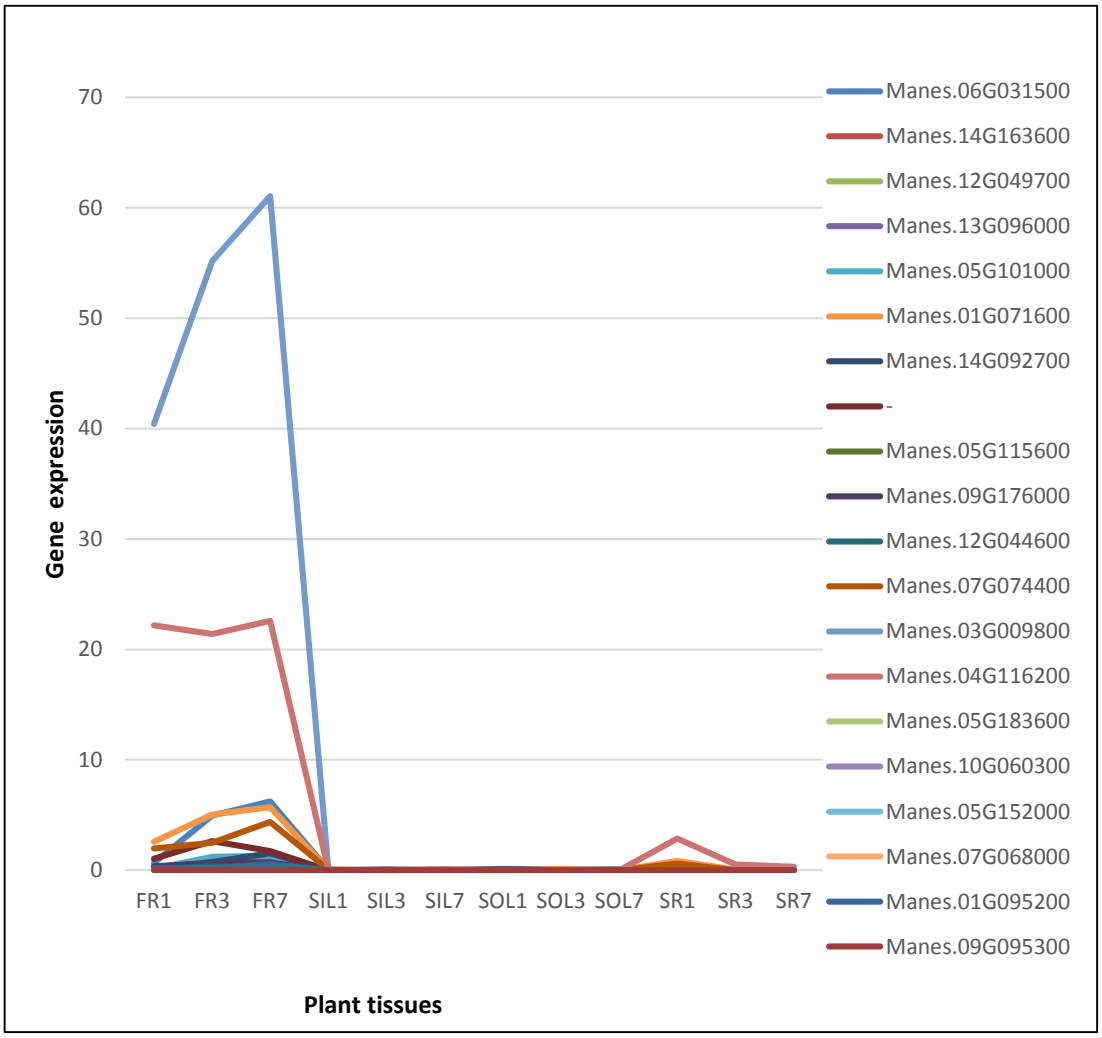

Figure 4.1.2: The gene expression levels for fibrous roots storage roots sink and source leaves at time point 1,3 and 7 consecutively from the RNA Seq. They are highly expressed in fibrous roots

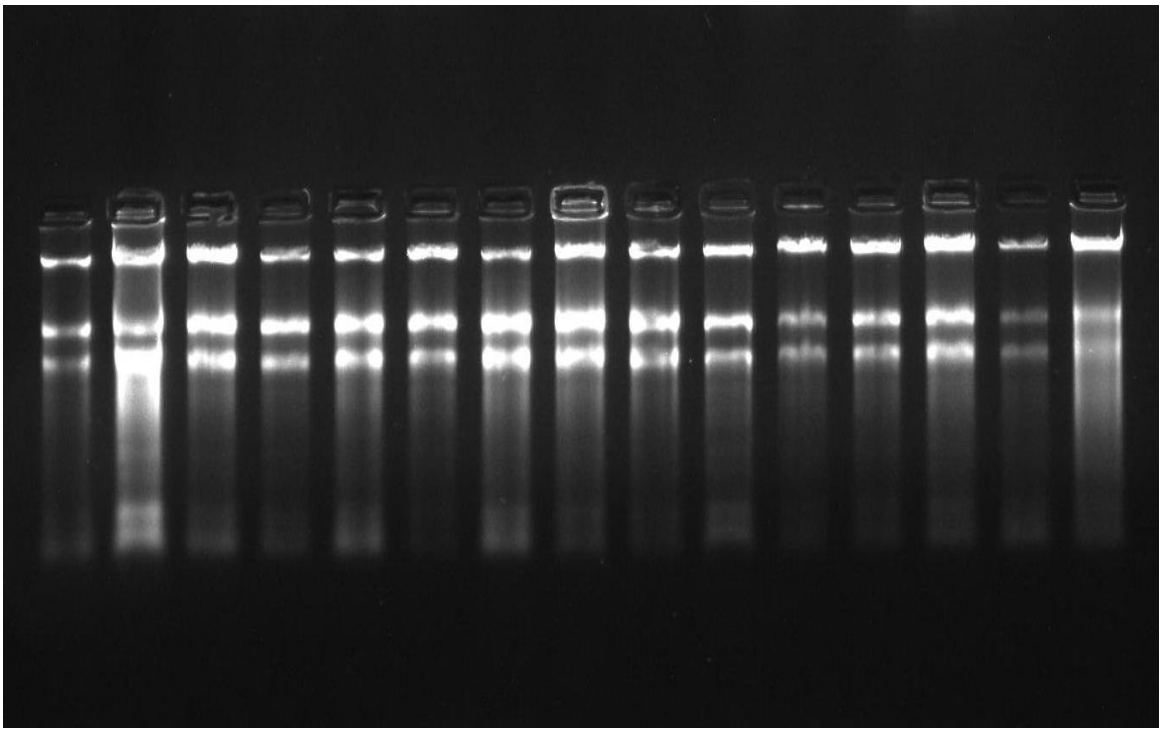

Figure 4.1.3: Gel electrophoresis image for Total Nucleic Acid showing the DNA molecules at the top and RNA at the mid of the band: due its low molecular weight RNA moves faster than DNA.

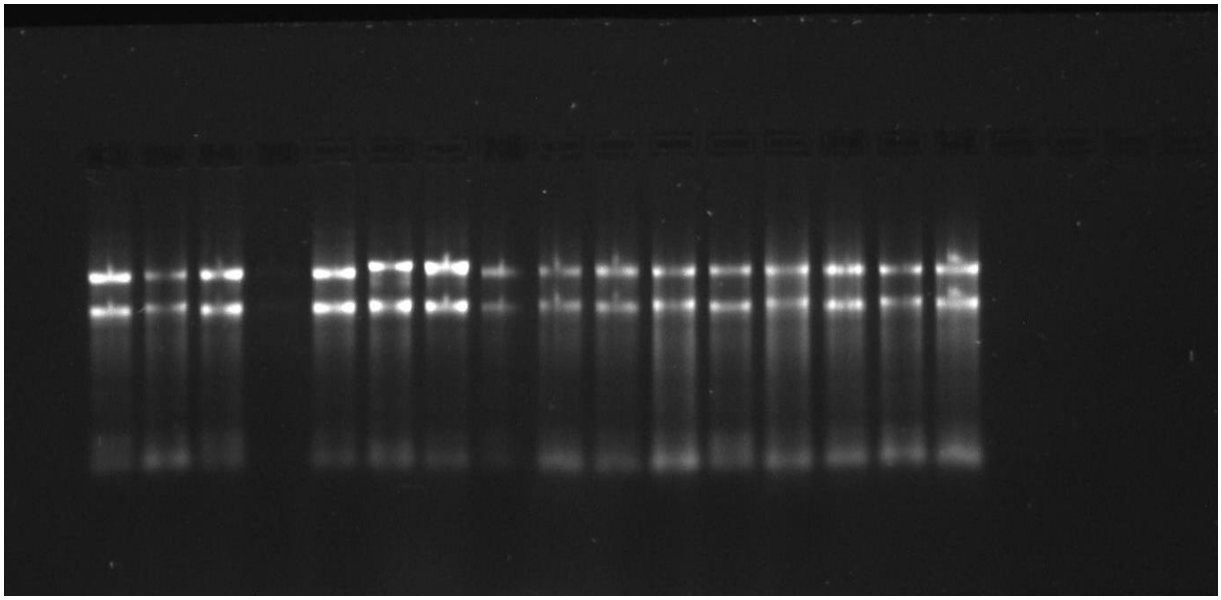

Figure 4.1.4: Gel electrophoresis image for RNA after isolation showing there is no RNA smearing and its good quality for cDNA synthesis.

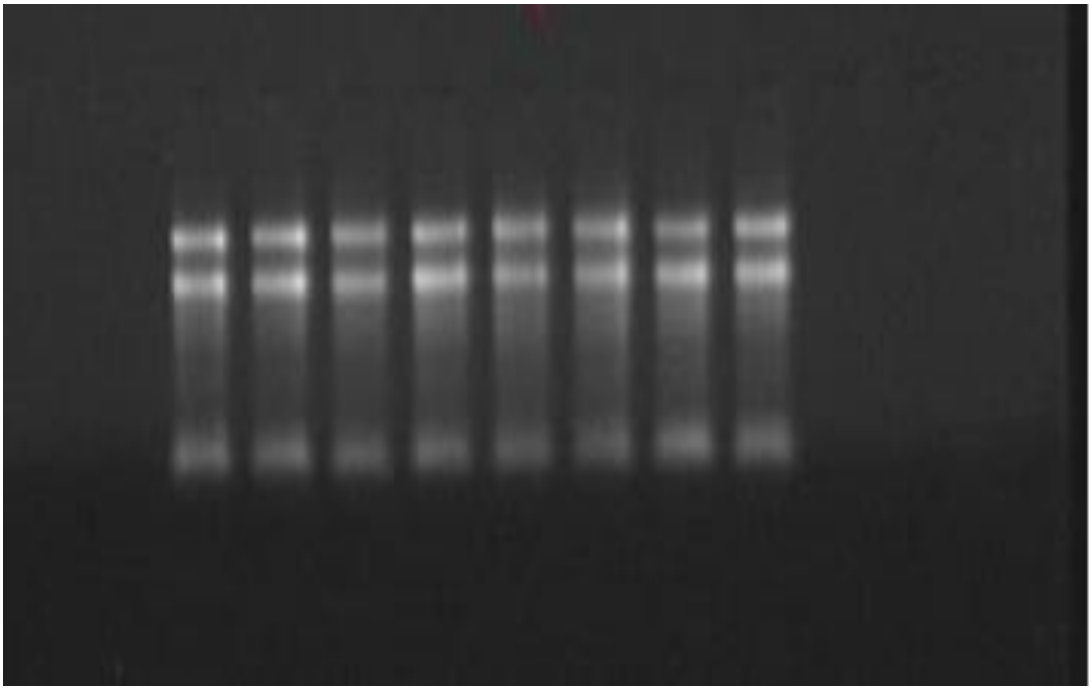

Figure 4.1.5: Gel electrophoresis image for RNA after isolation showing there is no RNA smearing and its good quality for cDNA synthesis.

4.2 Quantitative real-time PCR: Standard curves and melting curve

As stated previously, RT-qPCR is a technique that allows the relative quantification of specific cellular mRNAs of a given gene, and consequently we used it for quantification of the expression of the gene considered in relation to storage and fibrous root formation. The analysis of gene expression level at different experimental time point was held by a comparison of the root size and color.

Prior to relative RT-qPCR, the amplification efficiency of gene expression was evaluated by processing standard curves related to six fragments of gene sequence and three housekeeping genes protein phosphatase 2A (PP2A), MeActin and GTBp (Figures 4-9) as high and comparable efficiency values are the key for reliable quantification of target genes for samples using real time PCR. The standard curve analysis allowed verifying that the amplification efficiency of the sequences comparable; in particular, a difference of 10% between the efficiency values of the two amplicons to be compared through RT-qPCR is allowed. In our case, the efficiency values differ by less than 10%, so 9 couples sequences were used for the relative quantification of the gene expression of MeActin as a reference. The house keeping gene was chosen based on the standard curve and Melting curve to validate the product amplification products.

In the same way before continuing with the analysis of gene expression, the same approach allowed to discover that also the amplification efficiency of the genes, MeActin, was comparable in such a way as to be able to use MeActin as a gene reference to normalize the expression levels of the gene under investigation.

The melt curve analysis of qRT PCR to check for primer dimer or non-specific product formation, for all the primers assessed gave a single peak indicating the absence of primer dimer and a pure product.

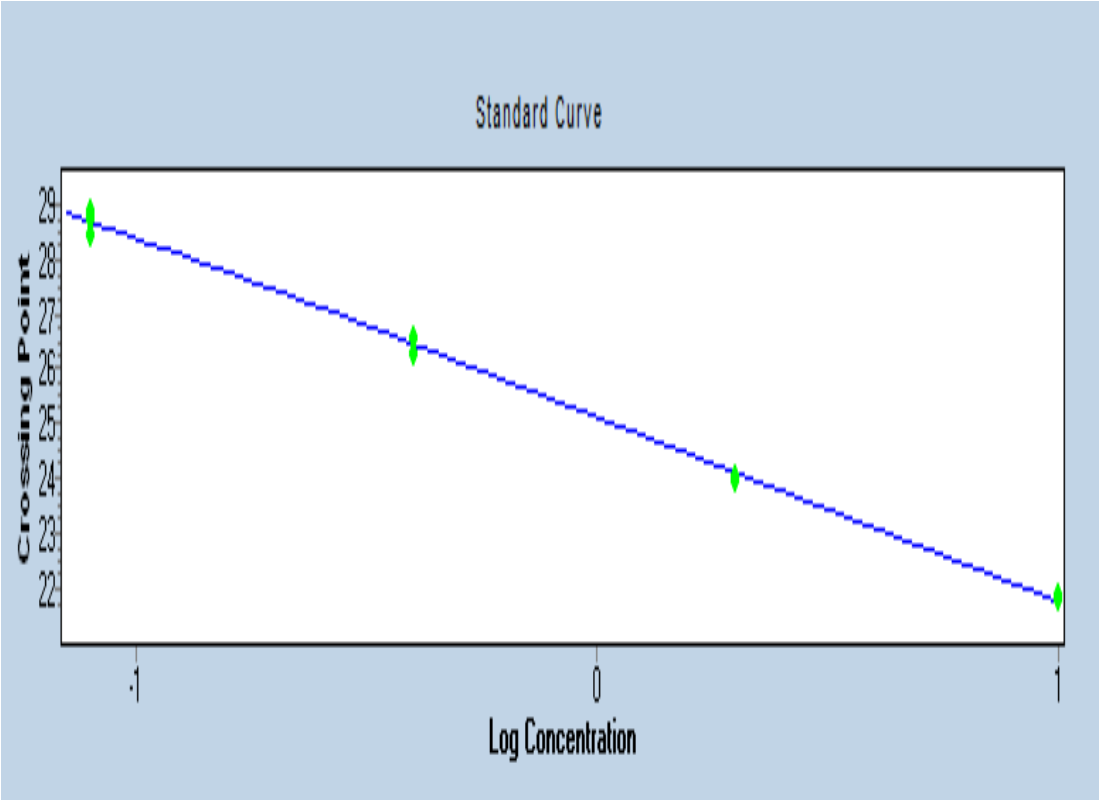

Figure 4.1.6 The Slope of the standard curve describing the kinetics of the RT-PCR amplification for the house keeping gene (MeActin) with the slope of 3.314 and efficiency of 1.97.

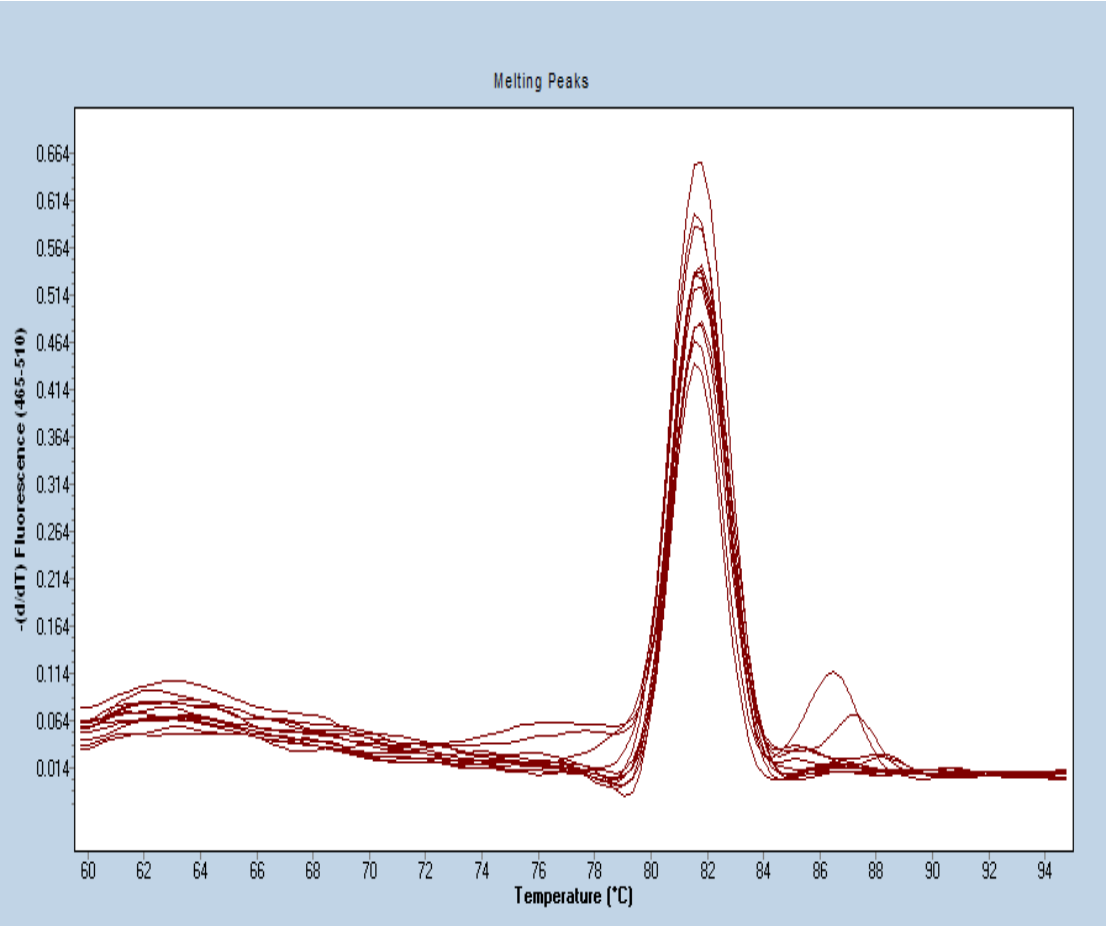

Figure 4.1.7: Melting curve analysis to check that primers are giving a specific PCR product and there is no aspecific amplification MeActin.

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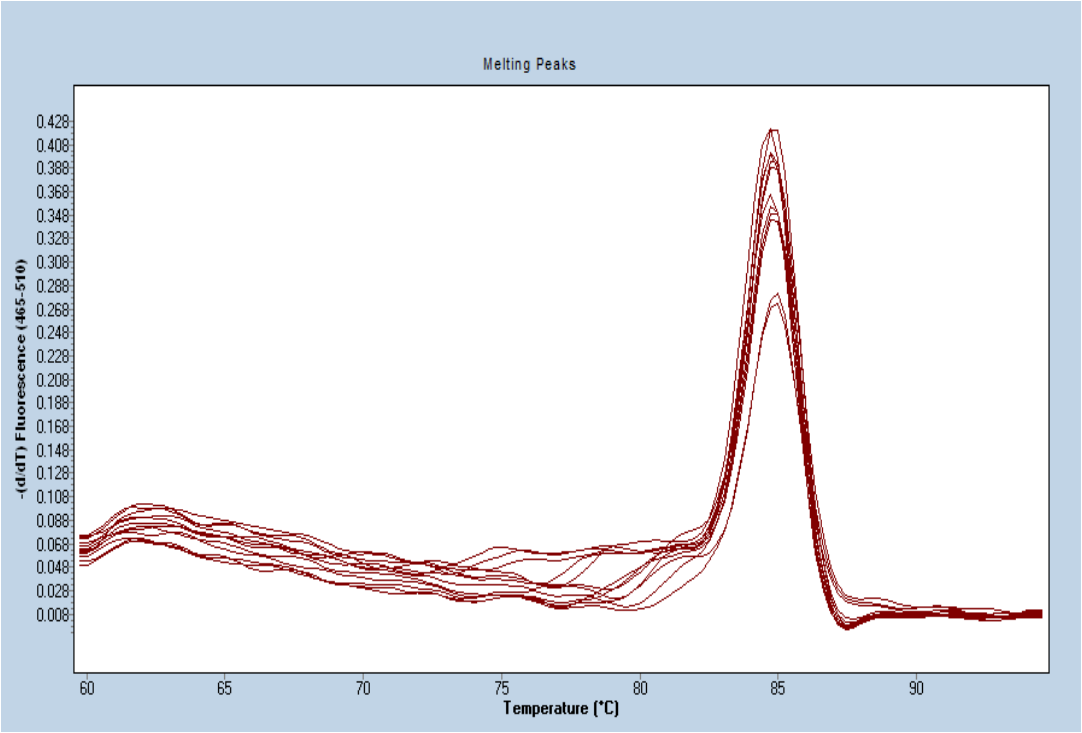

Figure 4.1.8: Melting curve analysis to check that primers are giving a specific PCR product and there is no non-specific amplification FR U1.

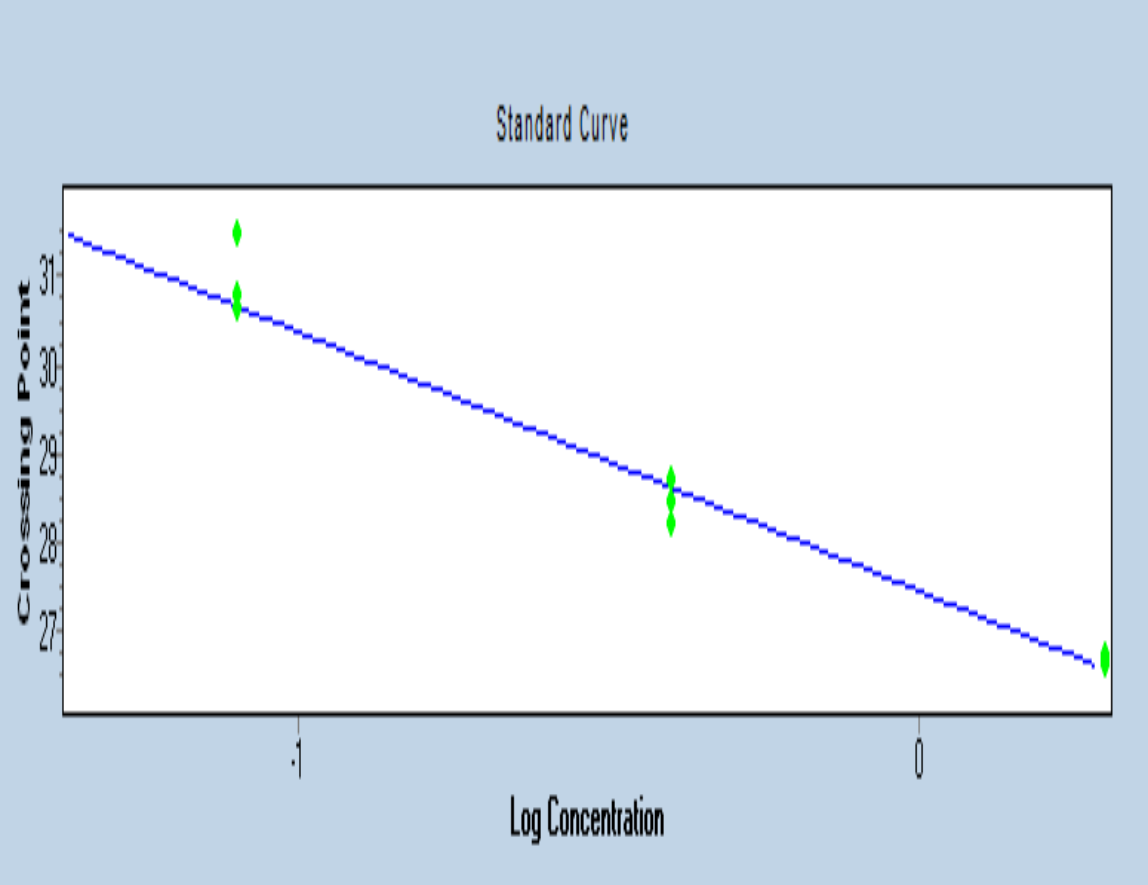

Figure 4.1.9: The Slope of the standard curve describing the kinetics of the RT-PCR amplification for the Fibrous root up (FR-U) with the slope of 1.769 and efficiency of 2.967.

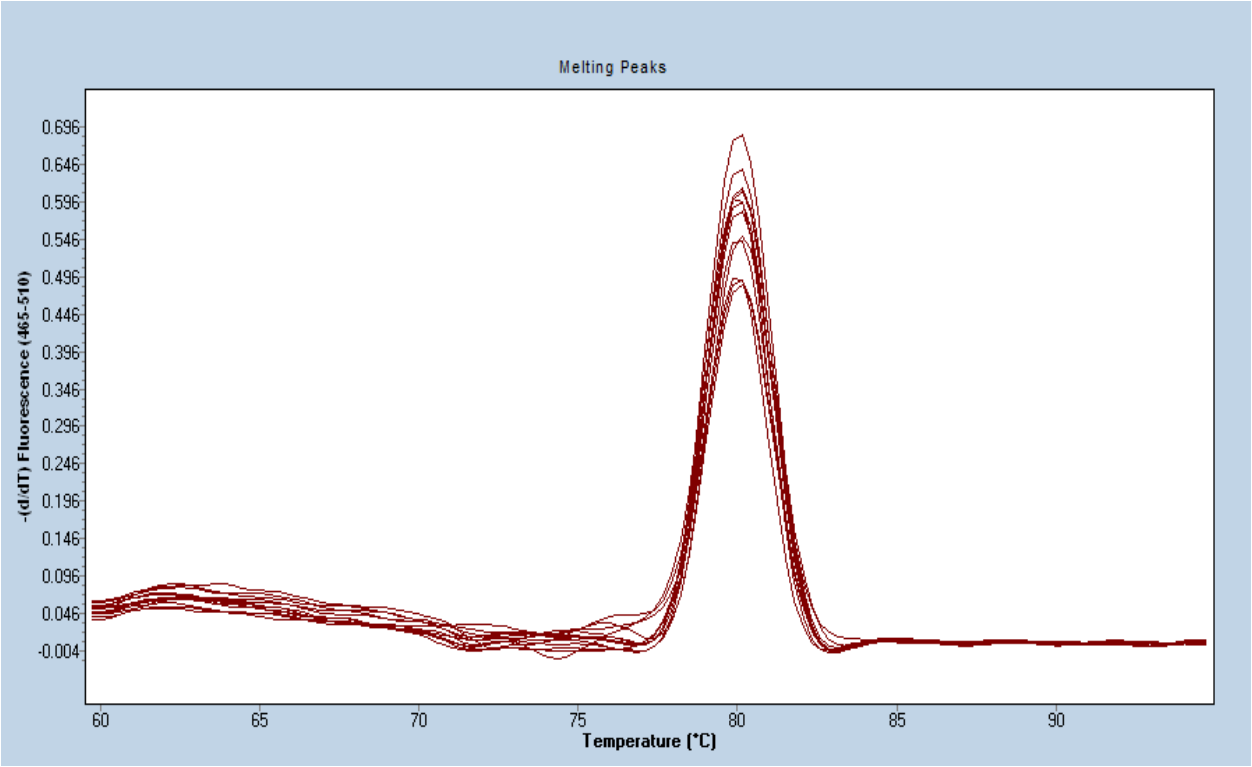

Figure 4.2.1: Melting curve analysis to check that primers are giving a specific PCR product and there is no non-specific amplification.

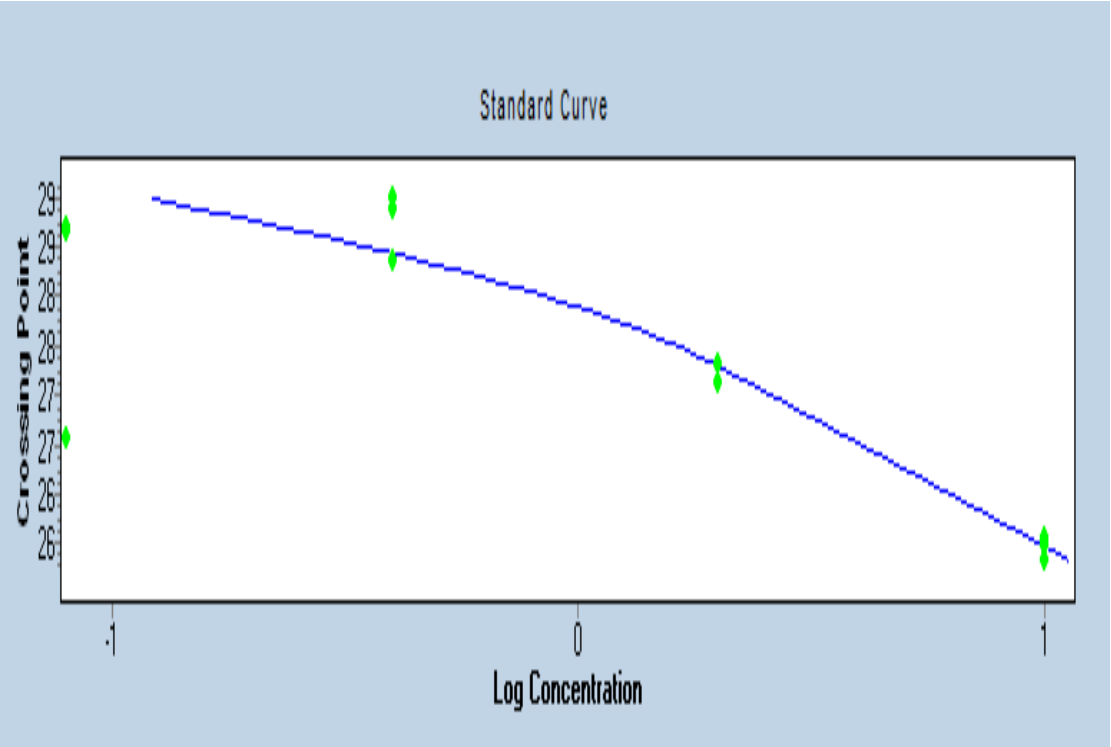

Figure4.2.2: The Slope of the standard curve describing the kinetics of the RT-PCR amplification for the storage roots down regulated (SR D1) with the slope of 1.769 and efficiency of 2.967.

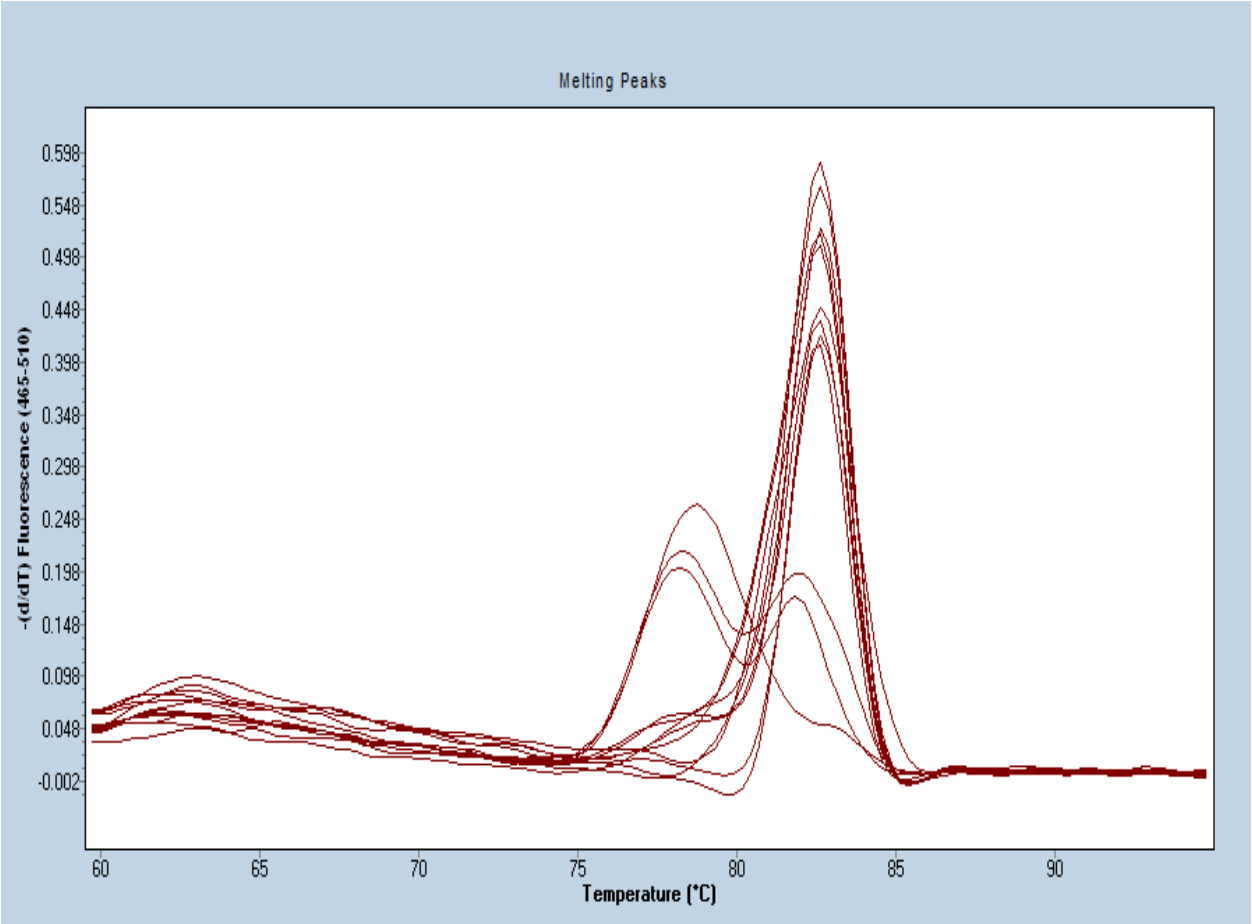

Figure 4.2.1: Melting curve analysis to check that primers are giving a specific PCR product and there is aspecific amplification. More than one peak shows non-specific amplification for storage roots down regulated up SR D1. With this primer the template was anneal

4.3 Confirmation of genes in reference genotype TMEB 419

To confirm the expression level of the selected genes we repeated the experiment planting the cassava genotype TMEB 419 used for the transcriptomic analysis and performed gene expression study by means of RT-qPCR. Primarily the aim of using this genotype is to investigate and elucidate whether the gene expression levels correlate with transcriptomics data analysis.

Three time points was used for gene validation with TMEB 419, 30, 42 and 52 days after planting. Six couples of primers (target) were also used in this confirmation study to amplify the above-mentioned genes.

FR- U Gene Unknown seed protein like 1 (USPL1) was highly expressed in both time point 2 and time point 5 but exceedingly higher in time point 5 and down regulated in time point one. The lower expression level in time point one might be attributed due error of selecting potential fibrous roots and storage roots at early time point since roots are just forming.

USPL1 proteins are expressed in storage organs. There is different kind of classification of SSPs as albumins or globulins are based on whether the proteins are water-soluble or soluble in salt solution respectively. The SSPs of crops also often have specific names. For example, glycinin, glutelin and zein represent specific SSP fractions from soybean, rice and maize, respectively. The Arabidopsis SSPs have also been named after those of *Brassica napus.* 12S globulin proteins are referred to as cruciferin, whereas the 2S albumins are referred to as either napin or arabin (Girke *et al.,* 2000). Both 12S globulins and 2S albumins are initially synthesized as a precursor and accumulate in protein bodies after processing of the pre-proteins.

SR-D2 NAD(P)-binding Rossmann-fold This was highly expressed in time point 2 fibrous roots and down regulated in both fibrous roots for time point 1and 5. In contrary to fibrous roots this was highly expressed in storage roots at time point 5and down regulated at time point one and two

SR-E GDSL-like lipase is up regulated in storage roots at 5^{th} time point and down regulated in the remaining storage roots for both 1^{st} time point and 2^{nd} time point. It's also down regulated for both fibrous roots at all-time point. This result is in contrary with results from the RNA Seq.

Lipase is one of the lipid hydrolyzing enzymes, distributed broadly throughout plants (Akoh *et al.,*2004). However, the knowledge about their roles in developmental processes and response to various stimuli are still very limited in rice. Microarray data analysis revealed tissue and developmental stage-specific expression patterns of several genes. Some genes were expressed in stigma and seed germination, several genes expressed exclusively in root and other genes were induced by any of the stresses. The analysis also suggests differential accumulation of cluster genes during these processes. It's also indicated that genes may have potential roles in rice development and abiotic stresses.
However, this results the results were obtained in the crop planted in uncontrolled field were the environmental factors were not controlled this include rainfall, flood, drought and soil fertility to mention few.

SR U3 Light-Dependent Short Hypocotyls was upregulated in storage roots at time point 5 and down regulated in fibrous roots for both time points. It slightly up regulated in fibrous roots at time point five.
SR U4 (Pt2L4 glutamic acid-rich protein) results have shown gene to be up regulated in storage roots at fifth time points and slightly down regulated in storage and fibrous for the other time point with exception of second time point in fibrous roots where it expression was very low

SR U5 (P54 glutamic acid-rich protein) This gene is highly expressed at time point five followed by 2nd time point in storage roots and downregulated in both fibrous roots for the first, second and fifth time point First time point behaves differently from the information that I have from the RNA Seq.
My study for this gene was in parallel with the study done by Cláudia R. *et al (*2006) which reported the isolation and characterization of a cDNA sequence (Mec1) coding for a glutamic acid-rich protein (Pt2L4) from cassava storage roots. Comparative sequence analysis showed a high identity of Pt2L4 with cassava protein C54, which is expressed in vascular tissues of storage roots. Northern blot analysis showed that the Mec1 transcript expression pattern might be related to the maturation of the storage parenchyma cells.
My results elucidated the up regulation of this gene at maturation of storage roots of cassava than the earlier stage This might explain the reason for the low expression of the gene.

4.4 Relative quantification for multiple genotypes

Nine couples of primers were used in this study for standard curve establishment 6 targets and 3 housekeeping genes. The primers for the confirmation study were MeSRE1 (constantly expressed RNA Seq), MeSRD1 (down regulated), MeSRU3 (up regulate for storage root), MeSRU4, (up regulated MeSRU5 (up regulated) MeFRU1 (up regulated FR) and Cassava MeActin as housekeeping gene (HKG).

MeSRU3 for light-dependent short hypocotyls. The target gene is highly expressed in storage roots for genotype 1090576 which is phenotypically characterized as having high dry matter, high root number, low root size and high root weight. The gene is down regulated in Kaleso and TMEB14 and slightly downregulated in fibrous roots for genotype 1090576. However as light regulates plant growth and development through a network of intrinsic factors, Root formation is also a product of plant photo assimilate, light-dependent short hypocotyls have showed hypersensitivity responses to continuous red, far-red, and blue, light and encodes a nuclear protein of a novel gene family that has homologues in rice Zhao *et al.*, (2004).

The difference in expression level in storage roots of different genotypes might be due to differential phytochrome sensitivity as light-dependent short hypocotyls is functionally dependent on phytochrome to mediate light regulation of seedling development.

SR U4 functionally annotated as Pt2L4 glutamic acid-rich protein was highly expressed in genotype 1090576 in similar way to sequenced genotype TMEB419 and down regulated in Kaleso and TMEB14 for both storage and fibrous roots

The transcript used to generate sequence information was from TMEB 419 its indicative that genotype 1090576 and TMEB 419 may have common metabolic and biochemical activities. The high identity of Pt2L4 with cassava protein C54 was found by Cláudia R. *et al (*2006*)* that expression pattern is related to the maturation of the storage parenchyma cells, specifically a function related to storage root formation.

Parenchyma cells are responsible for storage of photo assimilate (Stewart, 1983) this count for the low root size (RS), low root number (RN) and low shoot weight for the Kaleso genotype. Parenchyma cells, for the most part, resemble the undifferentiated cells produced by division of meristematic cells (Simpson, 2006). They vary in shape from elongate to isodiametric, although they can also be square or rectangular, as found frequently in the ray parenchyma of secondary xylem and phloem. Parenchyma cells are alive at maturity and

have the ability to dedifferentiate, which is particularly important during events such as injury. The tissue forms the most underground part of cassava plant.

FR U1 (Unknown seed protein like 1 (USPL1) is highly expressed in fibrous roots in Kaleso and has low expression level in TMEB14 and 1090576 for fibrous roots. The expression level is consistently low in storage roots of all genotypes. There are many typical genes associated with that have been reported in other crops like legumes, including the gene At1g49320, which encodes a protein with strong similarity to the Unknown Seed Protein (USP) of *Vicia faba*, described in the introduction. In this study this gene is named AtUSPL1. A second gene, At5g25610 named AtRD22 encodes the previously described gene product responsive to droughtness AtRD22 in which they are responsible for responsiveness of crop to droughtness which goes parallel with the absorption property of fibrous roots.

SR – D2 NAD (P)-binding Rossmann-fold is highly expressed in Kaleso for both storage and fibrous roots but slightly higher in fibrous roots, while it differentially expressed in fibrous roots and storage roots in 1090576 highly upregulated in fibrous roots and down regulated in storage roots. The gene expression was in contrast also to TMEB419 genotype where is upregulated ion storage roots and down regulated in fibrous roots.

However studies conducted on Arabidopsis has revealed function the protein domain as oxidoreductase activity, binding, catalytic activity; involved in oxidation reduction, metabolic process, located and located unknown cellular component, expressed in male gametophyte, flower expressed during mature pollen stage, germinated pollen stage, protein binding domain, glucose/ribitol dehydrogenase, short-chain dehydrogenase/reductase, best *Arabidopsis thaliana* protein match binding Rossmann-fold superfamily protein.

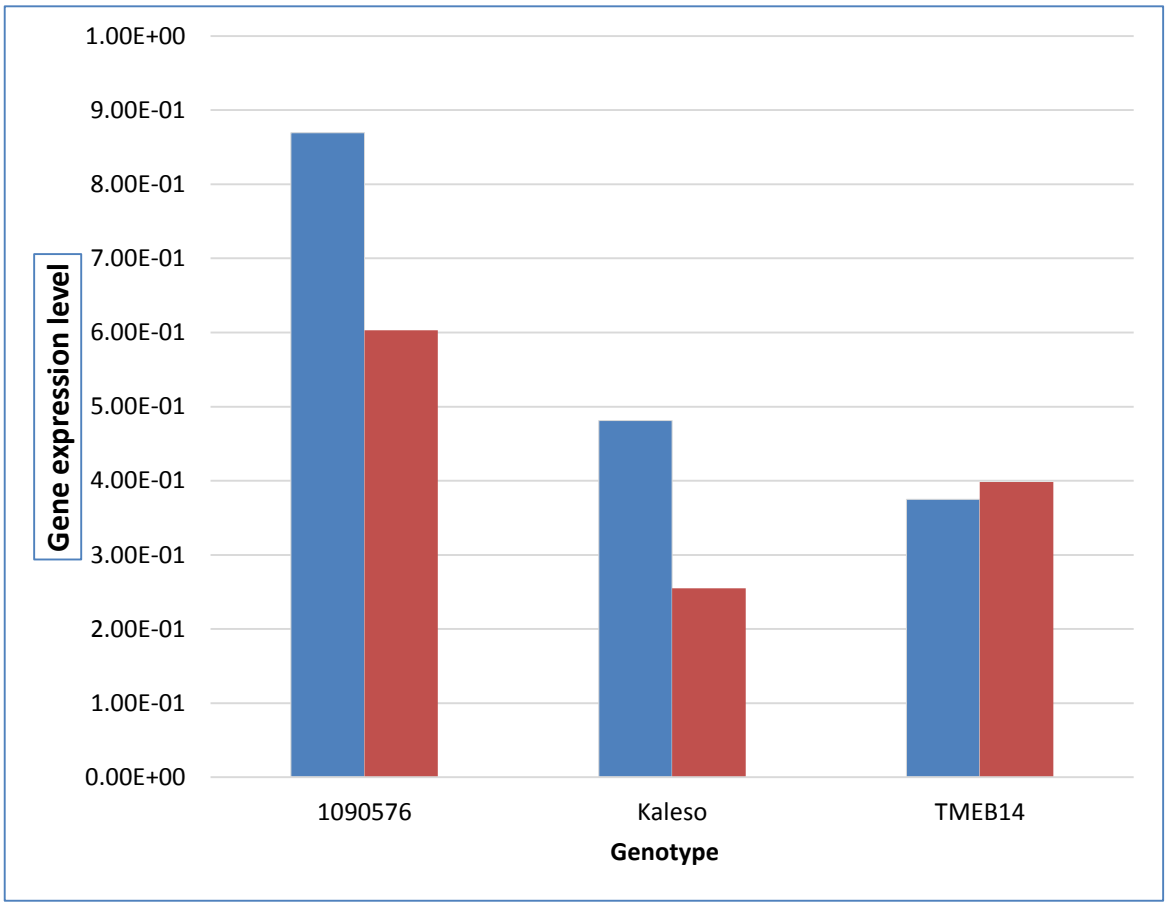

Figure 4.2.5: Histogram showing gene expression levels, storage roots upregulated (SR-U3) in storage roots and fibrous roots from, Kaleso, TMEB14 and 1090576 genotypes

Key

=Storage roots

=Fibrous roots

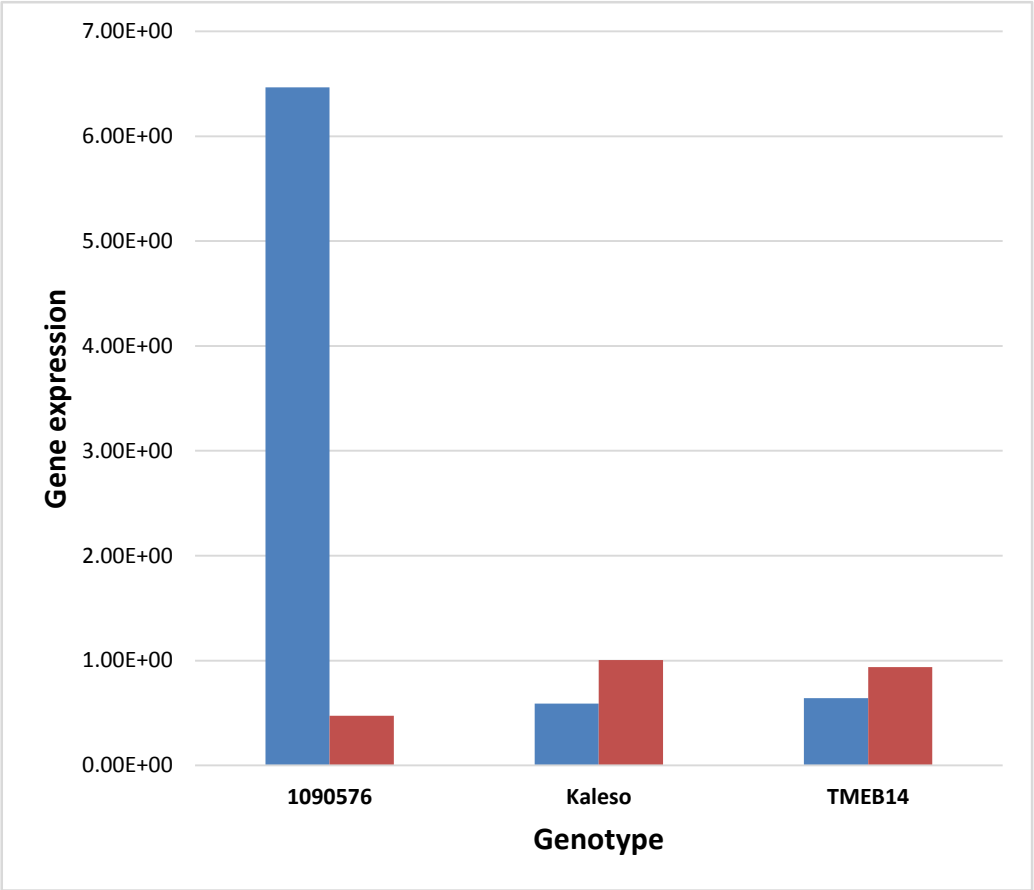

Figure 4.2.6: Histogram showing gene expression levels, storage roots upregulated (SR-U4) for storage roots and fibrous roots from Kaleso, TMEB14 and 1090576 genotyupes.

Key

=Storage roots

=Fibrous roots

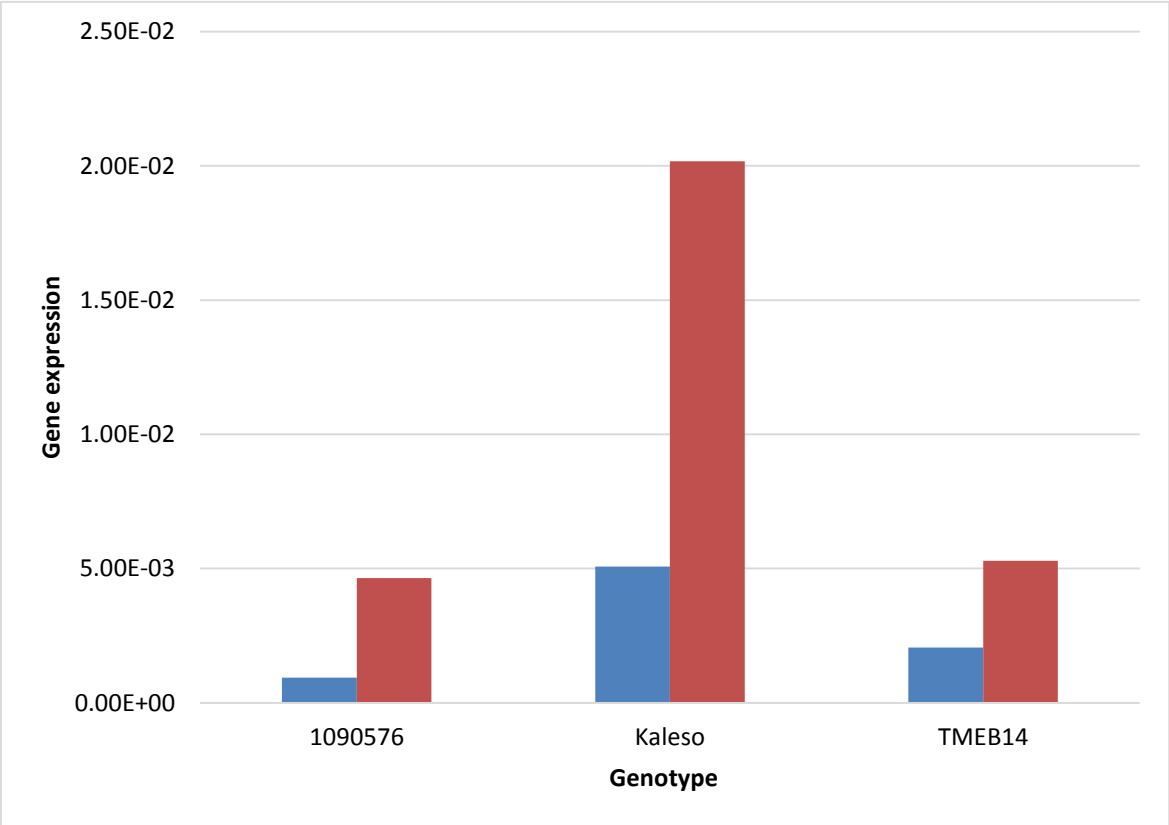

Figure 4.2.7: Histogram showing gene expression levels, fibrous roots up regulated (FR U1) in storage roots and fibrous roots from Kaleso, TMEB14 and 1090576.

Key

=Storage roots

=Fibrous

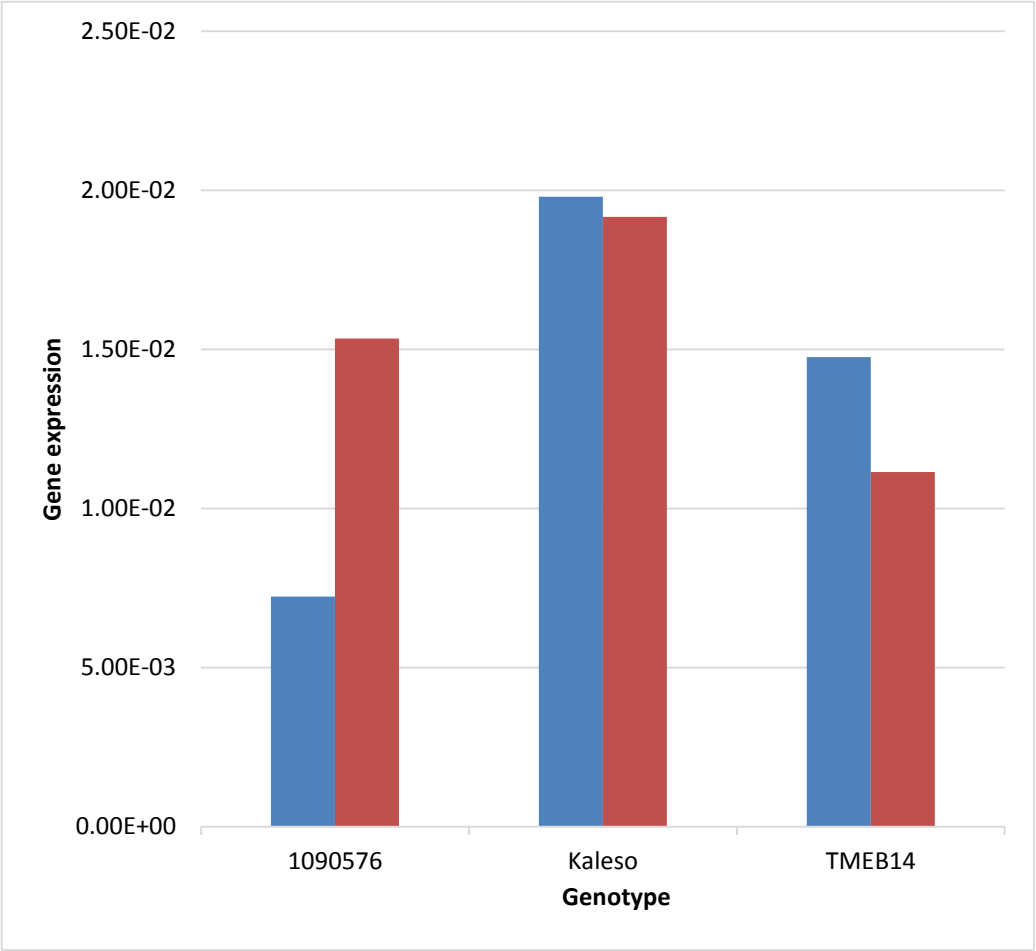

Figure 4.2.8: Histogram showing gene expression levels, storage roots down regulated (SR-D2) in storage roots and fibrous roots from Kaleso, TMEB14 and 1090576

Key

=Storage roots

=Fibrous

Figure 4.2.9: TMEB14 and Kaleso roots respectively 44 days after planting where is not easy to distinguish fibrous roots from storage roots by eyes but color and coarseness have been used to isolate each other. Whitish and soft roots are considered potential fibrous

Figure 4.3.1:1090576 and TMEB 419 roots respectively 44 days after planting where is not easy to distinguish fibrous roots from storage roots by eyes but color and coarseness have been used to isolate each other. Whitish and soft roots are considered potential fibro

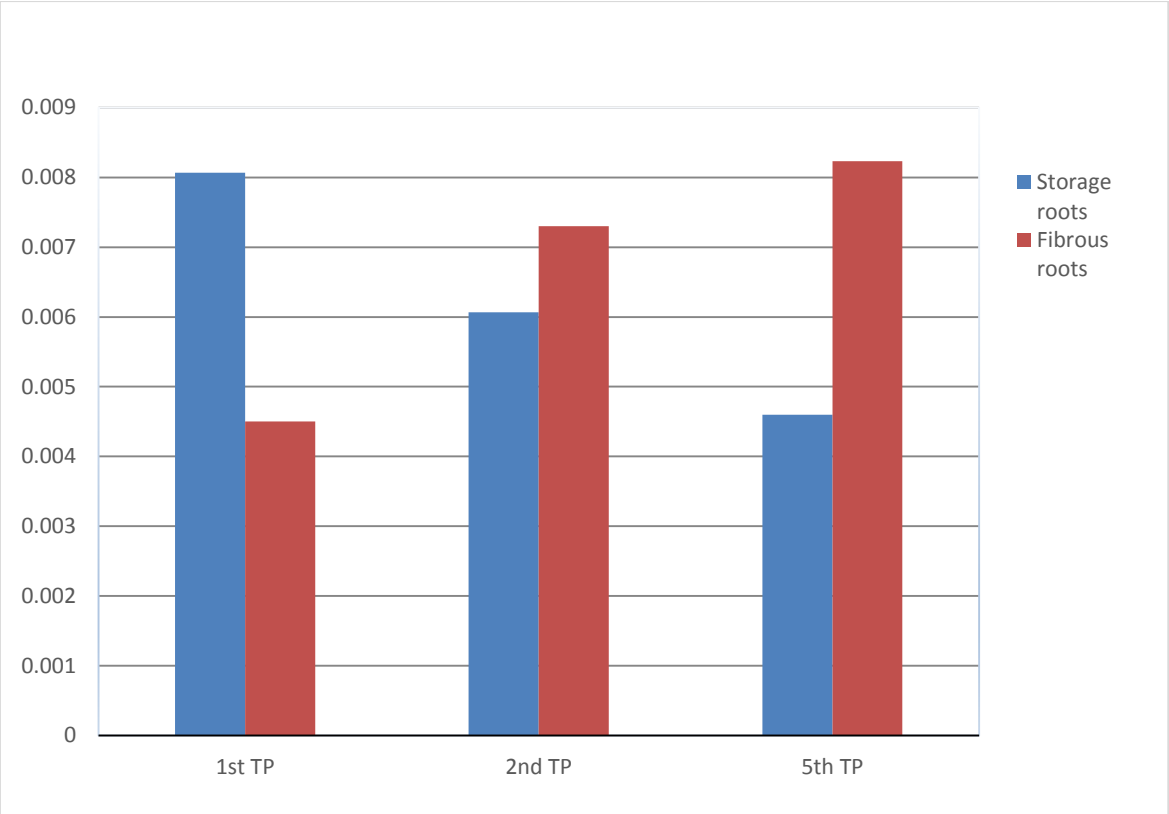

Figure 4.3.2: Histogram showing gene expression levels, Fibrous roots up- regulated (FR-U) in storage roots and fibrous roots from at first, second and fifth time point in TMEB 419

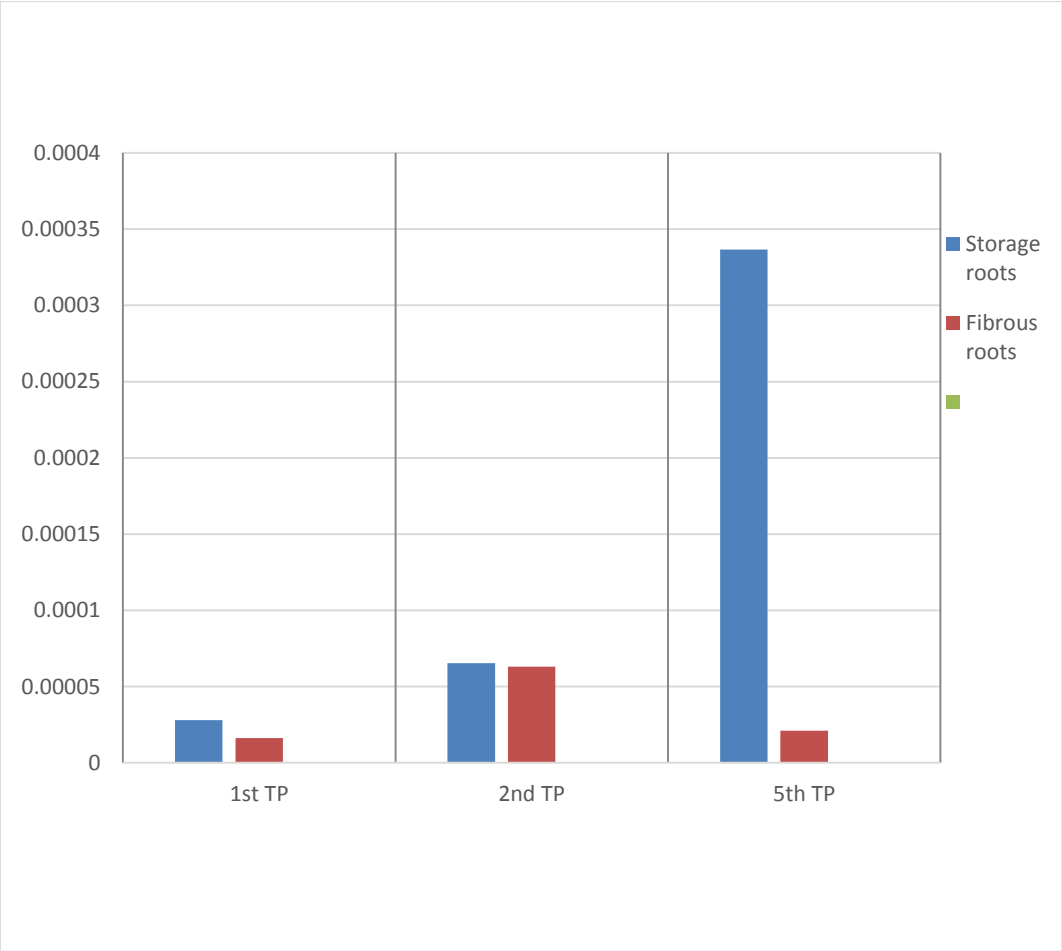

Figure 4.3.3: Histogram showing gene expression levels, Storage roots constantly expressed gene (SR-E) in storage roots and fibrous roots from at first, second and fifth time points in TMEB 419.

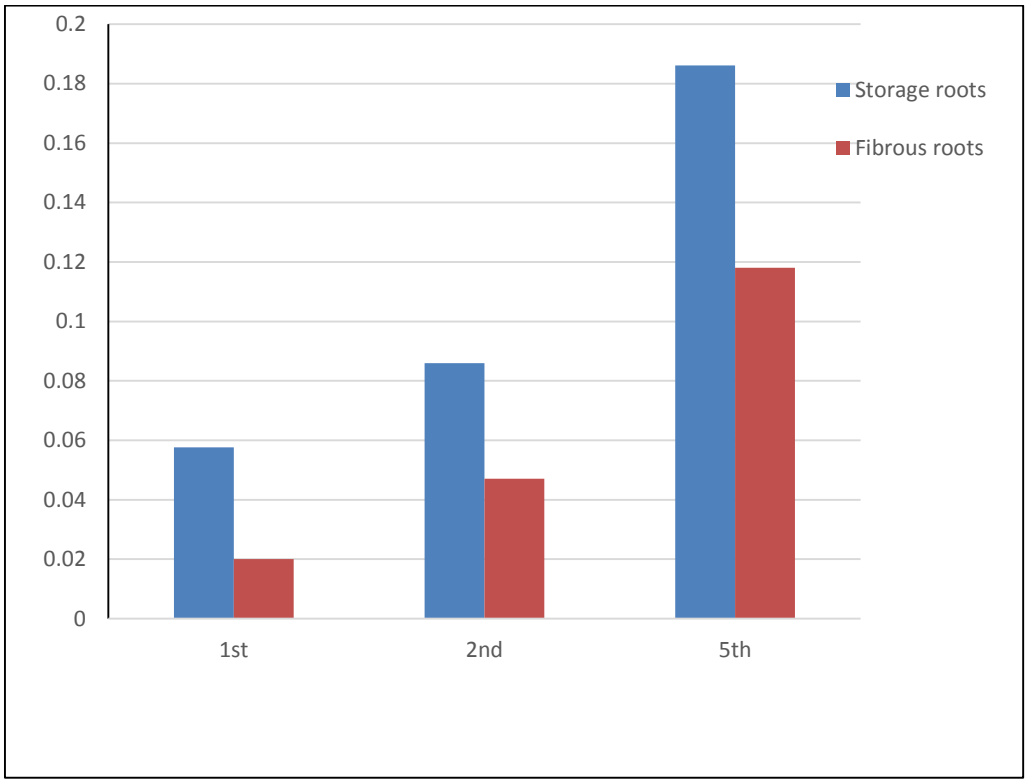

Figure 4.3.4: Histogram showing gene expression levels, Storage roots Up-regulated gene (SR-U3) in storage roots and fibrous roots from at first, second and fifth time points in TMEB 419.

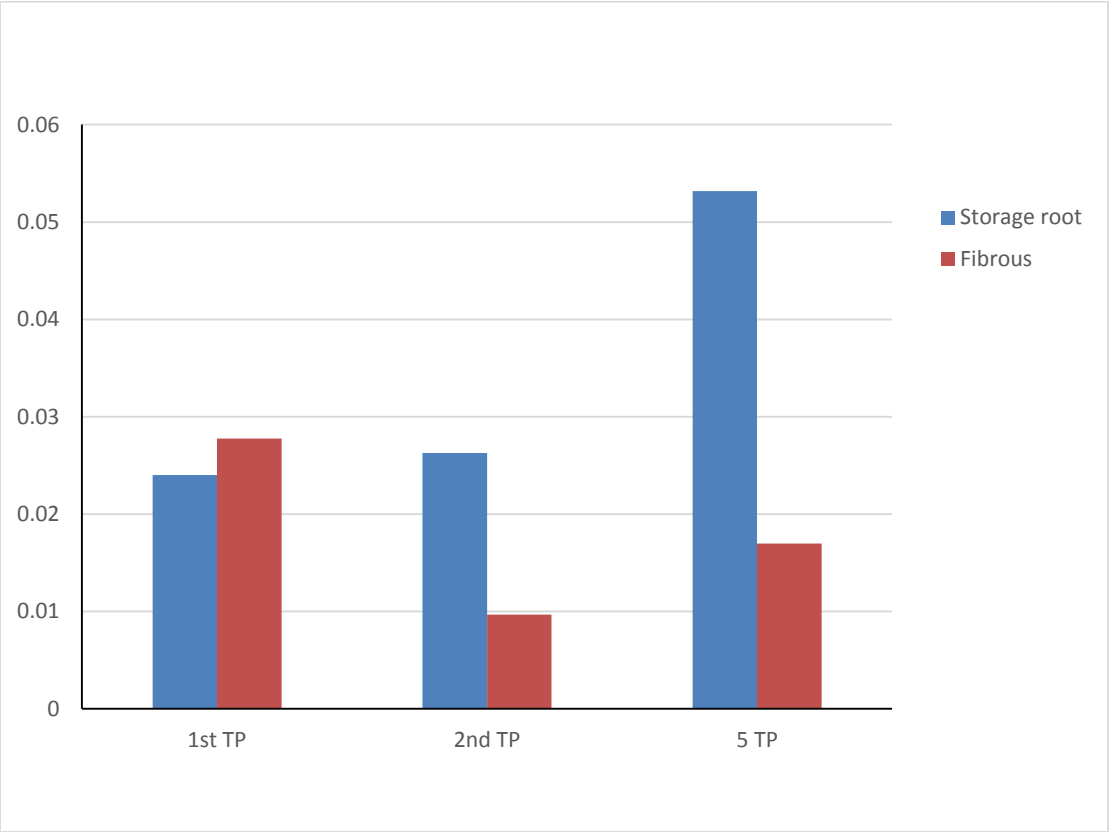

Figure 4.3.6: Histogram showing gene expression levels, Storage roots Up-regulated gene (SR-U4) in storage roots and fibrous roots from at first, second and fifth time points in TMEB 419.

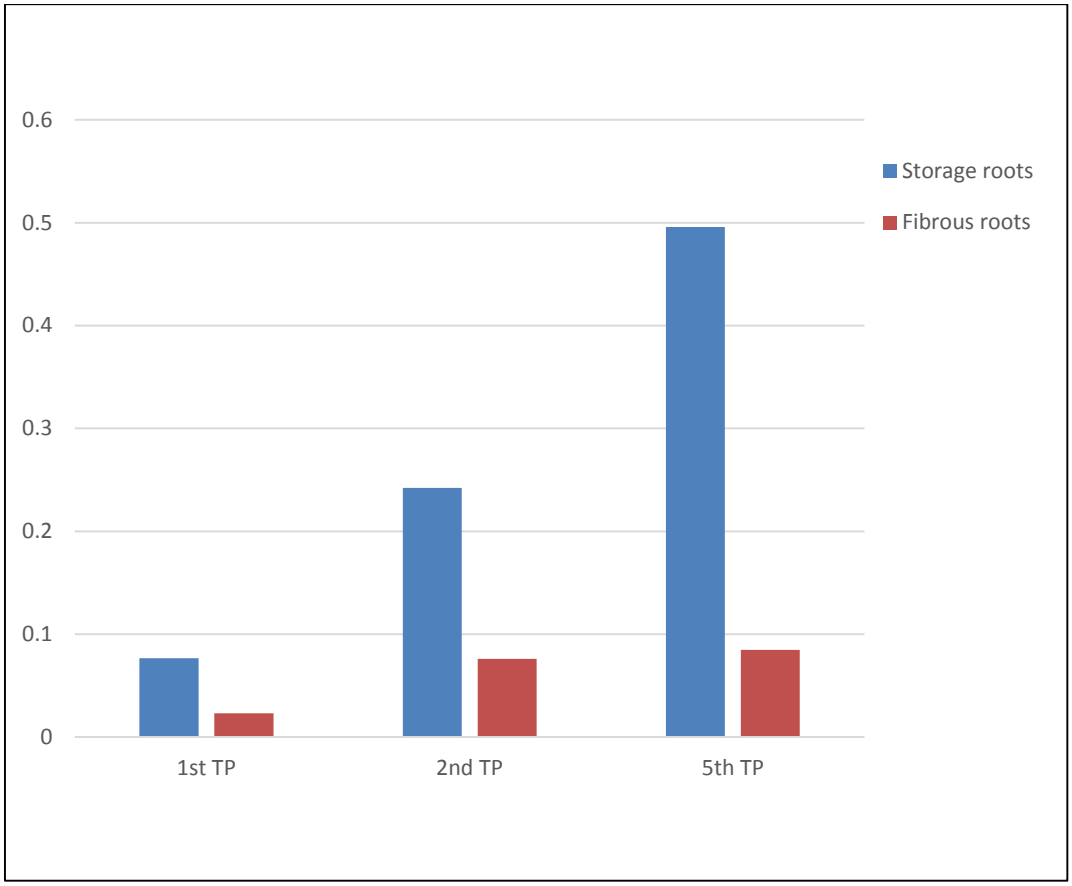

Figure 4.3.7: Histogram showing gene expression levels, Storage roots Up-regulated gene (SR-U5) in storage roots and fibrous roots from at first, second and fifth time points in TMEB 419.

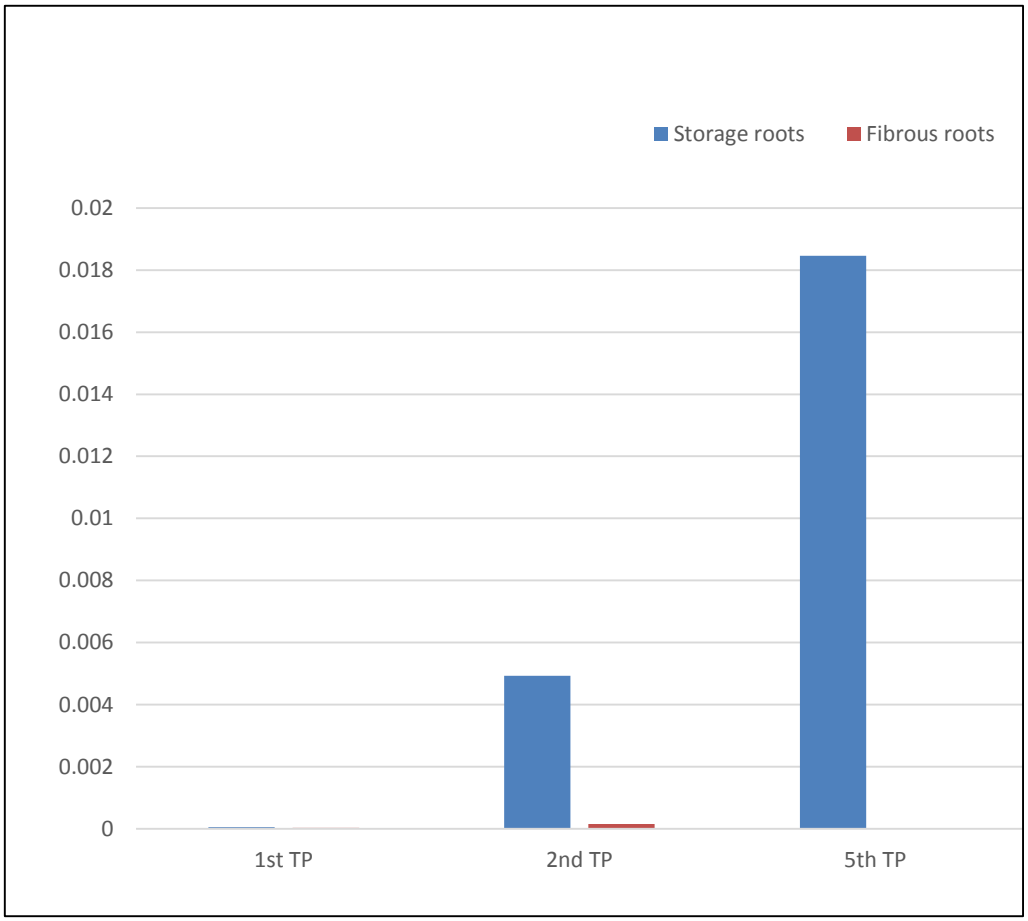

Figure 4.3.8: Histogram showing gene expression levels, Storage roots down-regulated gene (SR-D2) in storage roots and fibrous roots from at first, second and fifth time points in TMEB 419.

4.5 Cassava mosaic diseases (CMD)

CMD is widespread, occurring in all countries in Africa where cassava is grown. The disease is spread in cuttings, which may be symptomless, and by whiteflies. Disease management has concentrated on the use of resistant cultivars. While this is an important strategy to reduce yield losses, more attention should be paid to the use of clean planting material, early detection of CMD in fields and removal of plants. Cassava was the predominant food crop grown in many countries in the world with varying yield performance. The formation and development of storage roots were studied through monitoring the storage root and fibrous root development. This was done at different time point to confirm the gene expression levels for reference genotype and other selected genotype to see whether they have the same development pattern**.** Because of the extrinsic factors that may affect gene expression, cassava mosaic virus disease was also monitored.

Cassava mosaic disease was present in all genotypes except I102103, I051599, I071313 and I090454 which was clean from cassava mosaic disease at 54 days after planting other genotypes was affected by Cassava mosaic virus at varying degree for both severity and incidence. Disease incidences were significantly different ($P<0.050$) between genotypes (Table 4.1 & 4.2).

CMD moderate incidence was the highest record for genotype O8100045, for all other genotypes the CMD incidence was recorded low. Genotype TMBE 419 has moderate disease incidence but the level of severity was low. This situation might be explained by plant resistance to diseases.

Table 4.1: Analysis of variance for Cassava Mosaic Disease (CMD) incidence at 54 days after planting for 13 genotypes

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Rep	2	184.615385	92.307692	0.77	0.4747
Clone	12	4564.102564	380.34188	3.17	0.0078
Error	24	2882.051282	120.08547		
Total	38	7630.769231			

**Significant at 0.01

LSD 18.467

Table 4.2: Analysis of variance for Cassava Mosaic Disease (CMD) incidence at 54 days after planting for 13 genotypes

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
REP	2	–	–	0.46	0.6367
Clone	12	22.717	1.622	2.75	0.0167
Error	24	16.051	0.668		
Total	38	38.769			

**Significant at 0.01

LSD 1.3781

5. 0 SUMMARY CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

Cassava storage roots are used for food security and are increasingly important as an industrial commodity in developing economies. However, the biology of root formation in cassava is poorly understood. The main hypothesis of this research is identification of specific genes that are differentially expressed during root bulking in cassava at different time point and increase the knowledge of storage roots bulking to facilitate the early time point selection for root bulking trait.

To elucidate the mechanisms involved with storage root bulking in cassava, a transcriptomic study of cassava storage roots and fibrous roots formation was undertaken along with phenotypic information.

Therefore, in this study, we have verified the involvement of these genes in root bulking development by using quantitative real time PCR (RT-qPCR) to test the regulation of their expression levels at several time points during storage root development, and in different genotypes.

The gene expression levels in multiple genotypes Kaleso, TMEB14 and 1090576 were performed along with reference genotype 419 to investigate the regulatory genes related to the different stages involved in storage roots development.

RT-qPCR has shown to be effective technique to investigate and elucidate unknown molecular mechanisms of cassava bulking, which is a unique process among the major roots and tuber crop species.

This study was conducted to confirm the gene expression levels for cassava storage root and fibrous root formation. Some genes have been found to be differentially expressed through transcriptomic studies on cassava genotype TMEB 419.The genes are GDSL-like lipase, UDP-glucosyl transferase, NAD (P)-binding Rossmann-fold, KNOTTED 1-like protein, Light-Dependent Short Hypocotyls, Pt2L4 glutamic acid-rich protein, P54 glutamic acid-rich protein, Unknown seed protein like 1 (USPL1) and NAD(P)-binding Rossmann-fold

Cassava genotype TMEB 419 was sequenced in a series of time point, time point one (30 days after planting, time point two (42 days) and time point seven (60 days) after planting. At first time point some roots start to behave as potential storage roots and fibrous roots.

Some these roots were tagged and their development monitored and these expected to bulk have bulked and these expected to be fibrous did not bulk.

These roots were sampled for transcriptomic studies to study the gene expression levels, some gene were differentially expressed in fibrous roots and storage roots. Some of the genes were highly expressed in storage roots and fibrous roots while others are equally constantly expressed through all time points and, up regulated in fibrous root. This transcript was only from TMEB419 genotype to confirm the gene expression level study was conducted with multiple genotypes at 60 days after planting; TMEB 419 the root development was monitored. The study of the gene expression on storage root and fibrous roots formation was also supported by phenotypic information for multiple genotypes. The phenotypic information was used to select multiple genotypes for the analysis of the gene expression in which the criteria used were root size, root number, dry matter, root weight and sprouting.

In prior to relative RT-qPCR, the amplification efficiency of gene expression was evaluated by processing standard curves related to nine fragments of gene sequence and three housekeeping genes and 9 target genes but for the confirmation studies only 6 target genes and MeActin gene was used as housekeeping gene. The criteria for using the above gene were their good kinetics in melting curve slope and efficiency.

With respect to confirmation with multiple genotypes I found positive correlation between the results from RNA Seq with the genotype 1090576 where most of the genes were regulated similar to reference genotype. Confirmation with TMEB 419, in some cases has shown different results at early time point this may be associated with the sample selection error that some storage roots have been sampled as fibrous roots. Fifth time point has the similar expression pattern as indicated in RNA Seq.

5.2 Recommendations

This is the preliminary study for the analysis of the key genes responsible for storage roots formation and development in cassava and I found very interesting genes that are associated with the root bulking in multiple genotypes. This might be very important breeding strategy to develop a marker that will help breeders to select roots bulking traits at the early stage of crop development.

Moreover, I propose more traits associated to roots bulking to be involved for further research by using the traits I used in this thesis.

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