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THESIS

CLONING AND MOLECULAR CHARACTERIZATION OF *MeAMT2*, *MeNRT2*, *MePT1* AND *MeZIP* TRANSPORTER GENES IN THAI CASSAVA UNDER TISSUE CULTURE CONDITIONS

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Under tissue culture conditions, cassava (*Manihot esculenta* Crantz var. HB80) *MeAMT2*, *MeNRT2*, *MePT1* and *MeZIP* cDNAs' encoding for ammonium transporter (*AMT*), nitrate transporter (*NRT*), inorganic phosphate transporter (*PT*) and zinc transporter (*ZIP*) were isolated from developing fibrous roots. The cDNAs comprised 1,578 1,710 1,743 and 723 bp for *MeAMT2*, *MeNRT2*, *MePT1* and *MeZIP*, and the proteins were highly homologous with those from other plant species, respectively. DNA blot analysis indicated that at least three copies of *MePT1* and at least two copies of *MeAMT2*, *MeNRT2* and *MeZIP* are present in the cassava genome. For steady-state transcript accumulation of these transporter genes and some growth and development parameters under tissue culture conditions, 3 Thai cassava varieties, Huaybong 80 (HB80), Kasetsart 50 (KU50) and Rayong 1 (R1) were used for evaluation. It was found that at 8 weeks after cultured in MS media, total dry weight (TDW) was highest in +1E treatment in all three varieties and HB80 showed higher TDW than those of KU50 and R1 by 23.71% and 12.89%, respectively. In +1E treatment of all varieties, leaf, petiole, stem and fibrous root lengths were greater than those obtained from other treatments. In different plant parts and under different tissue culture mediums, levels of differential expression of *MeAMT2*, *MeNRT2*, *MePT1* and *MeZIP* genes was highest in stems, fibrous roots, stems and leaf, respectively. The result also showed that the expression levels of *MeAMT2*, *MeNRT2* and *MeZIP* genes were greater in +1E treatment when compared with others, reflecting the highest fresh weight, dry weight, leaf number, fibrous root number, leaf length, stem length, petiole length and fibrous root length, respectively. However, the *MePT1* gene showed decreasing expression trend when compared with control. Overall, levels of expression of these genes were most pronounced between 5-6 weeks after cultured which can be explained that the nutrients were highly utilized for starting growth and development during this period. When compared among the varieties, it was found that the expression levels of these genes in R1 were greater than those of KU50 and HB80, reflecting the highest total fresh weight in this observation.

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Tanawat Bamrungsetthapong

May, 2010

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LIST OF ABBREVIATIONS

AMT	=	Ammonium transporter
bp	=	Base pair
CDP-Star	=	Disodium 4-chloro-3-(methoxyspiro {1,2-dioxetane-3, 2'-(5'-chloro) tricyclo [3.3.1.1.3,7] decan}-4-yl)-1-phenyl phosphate
cm	=	Centimeter
dH_2O	=	Distilled water
DEPC	=	Diethylpyrocarbonate
DIG-11-dUTP	=	Digoxigenin-11-uridin-5'-triphosphate
DTT	=	Dithiothreitol
DNA	=	Deoxyribonucleic acid
EDTA	=	Ethylene diamine tetraacetic acid
g	=	Gram
hr	=	Hour
IPTG	=	isopropyl-beta-D-thylogalactopyranoside
kb	=	Kilobase
LB	=	Luria bertani broth
LBA	=	Luria bertani agar
µg	=	Microgram
µl	=	Microlitre
M	=	Molar
min	=	Minutes
mM	=	Millimolar
mg	=	Milligram
ml	=	Millilitre
N	=	Normal
ng	=	Nanogram
NRT	=	Nitrate transporter
OD	=	Optical density

LIST OF ABBREVIATIONS (Continued)

RACE	=	Rapid amplify cDNA end
PCI	=	Phenol: Chloroform: Isoamyl alcohol
PCR	=	Polymerase chain reaction
PT1	=	Inorganic phosphate transporter
RNA	=	Ribonucleic acid
RT	=	Room temperature
RT-PCR	=	Reverse transcriptase- polymerase chain reaction
rpm	=	Rounds per minute
SDS	=	Sodium dodecyl sulphate
SSC	=	Standard saline citrate
TAE	=	Tris acetate EDTA
TBE	=	Tris borate EDTA
UV	=	Ultraviolet
V	=	Volts
X-gal	=	5-bromo-4-chloro-3-indoyl-beta-D-galactopyranoside
ZIP	=	Zinc transporter

CLONING AND MOLECULAR CHARACTERIZATION OF *MeAMT2, MeNRT2, MePT1* AND *MeZIP* TRANSPORTER GENES IN THAI CASSAVA UNDER TISSUE CULTURE CONDITIONS

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is an important root crop grown in many tropical and subtropical countries. It is considered to be a source of starch from its tuberous roots. In Thailand, cassava is a major starch source, both for domestic consumption and export. The increase in both planting areas and production makes Thailand, currently, recognized as the world leader in cassava production. Thus, the intense effort to improve the starch yield derived from the combination of root yield and starch content, have been put in by researchers both in academic and industrial sectors. Research to elucidate the regulation of yield maximization of cassava tuber has become our main focus. The integration of basic knowledge in cassava physiology, biochemistry and molecular biology will develop a new body of knowledge to understand the regulation of nutrients uptake and will certainly assist the breeding program to develop the traits of interest in a shorter timeframe and lower costs. Thus, the nature of high production that reflects the differences of the nutrients uptake (macro- and micronutrient) of cassava has become an important consideration. Cassava researchers then put a tremendous effort to understand the processes involving in nutrients uptake.

In addition, the molecular identification and characterization of genes involving in nutrient transporter in cassava is our major goal. However, the mechanisms of nutrient uptake regulations at the physiological, biochemical and genetic levels are still unclear. Due to its complexity for the biochemical regulation of nutrient uptake and the unanswered molecular mechanisms, this research would emphasize on the investigation of the expression of macro- and micronutrient genes, controlling the major step of growth and development. This would lead to the understanding of the changes in the processes related to macro- and micronutrient

uptake during plant development, especially in the period of tuberous initiation and formation would put together another piece of jigsaw puzzle to reveal the major factors affecting the quantity and quality of cassava yield and starch.

Another major problem for the cassava tuber production is a low yield production and starch content. Therefore, the selection for cultivars which show uniformity and high yield and starch is necessary. Consequently, this study was performed to classify the genetic diversity of *Manihot esculenta* collected from germplasm based on starch component and molecular markers and to generate a database of *Manihot* collection, which can be used for crop improvement program.

OBJECTIVES

1. To clone, sequence and characterization transporter genes from Thai cassava.

2. To study expression of transporter genes at different stages of development and in different parts of cassava tissues compared with physiological characteristics.

LITERATURE REVIEW

Cassava

Cassava is one of the staple food-crops in the tropics. It is considered the largest carbohydrate producer per unit area. The standard carbohydrate content of cassava root is 30 percent with an average calories value of 109 calories per 100 g of roots and protein content of 0.9 percent (Illaco, 1981). A cassava diet, however, owing to the lack of other nutritive components, is rather unbalanced. Considerable research efforts are now being conducted in order to meet increasing food demand and generate income for the farmer in the tropics (Nestel and Cock, 1976). Cassava is a perennial shrub of the family Euphorbiaceae that includes Para rubber (*Heavea brasiliensis*) and caster bean (*Ricinus communis*). Cassava may be propagated either by stem cuttings or propagation from seed that is used only in breeding programs. The cuttings are commonly 10 to 30 cm long and are taken from the woody parts of mature plants (Cock, 1985). After planting, most of the auxillary buds on the cuttings begin to develop, but growth of the shoots at the proximal end of the cutting suppresses development of the other buds (Wholey, 1974).

The cassava plants originate in northeast Brazil. *M. esculenta* might have resulted from introgressive hybridization among a number of wild species and might have no direct wild correspondent, even though the Central American species *M. aesculifolia* is morphologically closely related to it. Allem (2002) identified a single South American wild species and two subspecies: *M. esculenta* subsp. *flabellifolia* and subsp. *peruviana.* Recent molecular data support Allem's hypothesis by revealing a greater affinity between cassava and the South America species, in particular *M. elabellifolia* and *M. peruviana* (Fregene *et al.,* 1994). Cassava began spreading in the Old World in the sixteenth century when Portuguese sailors supplied it to settlements along the coasts of Africa, India and the Philippines. Cassava was first grown in Thailand some time around 1850, primarily for human consumption. It has become popular in the eastern seaboard provinces during the past 50 years. Since 1956, cassava growing has spread to the provinces in the northeastern, western and

upper central parts of Thailand (Titapiwatanakun, 1984). This leads Thailand to be now entitled the leading cassava product exporter.

Currently there are many commercial cassava cultivars in Thailand. Rayong1 (R1) or native cultivar is the most important cultivars for industrial purpose. It was selected from local cultivars and had higher yields than all the introduced cultivars from Java and Virgin Islands. In 1975 the Department of Agriculture released it as the first recommended variety. R1 possesses excellent agronomic characteristics such as high yield, good germination, good plant type, wide adaptation, medium to high harvest index and moderate root starch content (Kawano *et al*., 1984). KU50 (Kasetsart 50) is currently the most popular variety (cultivated about 56% of the total cassava planting area). It contains higher starch percentage than R1. In the past decade, Kasetsart University consequently released two of becoming more popular, Huay Bong 60 (HB60) in 2003 and Huay Bong 80 (HB80) in 2007.

Kasetsart University research team reported that MKUC34-114-106 (HB80) has higher starch percentage (Sipunya *et al*., 2007) than KU 50 and R1 and becoming one of the needed varieties among farmers these days (Sarakarn *et al.,* 2002 and Rojanaridpiched *et al.,* 2002).

Cassava Starch

Starch serves as a storage carbohydrate in most plant species. Cassava starch is utilized as a component of human diet in the tropical and as feed stock (Jennings and Hershey, 1985). It is also becoming extremely important in the brewing and fermentation industries, and the paper and textile industries (Cooke and Cock, 1989; Ozbek and Yuceer, 2001). The alternate energy source derived from cassava, gasohol, is also another clear evidence of its increased importance.

In green tissues, starch is synthesized in the chloroplast as the product of photosynthesis and is degraded to provide substrate for respiration (Preiss and Levi, 1980). Starch is often accumulated in plant cell cultures, where it serves the

respiration needs for growth and organogenesis (Stitt and Steup, 1985). In non-green and storage organs such as fruits and roots, starch is existed as long-term storage (Steup, 1988). The starch synthesis in chloroplasts is called transitory or short-term storage of starch and that in amyloplasts is called reserve-term storage of starch. The turnover rate of the latter type of starch in non-photosynthetic tissues such as endosperm, roots and tubers is very low. It normally remains unchanged until the next growing season, when it is rapidly mobilized to support growth. In contrast, starch in the chloroplast turn over very rapidly. The reserve starch in cereals often represents 65-75 % and up to 80 % of dry weight (Martin and Smith, 1995). In cassava, most of the starch is stored within amyloplasts in the thickened roots commonly known as tubers. The starch content in tuber varies from 73.7-84.9 % of dry weight basis and also consists of about 20% amylose and containing a conical pit. An eighteen-month investigation on granule size variation by Munyikwa *et al*. (1994) indicated an increase in size of the granules up to the 6th month after planting. The starch quality is affected by the age of harvest roots and conditions during growth such as rainfall and temperature during growth and harvest (Santisopasri *et al*., 2000).

Plant Nutrients

The essential nutrients required by higher plants are exclusively inorganic, a feature distinguishing these organisms from man, animals and many species of microorganisms which additionally need organic foodstuffs to provide energy. In contrast, plants absorb light energy from solar radiation and convert it to chemical energy in form of organic compounds, whilst at the same time taking up mineral nutrients to provide the chemical elements which are also essential for growth.

The plant nutrients may be divided into macronutrients and micronutrients. Macronutrients are found and needed in plants in relatively higher amounts than micronutrients. The plant tissue concentration of the macronutrient N, for example, is over a thousand times greater than the concentration of the micronutrient Zn. Using this classification based on the element concentration in plant material, the following elements may be define as macronutrients; C, H, O, N, P, S, K, Ca and Mg, while

micronutrients are as follows; Fe, Zn, Mn, Cu, Mo, B, Cl and Ni. This division of the plant nutrients into macro- and micronutrients is somewhat arbitrary and, in many cases, differences between the concentration of macronutrients and micronutrients are considerably less well defined than the example cited above. The concentration of the micronutrients is also often far in excess of physiological requirements (Mengel and Kirkby, 2001).

For an element to be described as essential three criteria must be met. These criteria are as follows (Mengel and Kirkby, 2001);

1. A deficiency of the element makes it impossible for the plant to complete its life cycle.
2. The deficiency is specific for the element in question.
3. The element is directly involved in the nutrition of the plant, for example, as a constituent of an essential metabolite or required for the action of an enzyme system.

Nitrogen (N)

Nitrogen is one of the most widely distributed elements in nature. It presents in the atmosphere, the lithosphere and the hydrosphere (Delwiche, 1983). The soil accounts for only a minute fraction of lithospheric N, and of this soil N, only a very small proportion is directly available to plants. This occur mailnly in the form of NO_3^- or NH_4^+. Nitrogen is a very mobile element circulating between the atmosphere, the soil and living organisms. N, nevertheless, is an indispensable elementary constituent of numerous organic compounds of general importance, amino acids, proteins, nucleic acids and compounds of secondary plant metabolism such as the alkaloids. Higher plants are major contributors to the large amount of N which is continuously being converted from the inorganic to the organic form. The most important sources involved in this conversion are NO_3^- and NH_4^+ (Mengel and Kirkby, 2001).

In contrast to other plant nutrients nitrogen may be taken up in the form of a cation as NH_4^+ or anion as NO_3^-. Uptake rate of both depends mainly on the availability of these ions in the nutrient medium in general when one NH_4^+ may substitute for NO_3^-. Uptake rates are determined mainly by the physiological need of the plant and not so much on whether the source is a cation or anion (Mengel *et al*., 1983). Many species take up NH_4^+ preferentially to NO_3^- including those of forest trees (Marschner *et al*., 1991) and grasses.

Nitrate is a major source of nitrogen for plants. Indeed, most plants devote a significant portion of their carbon and energy reserves to its uptake and assimilation. Nitrate serves both as nutrient and signal and has profound effects on plant metabolism and growth. Plants have evolved intricate mechanisms to detect nitrate and to integrate its assimilation with photosynthesis and overall metabolism of nitrogen and carbon. These mechanisms allow plants to control growth rates, root architecture, carbon/nitrogen ratios, concentrations of reductants, and ionic and pH balances under diverse environmental conditions. As nitrate diffuses from the soil solution into the apoplast of the root, it is taken up by the epidermal and cortical cells. Plants utilize both high-affinity and low-affinity transport mechanisms to import nitrate. The high-affinity system is further divided into a constitutive component, which is expressed in the absence of nitrate, and an inducible component, which is induced by nitrate treatment. The low-affinity transport system is most observed at nitrate concentrations above 0.5 mM and usually displays nonsaturating uptake kinetics (Buchanan *et al*., 2000).

Nitrogen deficiency is characterized by a poor growth rate. The plants remain small, the stems have a spindly appearance, the leaves are small and the older ones often fall prematurely. Root growth is also affected and in particular branching is restricted. The root/shoot ratio, however, is usually increased by N deficiency. Nitrogen deficiency results in the collapse of chloroplasts and also in a disturbance of chloroplast development. Hence leaves deficient in N show chlorosis which is generally rather evenly distributed over the whole leaf. Necrosis of leaves or parts of the leaf occurs at a rather late and serves stage in the deficiency (Mengel and Kirkby,

2001). Deficiency symptoms of Fe, Ca and S are also similar to N deficiency being characterized by yellowish and pale leaves. In these deficiencies, however, the symptoms occur first in the younger leaves. These more general observations may be used to serve as a first means of distinguishing between these various nutrient deficiencies.

Phosphorus (P)

Phosphorus is an important plant macronutrient, making up about 0.2% of plant's dry weight. It is a component of key molecules such as nucleic acids, phospholipids and ATP. P availability is considered as one of the major growth-limiting factors for plants in many natural ecosystems (Barber *et al*., 1963) and is acquired by roots via high-affinity inorganic phosphate (Pi) transporters (Liu *et al*., 1998). Pi is also involved in controlling key enzyme reactions and in the regulation of metabolic pathways (Theodorou and Plaxton, 1993). Plants roots are capable of absorbing phosphate from solutions of very low phosphate concentrations of only a few mmol/m^3. Generally the phosphate concentration in the cytosol of plant cells is in the range of 5 to 8 mol/m^3 (Lauer *et al*., 1989) and, therefore, about a thousand folds greater than in the soil solution. Thus phosphate is taken up by plant cells against a very steep concentration gradient. Uptake is mediated by H^+ cotransport (Tanner and Caspari, 1996). Soil P is found in different pools, such as organic and mineral P. It is important to emphasize that 20 to 80% of P in soils is found in the organic form, of which phytic acid is usually a major component (Richardson, 1994).

The uptake of P poses a problem for plants, since the concentration of this mineral in the soil solution is low but plant requirements are rather high. The form of P most readily accessed by plants is Pi, therefore, plants must have specialized transporters at the root/soil interface for extraction of Pi from solutions of micromolar concentrations, as well as other mechanisms for transporting Pi across membranes between intracellular compartments, where the concentrations of Pi may be thousand folds higher than in the external solution. There must also be efflux systems that play a role in the redistribution of this precious resource when soil P is no longer available

or adequate (Schachtman *et al*., 1998). Under normal physiological conditions there is a requirement for energized transport of Pi across the plasma membrane from the soil to the plant because of the relatively high concentration of Pi in the cytoplasm and the negative membrane potential that is characteristic of plant cells. This energy requirement for Pi uptake is demonstrated by the effects of metabolic inhibitors, which rapidly reduce Pi uptake.

Plants suffering from P deficiency are small and stunted and have a rigid erect appearance. Young P-deficient plants have a bluish green colour in the early stages of growth. In cereal, tillering is decreased, while fruit trees show reduced growth rates of new shoots and flower initiation is impaired. The formation of fruits and seeds is especially depressed in plants suffering from P-deficiency. Thus not only low yields but also poor quality fruits and seeds are obtained from P deficient crops (Mengel and Kirkby, 2001). Generally the symptoms of P-deficiency appear in the older leaves which are often of a darkish green colour. The stems of many annual plant species suffering from P deficiency are characterized by a reddish colouration originating from an enhanced formation of anthocyanins. The leaves of P deficient plant are frequently tinged with brownish colour and senesce prematurely (Bergmann, 1992). One of the earliest symptoms of P deficiency is a specific inhibition of leaf expansion and leaf surface area (Fredeen *et al*., 1989). The photosynthetic CO_2 assimilation capacity of the leaves is also decreased. Inadequate supply of phosphate prevents the export of triosphosphates from chloroplasts (Walker, 1980) and therefore the synthesis of sucrose. In the roots, under P deficiency, they act as a dominant sink for photosynthates (Fredeen *et al*., 1989) and P from mature leaves. Dry weight shoot/root ratios are therefore typically decreased by the increased partitioning of carbohydrates to the root where sucrose can accumulate (Khamis *et al*., 1990).

Zinc (Zn)

Zn is an essential catalytic component of over 300 enzymes, including alkaline phosphatase, alcohol dehydrogenase, Cu-Zn superoxide dismutase and carbonic anhydrase. Zn also plays a critical structural role in many proteins. For example,

several motifs found in transcriptional regulatory proteins are stabilized by Zn, including the Zn finger, Zn cluster and RING finger domains (Grotz *et al*., 1998). Inside cells, Zn is neither oxidized nor reduced; thus, the essential role of Zn in cells is based largely on its behavior as a divalent cation that has a strong tendency to form stable tetrahrdral complexes (Berg and Shi, 1996). Studies of Zn uptake in plant mainly have been focused on hyperaccumulators, i.e., plants that can grow in soils containing high levels of Zn and accumulate high concentrations of Zn in their shoots. The uptake of Zn is controlled by regulating the number of active transporters in the membrane (Lasat *et al*., 1996). Once in the root, Zn is believed to be transported into the xylem, taken up by leaf cells and then stored in the vacuoles of leaf cells thus preventing the buildup of toxic levels in the cytoplasm.

Zn containing enzymes are also involved in the carbohydrate metabolism. The Zn containing enzymes aldolase and fructose 1,6-bisphosphatase have the same substrate, namely 1,6-fructose bisphosphate. Aldolase splits 1,6-fructose bisphosphate into two molecules of triosphosphate and thus “opens” the glycolytic pathway while the 1,6-fructose bisphosphatase dephosphorylates the 1,6-fructose bisphosphate to 6-fructose monophosphate which is the precursor for the synthetic pathway of carbohydrates (Mengel and Kirkby, 2001).

For zinc as an essential micronutrient for plant growth, there are relatively few studies which examined the mechanisms and regulation of Zn uptake by roots. Zn is taken up from the soil solution as a divalent cation. Currently, there is little agreement as to whether uptake is via ion channels or via a divalent cation carrier protein and whether there is a link between uptake and metabolic energy transduction. Zn is taken up as Zn^{2+} and currently it is not yet clear whether uptake is as facilitated diffusion through membrane channels specific for Zn^{2+} or whether it is mediated by specific transporters (Fox and Guerinot, 1998). Possibly both systems function for transporting Zn^{2+} across the plasma membrane. In both cases the uptake is driven by the plasmalemma H^{+} pump and hence related to the provision of ATP. Fox and Guerinot (1998) reported that there are genes for specific Zn trasporters mediating a Zn concentration dependent uptake of Zn^{2+} with Km values between 10 and

100 mmol/m^3. These values correspond to the Zn^{2+} concentrations prevailing in the rhizophere (Norvell and Welch, 1993). Interestingly, the proteins found for the specific Zn uptake did not show any activity for the uptake of Fe (Fox and Guerinot, 1998).

Plants suffering from Zn deficiency often show chlorosis in the interveinal areas of the leaf. These areas are pale green, yellow or even white. In the monocots chlorotic bands form on either side of the midrib of the leaf appears which later become necrotic. The internodes are shot and frequently the grain formation is affected (Brennan, 1992). In fruit trees leaf development is adversely affected. Unevenly distributed clusters or rosettes of small stiff leaves are formed at the ends of the young shoots. Frequently the shoots die off and the leaves fall prematurely (Mengel and Kirkby, 2001). Symptoms of Zn-deficiency in vegetable crops are more species-related than are deficiency symptoms of other plant nutrients. In most cases, however, Zn-deficiency is characterized by short internodes and chlorotic areas in older leaves (Maynard, 1979).

Characterization and expression analysis of transporter genes

Ammonium transporter (*AMT*)

Of all mineral elements required by plants, nitrogen is quantitatively the most important and thus often growth-limiting factor for many plants. Nitrogen is found in many organic compounds such as amino acids, nucleic acid and consequently in proteins, nucleic acids, etc. In most soils, nitrogen is heterogeneously distributed and found in various forms such as ammonium, nitrate, urea, amino acids, peptides and water-insoluble fractions (Jackson and Bloom, 1990). Due to the impermeability or poor permeability of the lipid bilayer of the plasma membrane to most of nutrients including nitrogen compounds, uni- and multicellular organisms possess various transporters. In 1979 electrophysiological studies in the unicellular algae *Chlara austrialis* showed a positive inward current across the plasma membrane after ammonium supply (Walker *et al.*, 1979). The first ammonium transporter, *AtAMT1;1*

from *Arabidopsis*, was consequently cloned only in 1992 by yeast complementation (Ninnemann *et al.*, 1994). Since then, many para- and orthologs were isolated either by yeast complementation or via homology cloning. Homologs were found to be present in bacteria, fungi, plants and animals (Marini *et al.*, 2000). The transport of the charged form of ammonium (NH_4^+) was further confirmed by electrophysiological characterization of heterologously expressed plant ammonium transporter in *Xenopus* oocytes (Ludewig *et al.*, 2002, 2003; Wood *et al.*, 2006). Ammonium transporters are divided in three subfamilies, Rhesus, *Mep*/*AMTB* and *AMT1* and in plant ammonium transporters are distributed in 2 subclasses *AMT1* and *AMT2* (Sohlenkamp *et al.*, 2002). The *AMT2* members are sequence-wise more closely related to fungal and bacterial ammonium transporters (*Mep* and *AMTB*) forming the *Mep*/*AMTB* subfamily (Ludewig *et al.*, 2001). In 2004, the first ammonium transporter, the bacterial ammonium transporter *AMTB*, was crystallized as trimeric protein (Khademi *et al.*, 2004). In most of *AMTs* characterized so far, the first 20-25 amino acids of the cytosolic C-terminus were highly conserved and were shown to be responsible of the allosteric regulation of trimeric complex (Loqué *et al.*, 2007, Severi *et al.*, 2007).

Nitrate transporter (*NRT*)

Plants have multiple nitrate carries with distinct kinetic properties and regulation. Two nitrate transporter gene families, *NRT1* and *NRT2*, have been disordered so far. The *NRT2* family encodes transporters that contribute to the inducible high-affinity uptake system. The *NRT1* family is more complex, including nitrate transporter with dual affinity (both low and high Km) or low affinity. The *AtNRT1* (*CHL1*) gene is expressed in the outer cell layers of the root (epidermal, cortical and endodermal cells), and concentrations of its mRNA are increased by treating plants with nitrate or solutions at slightly acidic pH. The second group of nitrate transporters found in plants is the *NRT2* family, which comprises gene that is important components of the inducible high-affinity nitrate uptake system. Genes from this family are conserved among fungi, algae and plants but are not similar to *CHL1* (*AtNRT1*). The first member of this family to be identified was the *crnA* gene from *Aspergillus*, followed by several *NRT2* genes from algae and plants. The *NRT2*

genes all show induction by nitrate and are down-regulated by several from of induced nitrogen, including ammonium and glutamine. This regulation allows plants to adjust the concentrations of this important class of transporters in response to the form of nitrogen in the soil solution and the nitrogen needs of the plant (Buchanan *et al*., 2000).

Inorganic phosphate transporter (*PT*)

The cloning of plant genes encoding Pi transporters has opened up new avenues of research into the molecular regulation of plant Pi acquisition and nutrition. A detailed study on the regulation of Pi transport in tomato indicated that in P-sufficient plants, mRNA transcripts for two transporters (*LePT1* and *LePT2*) are present only in roots. However, P starvation rapidly increases the abundance of both mRNAs in roots and *LePT1* mRNA in leaves. Provision of P to previously P-starved plants decreases the amount of *LePT1* and *LePT2* transcripts to that seen in P-sufficient plants. An antibody specific to *LePT1* was used to show that the P-deficiency brought about an increase in transporter abundance in roots. Additionally, *in situ* mRNA localization of *LePT1* and *LePT2* in P-starved plants demonstrated that these genes are expressed in the root epidermis, and, furthermore, immunocytochemical studies with antibodies to *LePT1* indicated that the protein is localized to the root cell plasma membrane, confirming a role for these transporters in P-acquisition from the soil (Buchanan *et al*., 2000).

The correlation between the levels of *PT* transcripts, protein activities suggest the regulatory role of high affinity *PTs* in Pi uptake and the transcriptional control of *PT* activity. For example, it was found that increased *PT* transcript levels in tomato under Pi starvation leads to a concurrent increase in the transporter protein and Pi acquisition (Muchhal and Raghothama, 1999; Raghothama and Karthikeyan, 2005). The Pi uptake rate was increased over 2.5-fold in the transgenic rice cells overexpressing a barley gene for high-affinity *PT* (Rae *et al*., 2003). Similarly, overexpression of an *Arabidopsis* PT gene in tobacco increased Pi uptake and cell growth (Mitsukawa *et al*., 1997). Recent studies suggested an alternative regulatory

mechanism of *PT* activity other than the transcriptional control. For example, the uptake rate of Pi was not enhanced in the transgenic barley plants with the increased mRNA from a *PT* transgene (Rae *et al*., 2004). The conserved regions for phosphorylation or glycosylation among Pht1-type *PTs* and their orthologs from non-plant species also indicate possible post-transcriptional regulatory mechanisms (Bucher *et al*., 2001). High-affinity *PTs* have been suggested as the potential targets for improving Pi uptake (Mitsukawa *et al*., 1997; Rae *et al*., 2003; Vance *et al*., 2003). However, attempts to test the potential of *PTs* by transgenic means have resulted only in limited success. Moreover, no convincing evidence has been reported to demonstrate the potential of *PTs* in whole plants.

Zinc transporter (*ZIP*)

The first gene family of Zn transporter from plants is *ZIPs* genes and that have been identified in Arabidopsis. In the plant, *ZIP1* and *ZIP3* are expressed in roots in response to Zn deficiency, suggesting that they transport Zn from the soil into the plant and *ZIP4* is expressed in shoots and roots of Zn-limited plants. Thus, *ZIP4* may transport Zn intracellularly or between plant tissues (Grotz *et al*., 1998).

The *ZIPs* are involved in the transport of Fe, Zn, Mn and Cd with family members differing in their substrate range and specificity (Guerinot, 2000; Mäser *et al*., 2001). About 85 *ZIP* family members have now been identified from bacteria, archea and all types of eukaryotes, including 15 genes in *A. thaliana* (Mäser, 2001). The first member *ZIP* family identified from plant (Eide *et al*., 1996), was *AtIRT1* (Iron-regulated transporter 1), cloned from *A. thaliana* and identified by functional complementation of the Fe-uptake-deficient yeast double mutant *fet3 fet4*. *AtIRT1* is now thought to be the major transporter for high affinity Fe uptake by roots (Connolly *et al*., 2002; Vert *et al*., 2002).

Based largely on yeast complementation studies, further information is available on the functional properties of other plant *ZIP* transporters. The *ZIP1-3*

transporters from *A. thaliana* restore Zn uptake in the yeast Zn uptake double mutant *zrt1 zrt2* and were proposed to play a role in Zn transport (Grotz *et al*., 1998; Guerinot, 2000). *ZIP1*, *ZIP3* and *ZIP4* are expressed in the roots of Zn deficient plants, while *ZIP4* is also expressed in the shoots (Grotz *et al*., 1998; Guerinot, 2000). Wintz *et al*. (2003) demonstrated that two *ZIP* genes; *AtZIP*2 and *AtZIP4* are involved in copper transport. The proposed role of *ZIP* transporters in Zn nutrition is supported by the characterization of homologues from other species. A member of the *ZIP* family, *GmZIP1*, has now been identified in soybean (Moreau *et al*., 2002). By functional complementation of the *zrt1 zrt2* yeast cells, *GmZIP1* was found to be highly selective for Zn, while yeast Zn uptake was inhibited by Cd. *GmZIP1* was specifically expressed in the nodules and not in roots, stems or leaves, and the protein was localized to the peribacteroid membrane, indicating a possible role in the symbiosis (Moreau *et al*., 2002). The yeast *ZIPs*, *ZRT1* and *ZRT2*, were shown to be high and low affinity Zn transporters, respectively (Eide, 1998; Guerinot, 2000); *ZRT1* is glycosylated and present at the plasma membrane. The third *ZIP* homologue in yeast, *ZRT3*, is proposed to function in the mobilization of stored Zn from the vacuole (MacDiarmid *et al*., 2000).

MATERIALS AND METHODS

Materials

1. General solutions

- Agarose gel loading buffer (6X): 0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol.
- Ampicillin 100 mg/ml, filter sterilized.
- 10% Blocking reagent (w/v) stock solution: 10 grams of blocking reagent (Roche) in 100 ml of maleic acid buffer.
- Chloroform: isoamyl alcohol (24:1).
- Depurination solution: 250mM HCl.
- Denaturation solution: 0.5N NaOH, 1.5M NaCl.
- Detection buffer: 100mM Tris- HCl, 100mM NaCl, pH 9.5.
- 0.01% Diethyl pyrocarbonate (DEPC) in dH_2O.
- DNA extraction buffer: 2M NaCl, 1M Tris-HCl, 0.5M EDTA, pH 8.0 and 10mM 2-mercaptoethanol.
- 70% ethanol.
- High SDS concentration hybridization buffer: 7% SDS, 50% deionized formamide, 5X SSC, 2% blocking reagent, 50mM sodium phosphate, pH 7.0, 0.1% N-lauroylsarcosine.
- Hydrolysis buffer: 50mM NaOH, 10mM NaCl.
- LB Broth: 1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl.
- LBA: 1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl and 1.5 % bacto-agar.
- 10M Lithium chloride stock solution.
- Maleic acid buffer: 0.1M maleic acid, 0.15M NaCl, pH 7.5.
- 10X MOPS buffer: 0.2M MOPS, 0.05M sodium acetate, 0.001M EDTA, pH 7.0.

- 10% N-lauroylsarcosine (w/v) stock solution.

- Neutralization buffer: 0.5M Tris, pH 8.0, 1.5M NaCl.

- Phenol: chloroform: isoamyl alcohol (25:24:1).

- RNA extraction buffer (1): 2M NaCl, 1M Tris-HCl, 0.5M EDTA (pH 8.0), and 10 mM 2- mercaptoethanol.

- RNA extraction buffer (2): 4M guanidine thiocyanate, 1M Tris-HCl (pH 8.0), 25mM sodium citrate (pH 8.0), 0.5% N-lauryl sarcosine.

- RNA sample loading buffer: 1.5X agarose gel loading buffer, 1.5X MOPS, 9% formaldehyde, 60% deionized formamide.

- RNase (DNase free) was prepared by dissolving 100mg of RNaseA in 10 ml dH_2O, boiled for 20 min and stored at –20 °C.

- 10% SDS (w/v) stock solution.

- 20% SDS (w/v) stock solution.

- 20X SSC: 3M NaCl, 0.3M Sodium citrate, pH 7.0.

- Sodium acetate: 3M sodium acetate, pH 5.4.

- Solution I: 50mM glucose, 25mM Tris-HCl, pH8.0, 10mM EDTA, pH 8.0.

- Solution II: 0.2N NaOH, 1% SDS.

- Solution III: 3M potassium acetate, 5M glacial acetic acid.

- Standard hybridization buffer: 5X SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% blocking reagent.

- 50X TAE electrophoresis buffer: 40mM tris, 5.17% galcial acetic acid (v/v), 2mM EDTA, pH 8.0.

- 10X TBE buffer: 178mM Tris, 178mM boric acid, 4mM EDTA, pH 8.0.

- TE buffer: 10mM Tris, 1mM EDTA, pH 8.0.

- Washing buffer: 0.1M maleic acid, 0.15M NaCl, pH 7.5, 0.3% Tween 20.

- 2X Washing solution: 2X SSC, 0.1% SDS, pH 7.0.

- 0.5X Washing solution: 0.5X SSC, 0.1% SDS, pH 7.0.

- X-gal: 2% in dimethylformamide.

2. General equipment

- Centrifuge (Micro, Spectrafuge, Labnet/USA)

- Centrifuge (Refrigerated Bench Top Centrifuge)
- Electrophoresis System (Gelmate 2000, Toyobo)
- Gel Documentation (Model Gene Genius)
- Hot Plate Stirrer
- Incubator Shaker (Model Vortemp56, Labnet/USA)
- Mixer, Vortex-Genie II (Model G560, SI/USA)
- pH Meter (Consort/Belgium)
- Spectrophotometer (UV/Visible, Amershame Pharmacia Biotech)
- Transluminator (LKB 2011 Macro Vue)
- PCR (Perkin Elmer Cetus of Hybaid thermal cycles)
- PCR (PTC-200 DNA Engine)
- Waterbath with shaker

3. Plant materials

Cassava (*Manihot esculenta* Crantz cv. HB80, KU50, R1) tissues were grown under different MS media recipes as 0E, -1/2E, E, +1E and +2E treatment (0, 5, 10, 20 and 40 mM NH_4NO_3; 0, 0.3, 0.6, 1.2 and 1.8 mM KH_2PO_4; 0, 0.037, 0.075, 0.15 and 0.22 mM $ZnSO_4.7H_2O$, respectively). Other plant parts (young leaves, stems and fibrous roots) were obtained at 4-8 weeks after being subcultured. These plant tissues were immediately frozen in liquid nitrogen and kept at -80°C until use.

Methods

1. Cloning and characterization of transporter genes

Cloning and characterization of ammonium transporter (*AMT2*), nitrate transporter (*NRT2*), inorganic phosphate transporter (*PT1*) and zinc transporter (*ZIP*) genes were used in 5 consecutive steps as follows; total RNA extraction, RT-PCR, cloning, sequencing, and sequence analysis.

1.1 Total RNA extraction

Total RNA was extracted from cassava tubers at various development stages. The extraction method is modified from Dellaporta *et al.* (1990). Each tissue sample was ground in liquid nitrogen to fine powder. The ground material was transferred into a centrifuge tube containing 10 ml of RNA extraction buffer. After the addition of 1 ml of 20% SDS, the mixture was mixed by vortexing. The suspension was incubated at 65°C for 10 minutes. After that, 5 ml of 5M potassium acetate was added; it was then mixed by vortexing and incubated on ice for 20 minutes. The contents were separated by centrifugation at 13,500 rpm for 40 minutes. The supernatant was transferred to a new tube and 10M LiCl was added, mixed by inverting and kept at 4°C overnight. The RNA was pelleted by centrifugation at 12,300 rpm for 40 minutes at 4°C. The pellet was then dissolved using 700 µl TE buffer and transferred to a new microcentrifuge tube. One volume of chloroform: isoamyl alcohol (24:1) was added to the sample and stirred for 5 minutes before being centrifuged at 12,000 rpm for 30 minutes at 4°C. RNA in the aqueous phase was precipitated by adding 0.1 volume of 3M sodium acetate and equal volume of isopropanol. The suspension was then centrifuged at 12,000 rpm for 30 minutes at 4°C after incubation at –20°C for 2 hr. The pellet was then washed with 70% ethanol and air-dried. The RNA pellet was resuspended in RNase-free water and stored at -80°C.

Concentration and purity of the RNA was determined using spectrophotometer at 260 and 280 nm. The expected A260/A280 ratio would be in the range of 2.0.

1.2 Partial *AMT2*, *NRT2*, *PT1* and *ZIP* cDNA synthesis

The reverse transcriptase RT-PCR was used to amplify the partial of *AMT2*, *NRT2*, *PT1* and *ZIP* cDNA using the degenerate primers specific to these genes. Primers were designed based on highly conserved regions of *AMT2* from *Arabidopsis thaliana* (GenBank accession AF182039), *Populus trichocargo* (EEE92674) and *Lotus japonicus* (AAL08212); *NRT2* from *Zea mays* (NP_001105780), *Lotus japonicus* (CAC35729) and *Nicotiana tabacum* (CAD89799); *PT1* from *Solanum tuberosum* (CAA67395), *Lycopersicon esculenta* (AAB82146) and *Nicotiana tabacum* (AAF74025) and *ZIP* from *Glycine max* (AY029321), *Fragaria x ananassa* (AY805423) and *Medicago truncatula* (AY339056). The primers used in this study are shown in Table 1.

Reverse transcription of total RNA, first-strand cDNA synthesis was performed using the Super Script III one step RT–PCR (Invitrogen). The RT-PCR (50 µl) was conducted using 25 µl 2X reaction mix, 1 µl template RNA (0.01 pg - 1 µg), 1 µl forward primer, 1 µl reverse primer, 2 µl Super Scrip III RT/Platinum Taq and 20 µl of DEPC-treated dH_2O. The PCR was performed as follows; cDNA synthesis at 50°C for 30 min, pre-denaturing at 94°C for 2 min, followed by 40 cycles at 95°C for 1 min, 58°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 10 min. The products were analyzed by electrophoresis on 1% (w/v) agarose gels in 1XTAE buffer at 100 V for 30 min, stained with ethidium bromide for 10 min and visualized under UV light.

Table 1 Primers used for the isolation of *AMT2*, *NRT2*, *PT1* and *ZIP* coding sequences from total RNA of cassava.

Primers	Sequences
AMT2	
MeAmt2F	5'-CGCTGCTTACTGGGTAGGAC-3'
MeAmt2R	5'-CCATCTCCCCAAAGAGCATA -3'
MeAmt2HF	5'-AAAATGGCCGGAGCTTACG-3'
MeAmt2HR	5'-AGACCAGCCCATCCACAG-3'
MeAmt2TF	5'-CATACCCTTGAGAATGCCG-3'
NRT2	
MeNrt2F	5'-ACAAGAACGTTGTGGGAACC-3'
MeNrt2R	5'-ATCCAGCGACAAGTCCAAAC-3'
MeNrt2HF	5'-GCAGCAATGGCGGCCGTCGG-3'
MeNrt2HR	5'-AACAGAAGCAACACCAGCAT-3'
MeNrt2TF	5'-GACAACGTCATCGCCGAG-3'
PT1	
MePt1F	5'-TGCTGGACAGTTGTTCTTCG-3'
MePt1R	5'-GTTCATGGTTTGTGCTGGTG-3'
MePt1HF	5'-AGTTTAGTCATGGCGAACGA-3'
MePt1HR	5'-GATAAATGCCCCACGAGT-3'
MePt1TF	5'-CATGGGGTTCACTTGCTTG-3'
ZIP	
MeZIPF	5'-TTGCCTGAAGGTTACGAGAG-3'
MeZIPR	5'-CTCTTCTTGTTGGAGCA -3'
M13-Oligo(dT_{18})	5'-CATGGTCATAGCTGTTTCCTG- oligodT_{18}-3'
M13R	5'-CAGGAAACAGCTATGACCATG-3'

1.3 Determination the 3’ end of *AMT2*, *NRT2*, *PT1* and *ZIP* genes

The 3' end of *AMT2*, *NRT2*, *PT1* and *ZIP* genes were determined using RT-PCR with adaptor M13-oligo-(dT_{18}) primer and *AMT2*, *NRT2*, *PT1* and *ZIP* specific primer 3' end. First-strand cDNA was synthesized by mixing 5 µg of total RNA of cassava fibrous roots, 30 µM of adaptor M13-oligo-(dT_{18}) primer, and adding DEPC-treated dH_2O to make the volume up to 12.5 µl. The reaction mixture was then incubated for 5 min at 70°C, and then chilled on ice. After that, 4 µl of 5X reaction buffer (250 mM Tris-HCl (pH 8.3 at 25°C), 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT), 2 µl of 10 mM dNTPs mix, and 0.5 µl of 20U RNase inhibitor were added to the reaction mixture. The reaction was then incubated at 37°C for 5 min to allow the M13-oligo-(dT_{18}) to anneal to RNA template. Then, 200U RevertAid^{TM} M-MuLV reverse transcriptase (Fermentas) was added, and incubated at 42°C for 60 min. After incubation, the enzyme was inactivated by heating at 70°C for 10 min and then chilled on ice. A 1/10 of first-strand cDNA reaction mixture was used as cDNA template for amplification of 3’ end. Ten µM of *AMT2*, *NRT2*, *PT1* and *ZIP* specific primer 3' end and reverse M13 were used for PCR amplification. The cycle of PCR reaction was as followed; 1 cycle of 94°C for 2 min, 35 cycles of 94°C for 30 sec, 58°C for 1 min and 72°C for 1 min and followed by 1 cycle of 72°C for 10 min. The PCR products was determined by electrophoresis on 1% (w/v) agarose gels in 1XTAE buffer at 100V for 30 min, stained with ethidium bromide for 10 min and visualized under UV light.

1.4 Determination the start condons of *AMT2*, *NRT2*, *PT1* and *ZIP* coding sequence

First-strand cDNA was synthesized by mixing 5 µg of total RNA of cassava fibrous roots, 30 µM of oligo-(dT_{23}) primer, and adding DEPC-treated dH_2O to make the volume of 12.5 µl. The reaction mixture was then incubated for 5 min at 70°C, and then chilled on ice. After that, 4 µl of 5X reaction buffer (250 mM Tris-HCl (pH 8.3 at 25°C), 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT), 2 µl of 10 mM dNTPs mix, and 0.5 µl of 20U RNase inhibitor was added to the reaction mixture.

The reaction was incubated at 37°C for 5 min to allow the M13-oligo-(dT_{18}) to anneal to RNA template. Then, 200U RevertAid^{TM} M-MuLV reverse transcriptase (Fermentas) was added, and incubated at 42°C for 60 min. After incubation, the enzyme was inactivated by heating at 70°C for 10 min and then chilled on ice. A 1/10 of first-strand cDNA reaction mixture was used as cDNA template for amplification. The *AMT2*, *NRT2*, *PT1* and *ZIP* coding sequence was amplified using degenerated primer and gene-specific primer. The cycle of PCR reaction was as followed; 1 cycle of 94°C for 2 min, 35 cycles of 94°C for 30 sec, 58°C for 1 min and 72°C for 1 min and followed by 1 cycle of 72°C for 10 min. The PCR products was determined by electrophoresis on 1% (w/v) agarose gels in 1XTAE buffer at 100V for 30 min, stained with ethidium bromide for 10 min and visualized under UV light.

1.5 Isolation of DNA from agarose gels

DNA bands were excised from agarose gels and extracted using QIAquick Gel Extraction Kit (QIAGEN) using a protocol recommended by the manufacturer. The DNA fragments from agarose gel were weighted and 3 volumes of buffer QG were added. The mixtures were incubated at 50ºC for 10 min or until the gels slice was completely dissolved. After the addition of 1 volume of isopropanol, the sample was loaded into a QIAquick spin column. The column was centrifuged at 14,000 rpm for 1 min, and then washed with 0.75 ml of buffer PE followed by centrifugation at 14,000 rpm for 1 min. After an additional centrifugation at 14,000 rpm for 1 min, 50 µl of buffer EB (10 mM Tris-Cl, pH 8.5) was added to the columns to elute the DNA. To ensure the DNA recovery, the column was incubated at room temperature for 1 min before centrifuged at 14,000 rpm for 1 min.

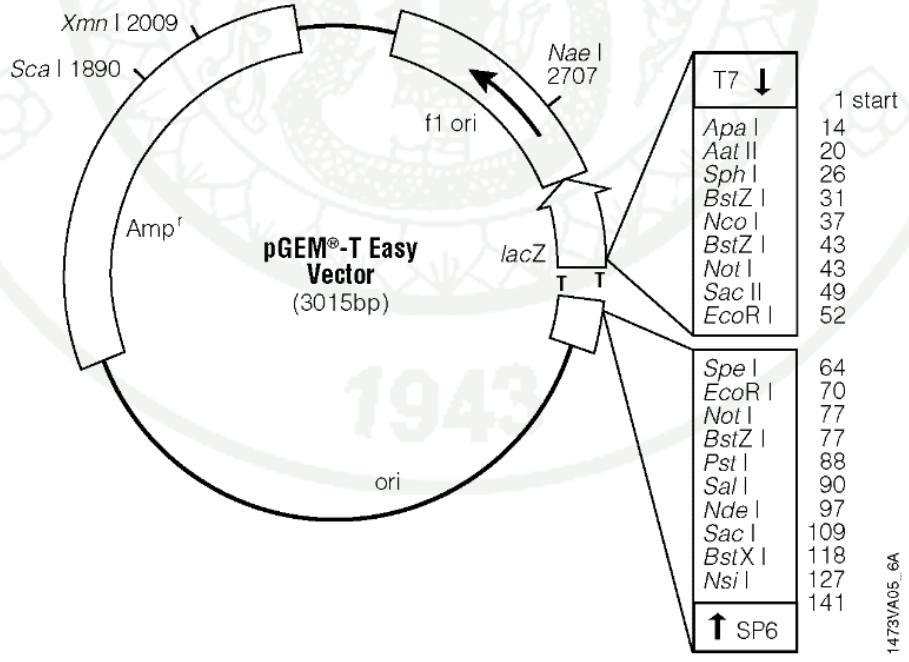
1.6 Purification of PCR product

PCR products were purified using MinElute PCR Purification Kit (QIAGEN) using protocol described by the manufacturer. First, the PCR sample was mixed with 1/5 volume of DNA-binding solution. The mixture was transferred to the

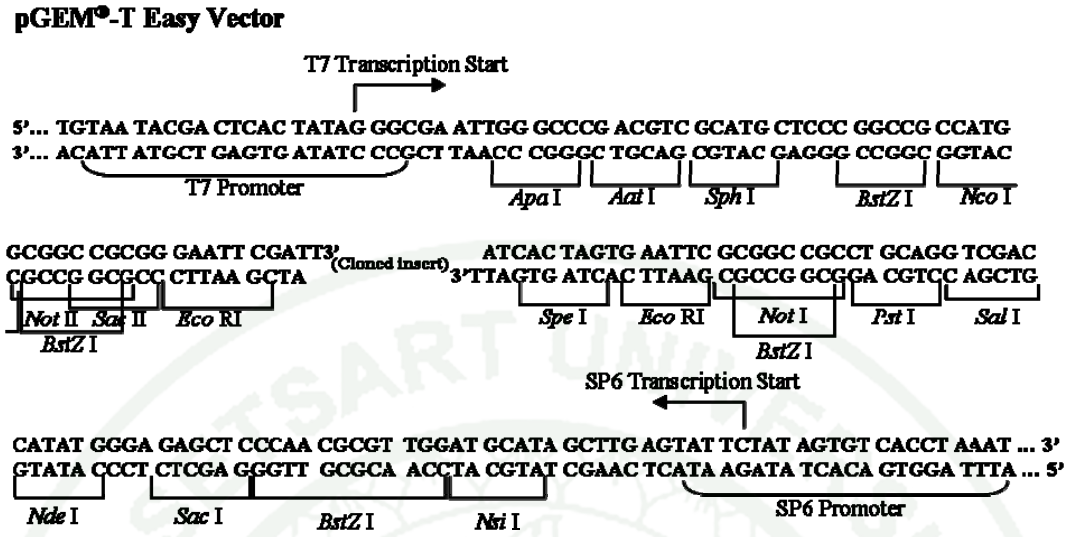
microspin cups that seated in a 2 ml receptacle tubes and centrifuged at 13,000 rpm for 1 min. The microspin cups were washed with 750 µl of PE buffer and centrifuged at 13,000 rpm for 1 min. To make sure that the washing buffer was completely removed, the tubes were further centrifuged at 13,000 rpm for 1 min. To elute the DNA, 10 µl of EB buffer (10 mM Tris-Cl, pH 8.5) was added, incubated at room temperature for 1 min, and then centrifuged at 13,000 rpm for 1 min.

1.7 Ligation of DNA fragments

PCR products were ligated to the vector pGEM-T Easy using a pGEM-T kit (Promega) followed the protocal recommended by the manufacturer. The reactions were performed in the 0.5 ml microcentrifuge tube. The 10 µl ligation reaction contained 3 µl PCR products, 50ng pGEM-T Easy vector, and 3 units T4 DNA ligase in 1X rapid ligation buffer (2X rapid ligation buffer, T4 DNA ligase). The ligation was then performed at 4ºC overnight



(A)



(B)

Figure 1 Structure of pGEM-T easy vector circle map (A) and (B) the promoter and multiple cloning sequence of the pGEM-T easy vector.

Source: Promega technical manual for pGEM-T easy vector system

1.8 Transformation of plasmid DNA

Transformation of the *E. coli* DH 5α was conducted using a protocol described by Sambrook *et al*., (1989). To prepare competent cells used in transformation experiments, the *E. coli* DH 5α was grown in LB broth at 37ºC until the OD600 reached 0.3. The culture was put on ice for 30 min, and then centrifuged at 8,000 rpm for 5 min at 4ºC. The cell pellet was resuspended in 10 ml of 50 mM $CaCl_2$. The cells were then incubated on ice for 30 min before being pelleted by centrifugation at 8,000 rpm for 5 min at 4ºC. The cells were resuspended in 1 ml of 50 mM $CaCl_2$ and stored at 4ºC.

To transform the competent cells, 4 µl of pGEM-T Easy ligation mixture was added to the 100 µl aliquots of *E. coli* DH 5α. The mixture was incubated on ice for 30 min, heat shocked at 42ºC for 90 sec, and placed on ice for 5 min. The cells

were resuspended in 1 ml of SOC medium and incubated in a shaking incubator at 37ºC for 90 min. Various dilutions of transformed cells were plated on LBA containing IPTG (20 mg/ml), X-gal (20 mg/ml) and amplicilin (10 mg/ml). The plates were incubated overnight at 37ºC. Recombinant clones were identified by blue/white selection. The white colonies were randomly picked and the cells were grown in LB cultures containing ampicillin (10 mg/ml). The transformants were analyzed by PCR to confirm the presence of inserts.

1.9 Plasmid extraction by alkaline lysis method

Plasmid DNA was extracted using a modification of the method of Sambrook *et al*., (1989). Recombinant clone was grown overnight at 37ºC with vigorous shaking in 1 ml of LB containing 10 mg/ml of amplicillin. The cell suspension was collected by centrifugation for 5 min at 10,000 rpm. The pellet was resuspended in 100 µl of chilled solution I (50 mM glucose, 10 mM EDTA and 25 mM tris-HCl, pH 8.0) and incubated on ice for 5 min. Two hundred microliters of freshly prepared solution II (0.2 M NaOH, 1% SDS) was added, and the content was mixed by inverting 2-3 times. The mixture was then neutralized with the addition of 150 µl of ice-cold solution III (3M potassium acetate, 5M galcial acetic acid) and incubated on ice for 5 min. The samples were centrifuged at 12,000 rpm for 5 min and the supernatant was extracted with equal volume of PCI. The DNA was precipitated with 2.5 volume of ice-cold ethanol and incubated at -20ºC for 30 min. The DNA was pelleted by centrifugation at 12,000 rpm for 15 min, washed with 70% ethanol, centrifuged at 12,000 rpm for 5 min and air-dried. The pellet was resuspended in 30 µl TE buffer containing 10 µg/ml RNaseA and incubated at RT for 30 min.

1.10 Checking the insertion by digestion with restriction enzyme

To check the insertion of gene in the plasmid, the recombinant plasmids extracted by alkaline lysis method were then subjected to digestion with restriction enzymes. The 10µl digestion reaction contained 3 µl recombinant plasmids, 2 units

E*co*RI in 1X E*co*RI buffer (50mM Tris-HCl (pH 7.5 at 37ºC), 10mM $MgCl_2$, 100mM NaCl, 0.02% Triton X-100 and 0.1 mg/ml BSA). Reaction mixes were then incubated at 37ºC overnight. The E*co*RI-digested fragments were visualized by electrophoresis on 1% agarose gel.

1.11 Confirming the insertion with PCR

To confirm the insertion of gene, the recombinant plasmids extracted by alkaline lysis method could be used as a template in PCR reactions. The PCR condition was performed as previously described. The size of the PCR products was determined by electrophoresis on 1% agarose gel at 100 V for 40 minutes.

1.12 Plasmid preparation for sequencing

Plasmid DNA was extracted by using a QIAprep Spin Miniprep Kit (QIAGEN) method as recommended by the manufacturer. Recombinant clone was grown in 5 ml of LB containing 10 mg/ml amplicillin overnight at 37ºC with vigorous shaking. The cells were collected by centrifugation at 4,000 rpm for 10 min and then resuspended in 250 µl of P1 buffer. Two hundred and fifty microliters of P2 buffer followed with was 350 µl of N3 buffer were added and the content was mixed by inverting. The suspension was centrifuged at 10,000 rpm for 10 min. The supernatants were transferred to the QIAprep column and centrifuged at 10,000 rpm for 1 min. The column was washed, firstly with 500 µl PB buffer and centrifuged at 10,000 rpm for 1 min, and secondly with 750 µl PE buffer and centrifuged at 10,000 rpm for 1 min. To make sure that the washing buffer was completely removed, the tubes were further centrifuged at 10,000 rpm for 1 min. The QIAprep spin column was transferred to the 1.5 ml microcentrifuge tubes and the DNA was eluted from the microspin cup by adding 50 µl of EB buffer (10 mM Tris-Cl, pH 8.5) and incubated at room temperature for 1 min before centrifuged at 10,000 rpm for 1 min.

1.13 DNA sequencing

Plasmid pGEM-T easy was sequenced to confirm the direction and the correction of the insert using ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystem). The sequencing reaction was analyzed at 1st BASE, Malaysia.

1.14 Nucleotides sequence analysis

GenBank nucleotides databases were searched using BLASTN and BLASTX program (NCBI) for sequences having homology to ammonium transporter, nitrate transporter, inorganic phosphate transporter and zinc transporter sequences. Comparisons of all nucleotide sequences were performed using the CLUSTALW program. The sequences from the overlapping cDNA fragments were assembled into a contiguous sequence using GeneDoc program (Nicholas and Nicholas, 1997).

2. Differential expression of transporter genes in cassava plant

Expression of *AMT2*, *NRT2*, *PT1* and *ZIP* were used in 2 consecutive steps, i.e. total RNA extraction and real time PCR analysis.

2.1 Total RNA extraction

Total RNA was extracted from cassava tubers at various development stages. The extraction method is modified from Dellaporta *et al.* (1983). Each tissue sample was ground in liquid nitrogen to a fine powder. The ground materials were transferred into a centrifuge tube containing 10 ml of RNA extraction buffer. After the addition of 1 ml of 20% SDS, the mixture was mixed by vortexing. The suspension was incubated at 65°C for 10 minutes. Afterward, 5 ml of 5M potassium acetate was added, mixed by vortexing and incubated on ice for 20 minutes. The contents were separated by centrifugation at 13,500 rpm for 40 minutes. The supernatant was transferred to a new tube and 5 ml of 10M LiCl was added, mixed by

inverting and kept at 4°C overnight. The RNA was pelleted by centrifugation at 12,300 rpm for 40 minutes at 4°C. The pellet was then dissolved with 700 µl TE buffer and transferred to a new microcentrifuge tube. One volume of chloroform: isoamyl alcohol (24:1) was added to the sample and stirred for 5 minutes before centrifugation at 12,000 rpm for 30 minutes at 4°C. RNA in the aqueous phase was precipitated by adding 0.1 volume of 3M sodium acetate and equal volume of isopropanol. The suspension was centrifuged at 12,000 rpm for 30 minutes at 4°C after incubated at –20°C for 2 hr. The pellet was washed with 70% ethanol and air-dried. The RNA was resuspended in RNase-free water and stored at -80°C.

Concentration and purity of the RNA was determined using spectrophotometer at 260 and 280 nm. The expected A260/A280 ratio would be in the range of 2.0.

2.2 Real time PCR analysis

For Real time PCR analysis, about 500 ng of total RNA from other cassava tissues (young leaves, stems and fibrous roots) obtained from different MS media (0E, -1/2E, E, +1E and +2E treatments) were used.

The LightCycler® 480 system PCR run with the LightCycler® 480 RNA Master Hydrolysis Probes using a LightCycler® 480 Multiwell Plate 96 were performed. Using LightCycler® 480 RNA Master Hydrolysis Probes kit, the reaction contained up to 500 ng of total RNA, 1 µl of 10 mM of the specific primers (forward and reward), 0.25 µl of 10 nM of the specific probe, 0.7 µl of activator, 0.5 µl of enhancer, 3.7 µl of LightCycler® 480 RNA Master Hydrolysis Probes and up to 10 µl of deionized water. Results were analyzed using LightCycler® 480 software (Roche), and calculated using the comparative threshold T cycle C method according to the manufacturers' instructions for data normalization. The NADH-plastoquinone oxidoreductase subunit 5 was used as gene internal control.

3. Physiological characteristics study of cassava plant

Cassava (cv. HB80, KU50, R1) tissues using buds were grown using 5 different MS media as 0E, -1/2E, E, +1E and +2E treatment (0, 5, 10, 20 and 40 mM NH_4NO_3; 0, 0.3, 0.6, 1.2 and 1.8 mM KH_2PO_4; 0, 0.037, 0.075, 0.15 and 0.22 mM $ZnSO_4.7H_2O$, respectively). So the experiment was arranged as Factorial in CRD with 4 replications. Other plant parts (young leaves, stems and fibrous roots) were obtained at 4-8 weeks after subculture. Data of agronomic characteristic was recorded as followed;

3.1 Biomass

Growth parameters for the plantlets were recorded at the beginning (4 weeks after culture) and the end of the experiment (8 weeks after culture). Leaves, stems and fibrous roots were then separated and the fresh weights were recorded. Plant material was dried at 65°C for 48 hr to retrieve constant weight and then leaves, stems and fibrous roots dry weights were determined (Smethurst and Shabala, 2003).

3.2 The partitioning study

Each plant parts were separated to study fresh and dry weights. The fresh and dry weights analysis of fibrous roots, stem and leaves was performed for the partitioning study.

3.3 Young leaf length, stem length, fibrous root length and leaf width

Leaf and fibrous root length were measured from the base to the tip of leaf and fibrous root; stem length was measured on the main stem from the ground to the first unexpanded leaf and leaf width was recorded by removing three fully expanded leaves and measuring their width at the middle of each leaf

.

3.4 Leaf and fibrous root number

Number of leaves and fibrous roots per plant were calculated by counting all leaves and fibrous roots of a plant.

3.5 Leaf chlorophyll content

From the beginning of the subculture treatment until just before the end of the experiment, sequential extractions of chlorophyll (Chl) *a* and *b* were made at weeks 4, 5, 6, 7 and 8. Leaf chlorophyll was extracted from two leaf discs (both 0.6 mm in diameter) excised from the youngest fully expanded leaf using 4 ml N, N-Dimethylformamide (DMF) and incubated at 4°C for at least 48 hr in the dark until the leaf disc color turned white. The absorbance was determined for each leaf extraction using a spectrophotometer at wavelengths of 647 and 664 nm. Ratios of Chl *a* to *b* were calculated and compared with the control treatment (Arnon, 1949).

An average value for each character was subjected to statistical analysis.

4. Determination of copy number of transporter genes

Determination of *AMT2*, *NRT2*, *PT1* and *ZIP* copy number was used in 4 consecutive steps, as follows; genomic DNA extraction, restriction endonuclease digestion, agarose gel electrophoresis, and Southern blot analysis.

4.1 Genomic DNA extraction

Young cassava leaves were used for genomic DNA extraction using a protocol described by Dellaporta *et al.* (1983). Two grams of frozen cassava leaves were ground in liquid nitrogen into fine powder. Fifteen milliliters of DNA extraction buffer and 1 ml of 20% SDS were added and mixed by vortexing. The suspension was incubated at 65°C for 10 minutes. Afterward, 5 ml of 5M potassium acetate was added, mixed by vortexing and the sample was incubated on ice for 20 minutes. The

contents were separated by centrifugation at 13,500 rpm for 20 minutes. The supernatant was transferred to new tube and 10 ml of isopropanol was added, mixed by inverting and kept at –20°C for 30 minutes. The DNA was pelleted by centrifugation at 12,300 rpm for 15 minutes at 4°C. The pellet was dissolved with 700 µl TE buffer and transferred to microcentrifuge tube. The DNA was precipitated again by the addition of 0.1 volume of 3M sodium acetate and 500 µl of isopropanol, and the sample was centrifuged at 13,000 rpm for 30 minutes at 4°C. The pellet was washed with 75% ethanol, dried at room temperature and resuspended in 100 µl TE buffer containing 20 µg/µl of RNaseA. The sample was incubated at 65°C for 15 minutes to eliminate RNA contamination. The sample was kept at 4°C for short-term storage or –20°C for long-term storage.

Concentration and purity of the DNA was determined using spectrophotometer at 260 and 280 nm.

4.2 Southern blot hybridization

Ten micrograms of genomic DNA were digested with 100 U of restriction endonuclease (*Bam*HI, *Eco*RV, *Hind*III, *Pst*I, *Sac*I and *Sal*I) at 37°C overnight in a total volume of 150 µl. The digested DNA was extracted with phenol: chloroform: isoamyl alcohol (25:24:1) mixture. The DNA in aqueous phase was precipitated by adding 2.5 volume of 100% ethanol and 0.1 V of 3M Sodium acetate and centrifuged at 12,000 rpm for 15 min at 4°C. The pellet was washed with 500 µl of 70% ethanol, air-dried and resuspended in 20 µl of dH_2O. The digested DNA was seperated on 1% agarose gel containing 1X TAE. The gel was stained with ethidium bromide to visualize DNA fragments.

The DNA was depurinated by soaking the gel in 0.25 M HCl for 10 min. Then, the DNA was denatured by slowly gel shaking in denaturing buffer for 2x15 min and neutralized by slowly gel shaking the gel in neutralization buffer for 2x15 min at room temperature. The DNA was transferred to a nylon membrane using a capillary transfer method in the presence of 10X SSC according to the manufacturer

(Roche) instructions. The DNA was cross-linked to the membrane by baking at 120°C for 30 min. The membrane was pre-hybridized in standard hybridization buffer at 65°C for at least 1 hr, and hybridized with ammonium transporter, nitrate transporter, phosphate transporter and zinc transporter probes. The hybridization was carried out overnight at 60°C in standard hybridization buffer. The membrane was washed with 2X washing solution at room temperature for 2x15 min and with 0.5X washing solution at 65°C for 2x15 min before the detection.

RESULTS AND DISCUSSION

1. Cloning and characterization of transporter genes

1.1 Ammonium transporter gene (*AMT2*)

AMT2 cDNA (*MeAMT2*) was isolated from fibrous root using reverse transcriptase (RT) PCR and RACE systems. The 1,578 bp cDNA sequence contained a 1,425 bp coding sequence that could encode a protein of 474 amino acids (Figure 2) which corresponds to a polypeptide with a predicted molecular weight (MW) of 51.1 kDa. The nucleotide sequences of *MeAMT2* gene was deposited in the GenBank database under an accession number GU248340.

The deduced amino acid sequence of *MeAMT2* was used to compare the amino acid composition of the polypeptide to the ones of other plants' *AMT*. The analysis revealed a high degree of sequence identity of 66% to 88% when compared to other plants and highly conserved when compared with various plant species (Figure 3). The phylogenic tree indicated that the amino acid sequence of *MeAMT2* was in separated clusters. *MeAMT2* appeared in the cluster with closer relationship to *Arabidopsis thaliana AMT* than to that of *Ricinus communis AMT* (Figure 4).

```
1    AAAATGGCCGGAGCTTACGATCCAAGCTTGCCGGAGGTTCCTGAATGGCTCAACAAAGGA
1       M  A  G  A  Y  D  P  S  L  P  E  V  P  E  W  L  N  K  G
61   GACAATGCGTGGCAGCTCACGGCAGCGACTCTGGTTGCCCTACAGAGTATGCCAGGTCTT
21    D  N  A  W  Q  L  T  A  A  T  L  V  A  L  Q  S  M  P  G  L
121  GTTATCCTCTATGCCAGCATCGTCAAGAAGAAATGGGCTGTGAATTCAGCTTTTATGGCT
41    V  I  L  Y  A  S  I  V  K  K  K  W  A  V  N  S  A  F  M  A
181  CTTTACGCTTTCGCCGCCGTTCTTCTCTGTTGGGTGCTATTCTGTTACAAAATGGCTTTT
61    L  Y  A  F  A  A  V  L  L  C  W  V  L  F  C  Y  K  M  A  F
241  GGAGAAGAGCTTTTGCCGTTTTGGGGAAAGGGTGGTCCAGCTTTCAACCAAGGATACCTT
81    G  E  E  L  L  P  F  W  G  K  G  G  P  A  F  N  Q  G  Y  L
```

Figure 2 Nucleotide and deduced amino acid sequences of cassava (*Manihot esculenta* Crantz.) cDNA fragment encoding full open reading frame of *AMT2* (*MeAMT2*).The start and stop codons are underlined.

```
301  AAGGGACAAGCAAAGATCCCAAATAGTAATGTGGCGGCGCCGTGGTATCCGATGGCGACG
101   K  G  Q  A  K  I  P  N  S  N  V  A  A  P  W  Y  P  M  A  T
361  TTGGTGTATTTTCAGTTCACATTCGCGGCGATAACGACGATACTTGTTGCTGGTTCTGTG
121   L  V  Y  F  Q  F  T  F  A  A  I  T  T  I  L  V  A  G  S  V
421  TTGGGGAGGATGAATATTAAAGCATGGATGGCTTTTGTGCCTCTGTGGTTGATCTTTAGC
141   L  G  R  M  N  I  K  A  W  M  A  F  V  P  L  W  L  I  F  S
481  TACACAGTNGGAGCTTATAGTATATGGGGAGGTGGGTTTCTGTATCAGTGGGGAGTTATT
161   Y  T  V  G  A  Y  S  I  W  G  G  G  F  L  Y  Q  W  G  V  I
541  GATTATTCCGGCGGTTATGTTATTCATCTCTCCTCAGGAGTTGCCGGTTTCGTCGCTGCT
181   D  Y  S  G  G  Y  V  I  H  L  S  S  G  V  A  G  F  V  A  A
601  TACTGGGTAGGACCAAGGCTAAAGAGCGATAGAGAAAGATTTCCTCCAAATAACGTGTTG
201   Y  W  V  G  P  R  L  K  S  D  R  E  R  F  P  P  N  N  V  L
661  CTGATGCTTGCCGGTGCTGGGCTGCTGTGGATGGGCTGGTCTGGCTTCAACGGAGGAGCA
221   L  M  L  A  G  A  G  L  L  W  M  G  W  S  G  F  N  G  G  A
721  CCGTATGCAGCTAATCTAAATGCTTCGATTGCGATATTAAACACCAACATAAGTGCAGCA
241   P  Y  A  A  N  L  N  A  S  I  A  I  L  N  T  N  I  S  A  A
781  ACAAGCCTGCTTGTGTGGACGTCGCTGGATGTTGTGTTCTTTGGTAAACCATCAGTGATC
261   T  S  L  L  V  W  T  S  L  D  V  V  F  F  G  K  P  S  V  I
841  GGGGCTGTTCAGGGTATGGTGACAGGACTAGCTTGCGTTACCCCAGGAGCAGGGCTGGTT
281   G  A  V  Q  G  M  V  T  G  L  A  C  V  T  P  G  A  G  L  V
901  CAATCGTGGGCGGCTATTGTGATGGGAGCTCTTTCTGGAAGCATTCCATGGGTGTCTATG
301   Q  S  W  A  A  I  V  M  G  A  L  S  G  S  I  P  W  V  S  M
961  ATGGTGCTTCACAAAAAGTCTTCGCTGCTACAGCAGGTGGACGACACACTAGGCGTGTTT
321   M  V  L  H  K  K  S  S  L  L  Q  Q  V  D  D  T  L  G  V  F
1021 CACACTCACGCGGTGGCTGGGCTATTAGGTGGCCTCCTCACAGGGCTTCTAGCAGAGCCA
341   H  T  H  A  V  A  G  L  L  G  G  L  L  T  G  L  L  A  E  P
1081 GATCTTTGCGACCTTATTCTACCGAAGAAAACACGAGGCGCATTTTACGGCGGAAATGGT
361   D  L  C  D  L  I  L  P  K  K  T  R  G  A  F  Y  G  G  N  G
1141 GGACGGCAATTCTTGAAGCAATTGGTTGCTGCTTGCTTTATTATAGTTTGGAACATAGTC
381   G  R  Q  F  L  K  Q  L  V  A  A  C  F  I  I  V  W  N  I  V
1201 TCCACCACCATAATCCTTTTAGCTATAAGATTGTTCATACCCTTGAGAATGCCGGAAGAG
401   S  T  T  I  I  L  L  A  I  R  L  F  I  P  L  R  M  P  E  E
1261 CAACTGGTAATCGGAGACGATGCCGTTCATGGAGAGGAAGCTTATGCTCTTTGGGGAGAT
421   Q  L  V  I  G  D  D  A  V  H  G  E  E  A  Y  A  L  W  G  D
1321 GGAGAGAAGTTTGATGCTACAAGGCATGTGCAACAGTTTGAGAGAGATCAAGAAGCTGCT
441   G  E  K  F  D  A  T  R  H  V  Q  Q  F  E  R  D  Q  E  A  A
1381 CAGTCTCCTTATGTTCATGGTGCTAGAGGTGTCACCATCAATCTATGATTTTTCTTCTTC
461   Q  S  P  Y  V  H  G  A  R  G  V  T  I  N  L  *
1441 TTTTTCTTATTTATTTTTTTGTGTATTGATTTCCTTATGACATTAGAGATGTGAATGAGT
1501 TATTTGTGTTCTGTATAGGTGGTAAAACTTCACTTTTCATATTTTTTGGTGTGTATTTGG
1561 AGTTAACAACCAAAAAAA
```

Figure 2 (Continued)

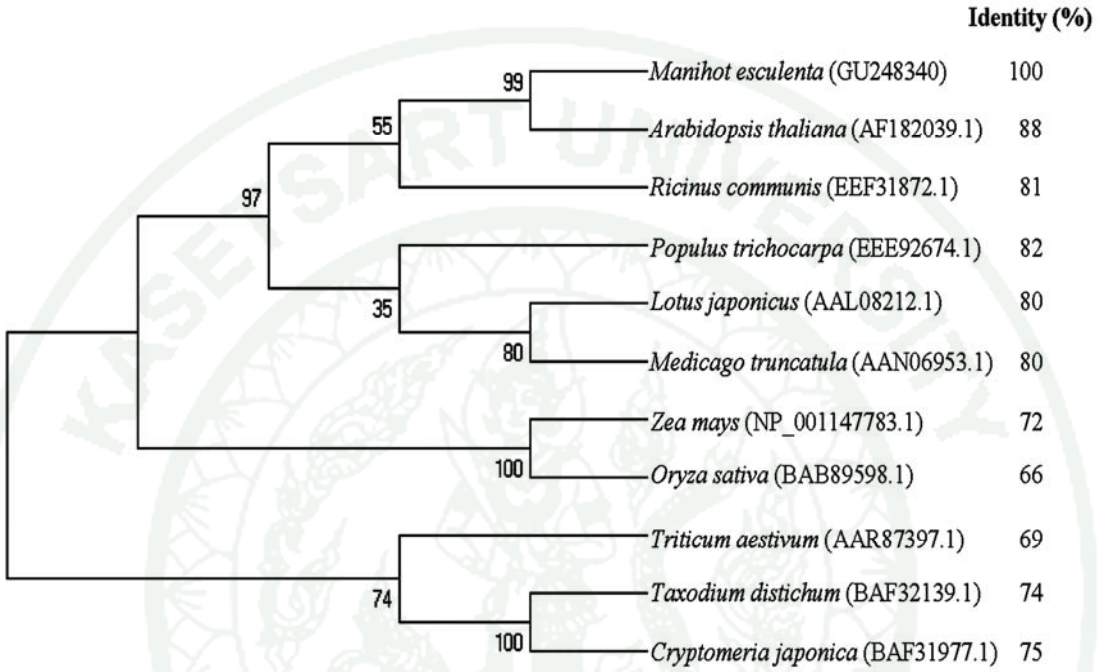


Figure 3 Phylogenetic analysis of aligned amino acid sequences deduced from *MeAMT*2 cDNA and other *AMT* genes. To estimate phylogenetic relationships from the deduced amino acid sequences, a bootstrap majority-rule consensus tree was generated after 1000 cycles of bootstrap replicates the Neighbor-Joining method (Saitou and Nei, 1987) of the MEGA 4 software program (Tamura *et al*., 2007). The numbers on the lines denote frequency of occurrence of bootstrapping.

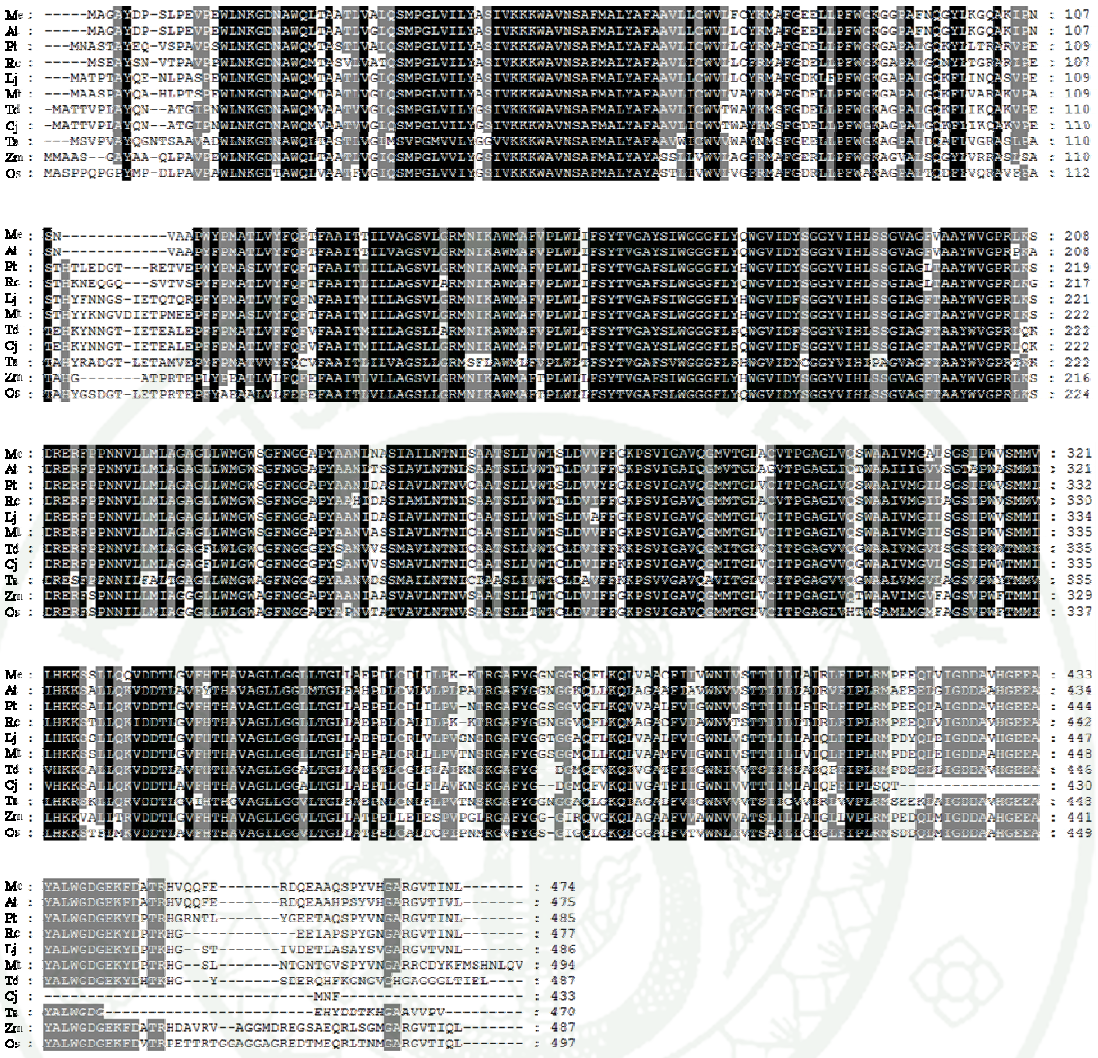


Figure 4 Alignment of amino acid sequences of *AMT*. Abbreviations of the species of each sequence are: Me, *Manihot esculenta*; At, *Arabidopsis thaliana*; Pt, *Populus trichocarpa*; Rc, *Ricinus communis*; Lj, *Lotus japonicus*; Mt, *Medicago truncatula*; Td, *Taxodium districhum*; Cj, *Cryptomeria japonica*; Ta, *Triticum aestivum* ; Zm, *Zea mays* and Os, *Oryza sativa*.

1.2 Nitrate transporter gene (*NRT2*)

NRT2 cDNA (*MeNRT2*) was isolated from fibrous root using reverse transcriptase (RT) PCR and RACE systems. The 1,710 bp cDNA sequence contained a 1,575 bp coding sequence that could encode a protein of 524 amino acids (Figure 5)

which corresponds to a polypeptide with a predicted molecular weight (MW) of 56.4 kDa. The nucleotide sequences of *MeNRT2* gene was deposited in the GenBank database under an accession number GU248341.

The deduced amino acid sequence of *MeNRT2* was used to compare the amino acid composition of the polypeptide to the ones of the other plants' *NRT2*. The analysis revealed a high degree of sequence identity of 67% to 93% when compared to other plants and also highly conserved region when compared with various plant species (Figure 6). The phylogenic tree indicated that the amino acid sequence of *MeNRT2* was in separated clusters. *MeNRT2* appeared in the cluster with closer relationship to *Zea mays NRT* than to that of *Oryza sativa NRT* (Figure 7).

```
1    GCAGCAATGGCGGCCGTCGGCGCTCCGGGCAGCTCTCTGCACGGAGTCACGGGGCGCGAG
1          M  A  A  V  G  A  P  G  S  S  L  H  G  V  T  G  R  E
61   CCGGCGTTCGCCTTCTCCACGGAGCACGAGGAGGCGGCGAGCAATGGTGGCAAGTTCGAC
21    P  A  F  A  F  S  T  E  H  E  E  A  A  S  N  G  G  K  F  D
121  CTGCCGGTGGACTCAGAGCACAAGGCGAAGAGCGTCCGTCTCTTCTCCGTGGCGAACCCA
41    L  P  V  D  S  E  H  K  A  K  S  V  R  L  F  S  V  A  N  P
181  CACATGCGCACCTTCCACCTCTCCTGGATCTCCTTCTTCACCTGCTTCGTCTCTACTTTC
61    H  M  R  T  F  H  L  S  W  I  S  F  F  T  C  F  V  S  T  F
241  GCTGCAGCTCCTCTTGTTCCTATCATTCGTGATAATCTCAATCTTACCAAAGTTGATATT
81    A  A  A  P  L  V  P  I  I  R  D  N  L  N  L  T  K  V  D  I
301  GGTAATGCTGGTGTTGCTTCTGTTTCTGGAAGCATCTTCTCTAGGCTTGCTATGGGTGCG
101   G  N  A  G  V  A  S  V  S  G  S  I  F  S  R  L  A  M  G  A
361  ATTTGCGACCTGTTAGGTCCAAGATATGGATGTGCGTTTCTCATAATGTTAACTGCTCCA
121   I  C  D  L  L  G  P  R  Y  G  C  A  F  L  I  M  L  T  A  P
421  ACTGTGTTTTGTATGTCTTTTGTGTCCTCAGCTGGAGGCTACATAGCCGTCAGATTCATG
141   T  V  F  C  M  S  F  V  S  S  A  G  G  Y  I  A  V  R  F  M
481  ATTGGATTCTCTCTTGCAACCTTTGTGTCTTGCCAGTACTGGATGAGCACTATGTTTAAT
161   I  G  F  S  L  A  T  F  V  S  C  Q  Y  W  M  S  T  M  F  N
541  AGCAAGATCATAGGGCTTGTCAATGGAACCGCAGCTGGTTGGGGGAACATGGGTGGTGGT
181   S  K  I  I  G  L  V  N  G  T  A  A  G  W  G  N  M  G  G  G
601  GCAACTCAGCTGATAATGCCTTTGGTCTATGATGTGATTCAGCGAGCTGGTGCAACTCCA
201   A  T  Q  L  I  M  P  L  V  Y  D  V  I  Q  R  A  G  A  T  P
```

Figure 5 Nucleotide and deduced amino acid sequences of cassava (*Manihot esculenta* Crantz.) cDNA fragment encoding full open reading frame of *NRT2* (*MeNRT2*).The start and stop codons are underlined.

```
661  TTTACTGCTTGGAGGATAGCATTTTTTGTTCCTGGATGTCTTCATGTGATCATGGGAATC
221   F  T  A  W  R  I  A  F  F  V  P  G  C  L  H  V  I  M  G  I
721  TTGGTCTTGACTCTAGGCCAAGATCTGCCTGATGGGAATCTCGGTGCCCTGCAGAAGAAG
241   L  V  L  T  L  G  Q  D  L  P  D  G  N  L  G  A  L  Q  K  K
781  GGTGATGTCGCCAGGGATAAGTTCTCCAAGGTTCTTTGGTATGCCATCACAAATTACAGG
261   G  D  V  A  R  D  K  F  S  K  V  L  W  Y  A  I  T  N  Y  R
841  ACTTGGATCTTTGTCCTTCTGTATGGAATCTCCATGGGCGTTGAACTCTCCACTGACAAC
281   T  W  I  F  V  L  L  Y  G  I  S  M  G  V  E  L  S  T  D  N
901  GTCATCGCCGAGTACATGTACGACCGCTTCGACCTCGACCTCCGCGTCGCTGGGACCATC
301   V  I  A  E  Y  M  Y  D  R  F  D  L  D  L  R  V  A  G  T  I
961  GCCGCCTGCTTCGGCATGGCCAACATCGTCGCACGCCCCATGGGCGGCATCATGTCCGAC
321   A  A  C  F  G  M  A  N  I  V  A  R  P  M  G  G  I  M  S  D
1021 ATGGGCGCGCGCTACTGGGGCATGCGCGCTCGCCTCTGGAACATCTGGATCCTCCAGACC
341   M  G  A  R  Y  W  G  M  R  A  R  L  W  N  I  W  I  L  Q  T
1081 GCCGGCGGCGCCTTCTGCCTCTGGCTGGGGCGCGCCAGCACCCTCCCCGTCTCCGTCGTC
361   A  G  G  A  F  C  L  W  L  G  R  A  S  T  L  P  V  S  V  V
1141 GCCATGGTGCTCTTCTCCTTCTGCGCGCAGGCGGCATGCGGCGCCATCTTCGGGGTTATC
381   A  M  V  L  F  S  F  C  A  Q  A  A  C  G  A  I  F  G  V  I
1201 CCCTTTGTCTCCCGCCGCTCCCTCGGCATCATCTCCGGCATGACGGGCGCCGGCGGCAAC
401   P  F  V  S  R  R  S  L  G  I  I  S  G  M  T  G  A  G  G  N
1261 TTCGGCGCCGGGCTCACGCAGCTGCTCTTCTTTACCTCCTCGACCTACTCCACGGGCAGG
421   F  G  A  G  L  T  Q  L  L  F  F  T  S  S  T  Y  S  T  G  R
1321 GGGCTGGAGTACATGGGCATCATGATCATGGCGTGCACGCTGCCCGTGGTGTTCGTGCAC
441   G  L  E  Y  M  G  I  M  I  M  A  C  T  L  P  V  V  F  V  H
1381 TTCCCTCAGTGGGGGTCCATGTTCTTTCCGCCCAGCGCCACCGCCGACGAGGAGGGCTAC
461   F  P  Q  W  G  S  M  F  F  P  P  S  A  T  A  D  E  E  G  Y
1441 TACGCCTCCGAGTGGAACGACGACGAGAAGAGCAAGGGACTCCATAGCGCCAGCCTCAAG
481   Y  A  S  E  W  N  D  D  E  K  S  K  G  L  H  S  A  S  L  K
1501 TTCGCCGAGAACAGCCGCTCAGAGCGCGGCAAGCGAAACGTCATCCAGGCCGACGCCGCC
501   F  A  E  N  S  R  S  E  R  G  K  R  N  V  I  Q  A  D  A  A
1561 GCCACGCCGGAGCATGTCTAAGTCTACTACTAAGATGGATCGATCGACGATCACCTATAC
521   A  T  P  E  H  V  *
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Figure 5 (Continued)

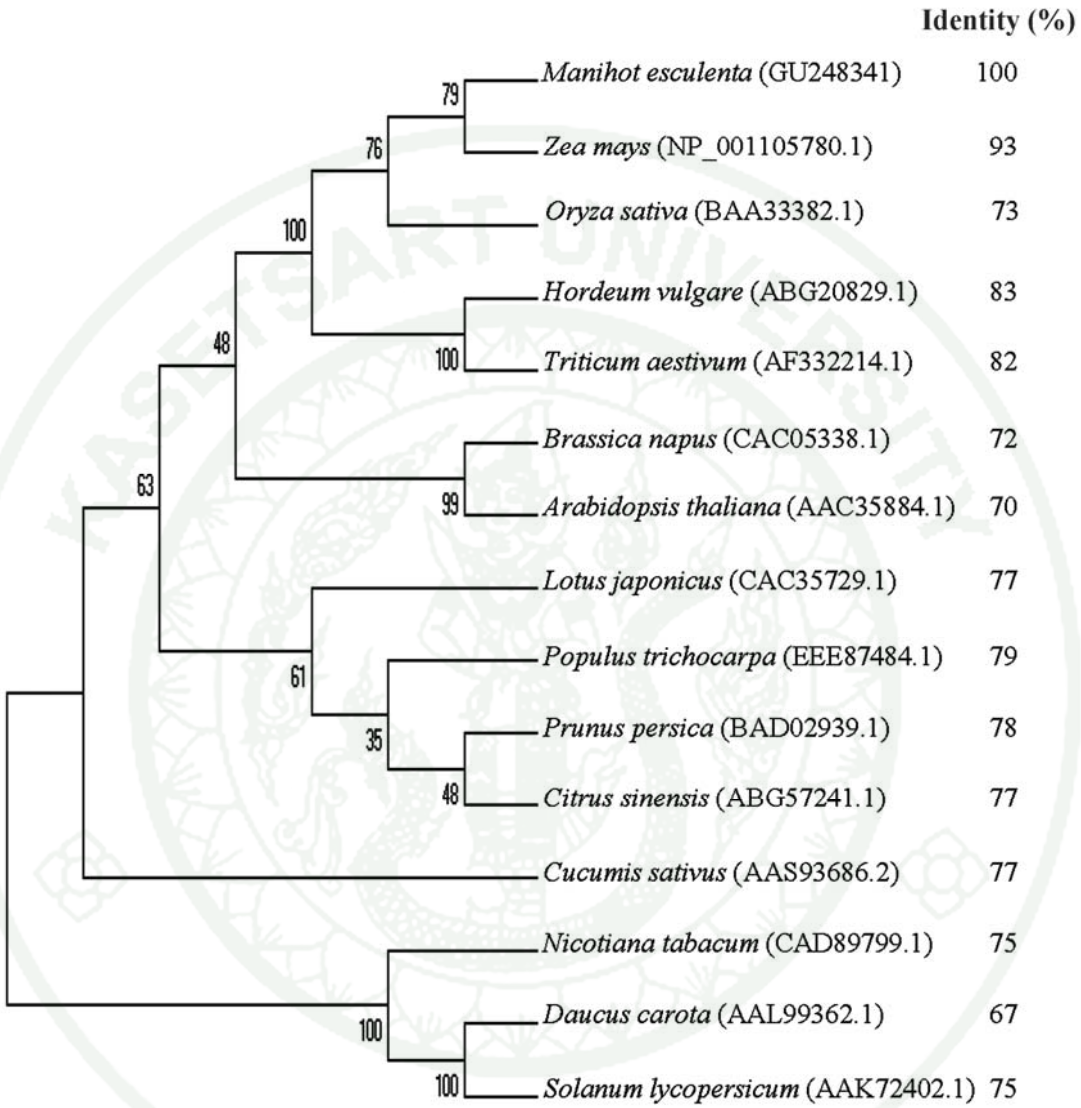

Figure 6 Phylogenetic analysis of aligned amino acid sequences deduced from *MeNRT2* cDNA and other *NRT* genes.

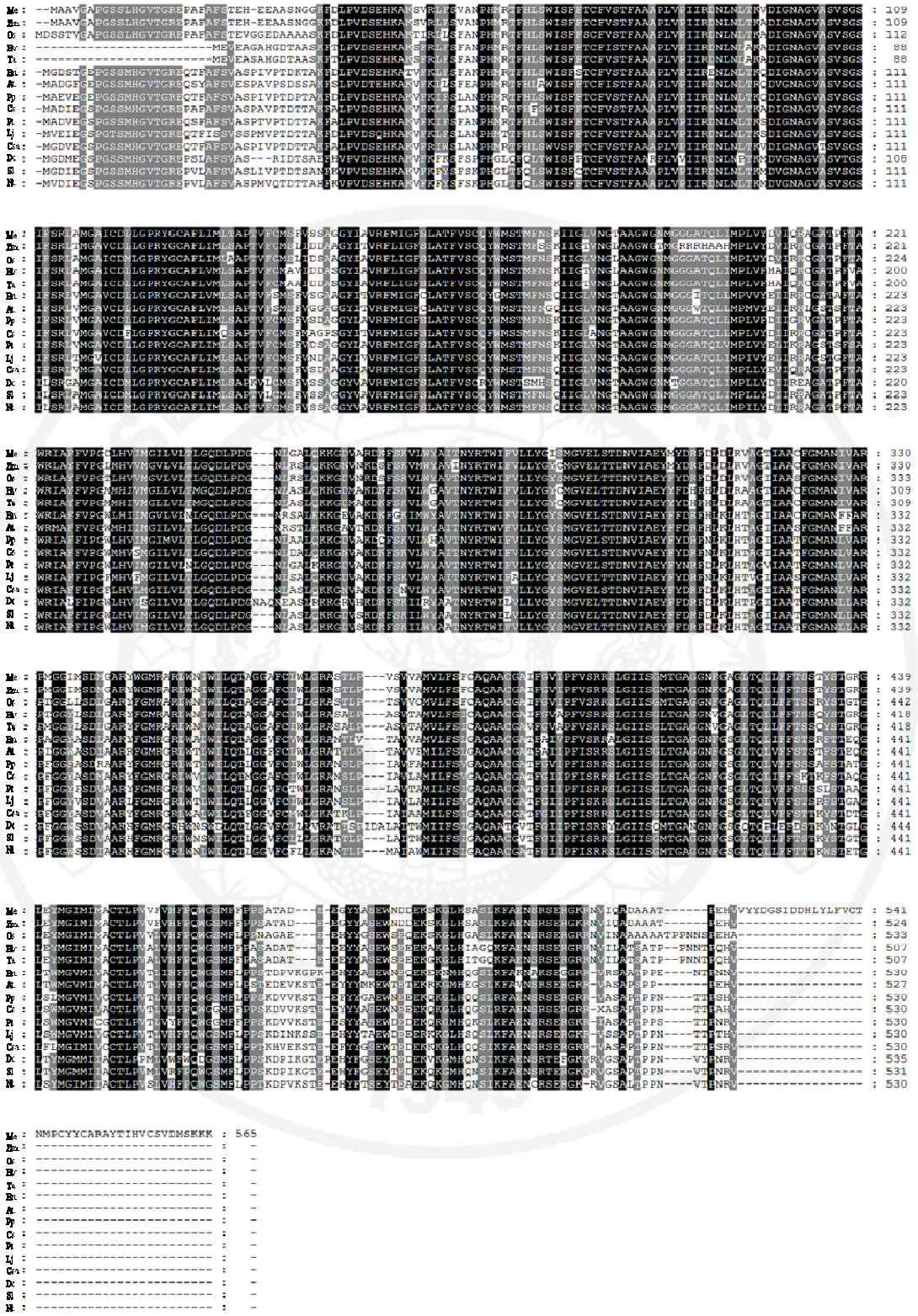

Figure 7 Alignment of amino acid sequences of *NRT*. Abbreviations of the species of each sequence are: Me, *Manihot esculenta*; Zm, *Zea mays*; Os, *Oryza*

sativa; Hv, *Hordeum vulgare*; Ta, *Triticum aestivum*; Bn, *Brassica napus*; At, *Arabidopsis thaliana*; Lj, *Lotus japonicus*; Pt, *Populus trichocarpa*; Pp, *Prunus persica*; Cs, *Citrus sinensis*; Csa, *Cucumis sativus*; Nt, *Nicotiana tabacum*, Dc, *Daucus carota* and Sl, *Solanum lycopersicum*.

1.3 Inorganic phosphate transporter gene (*PT1*)

A cassava *PT1* cDNA (*MePT1*) was isolated from fibrous root using reverse transcriptase (RT) PCR and RACE systems. The 1,743 bp cDNA sequence contained a 1,701 bp coding sequence that could encode a protein of 566 amino acids (Figure 8) which corresponds to a polypeptide with a predicted molecular weight (MW) of 62.2 kDa. The nucleotide sequences of *MePT1* gene was deposited in the GenBank database under an accession number GU248338.

The deduced amino acid sequence of *MePT1* was used to compare the amino acid composition of the polypeptide to the ones of the other plants' *PT1*. The analysis revealed a high degree of sequence identity of 78% to 85% when compared to other plants. The phylogenic tree indicated that the amino acid sequence of *MePT1*was in separated clusters. *MePT1* appeared in the cluster with closer relationship to *Populus tricocarpa PT1* than to that of *Lycopersicon esculentum PT1* (Figure 9).The proteins are extremely hydrophobic and are predicted to be integral membrane proteins containing 12 membrane-spanning domains. This is a common feature shared by proteins responsible for transport of substrates as diverse as sugars, ions, antibiotics, and amino acids (Griffith *et al*. 1992; Marger and Saier 1993). Among the sequences conserved in these 13 transporters, there are the motifs MKMPETARYTA and PESKGKSLEEL which are potential consensus sequences for phosphorylation by protein kinase C and casein kinase II, respectively (Figure 10). The *MePT1* polypeptide contains 12 membrane-spanning and the motifs highly conserved domains as previously reported in various plant species such as *Arabidopsis thaliana* (Muchhal *et al*. 1996); *Medicago truncatula* (Liu *et al*., 1998) and *Lycopersicon esculentum* L. (Liu *et al*., 1998).

```
1     AGTTTAGTCATGGCGAACGATTTGCAAGTGCTAAATGCACTAGATGTCGCGAAGACACAA
1              M  A  N  D  L  Q  V  L  N  A  L  D  V  A  K  T  Q
61    CTGTATCACTTCACAGCGATTGTGATTGCTGGGATGGGGTTTTTTACTGATGCTTATGAC
21     L  Y  H  F  T  A  I  V  I  A  G  M  G  F  F  T  D  A  Y  D
121   CTTTTCTGCATTTCTATGGTCACTAAATTGATTGGTCGTCTTTACTACCATCATGACAAT
41     L  F  C  I  S  M  V  T  K  L  I  G  R  L  Y  Y  H  H  D  N
181   GCATTGAAACCTGGCTCTCTGCCCCCTAATGTTTCAGCAGCTGTTAATGGAGTCGCCTTC
61     A  L  K  P  G  S  L  P  P  N  V  S  A  A  V  N  G  V  A  F
241   TGTGGCACCCTTGCTGGACAGTTGTTCTTCGGATGGCTTGGTGACAAATTAGGCAGGAAA
81     C  G  T  L  A  G  Q  L  F  F  G  W  L  G  D  K  L  G  R  K
301   AAAGTGTATGGAATGACCCTCATGCTTATGGTGGTCTGTTCTGTTGCCTCAGGACTTTCG
101    K  V  Y  G  M  T  L  M  L  M  V  V  C  S  V  A  S  G  L  S
361   TTTGGACATTCTGCAAAGGGTGTTATAGCCACACTTTGTTTCTTCAGATTTTGGCTTGGT
121    F  G  H  S  A  K  G  V  I  A  T  L  C  F  F  R  F  W  L  G
421   TTTGGCATTGGAGGTGACTACCCTCTCTCTGCAACAATCATGTCTGAATATGCTAATAAA
141    F  G  I  G  G  D  Y  P  L  S  A  T  I  M  S  E  Y  A  N  K
481   AAGACTCGTGGGGCATTTATCGCCGCAGTGTTTGCAATGCAAGGATTTGGGATTCTAGCT
161    K  T  R  G  A  F  I  A  A  V  F  A  M  Q  G  F  G  I  L  A
541   GGTGGGATCGTTGCTCTGATTGTGTCGGCTTCCTTTGATCATGCCTACAGTGCCCCTACT
181    G  G  I  V  A  L  I  V  S  A  S  F  D  H  A  Y  S  A  P  T
601   TATGAAGTTGATCCGTTAGGCTCAACAGTGCCGGAAGCAGACTATATTTGGCGAATCATT
201    Y  E  V  D  P  L  G  S  T  V  P  E  A  D  Y  I  W  R  I  I
661   TTGATGTTTGGAGCCGTACCAGCAGCTATGACTTACTACTGGCGAATGAAGATGCCTGAG
221    L  M  F  G  A  V  P  A  A  M  T  Y  Y  W  R  M  K  M  P  E
721   ACAGCTCGTTACACAGCTCTGGTTGCGAAGAACGCTAAGCAAGCAGCTTCAGACATGTCT
241    T  A  R  Y  T  A  L  V  A  K  N  A  K  Q  A  A  S  D  M  S
781   AGAGTACTGCAGGTTGAGCTTGAAGCAGAAGAGCACAAGATAGAGCAGATATCTCAGGAC
261    R  V  L  Q  V  E  L  E  A  E  E  H  K  I  E  Q  I  S  Q  D
841   CCATCCAATTCATTTGGACTTTTTAGTAAGGAATTTGCTCGCAGACATGGGGTTCACTTG
281    P  S  N  S  F  G  L  F  S  K  E  F  A  R  R  H  G  V  H  L
901   CTTGGAACCACCGTGTGCTGGTTCTTACTAGACATAGCTTATTACAGTTCAAATCTTTTC
301    L  G  T  T  V  C  W  F  L  L  D  I  A  Y  Y  S  S  N  L  F
961   CAGAAGGATATCTTTAGCGCAATCGGTTGGATTCCACCTGCACAGACCATGAATGCAATT
321    Q  K  D  I  F  S  A  I  G  W  I  P  P  A  Q  T  M  N  A  I
1021  CATGAAGTATATGTGATTGCCAGAGCACAAACACTTATCGCCTTGTGTGGCACAGTTCCT
341    H  E  V  Y  V  I  A  R  A  Q  T  L  I  A  L  C  G  T  V  P
1081  GGATATTGGTTCACAGTGGCTCTCATTGATCGTATAGGGAGATTTTTCATCCAAGTGATG
361    G  Y  W  F  T  V  A  L  I  D  R  I  G  R  F  F  I  Q  V  M
1141  GGTTTCTTCTTCATGACTGTATTTATGTTTGCTCTGGCAATACCTTACCATCATTGGACA
381    G  F  F  F  M  T  V  F  M  F  A  L  A  I  P  Y  H  H  W  T
```

Figure 8 Nucleotide and deduced amino acid sequences of cassava (*Manihot esculenta* Crantz.) cDNA fragment encoding full open reading frame of *PT1* (*MePT1*).The start and stop codons are underlined.

```
1201  TTGAGGGATAACAGAATTGGGTTTCTGATCATGTACTCACTGACATTTTTCTTCGCCAAT
401    L  R  D  N  R  I  G  F  L  I  M  Y  S  L  T  F  F  F  A  N
1261  TTTGGACCAAATGCCACCACATTTGTTGTTCCAGCAGAGATTTTCCCTGCAAGGCTAAGG
421    F  G  P  N  A  T  T  F  V  V  P  A  E  I  F  P  A  R  L  R
1321  TCAACCTGCCATGGAATATCTGCAGCTTGTGGAAAGGCCGGGGCTATTGTCGGTTCTTTT
441    S  T  C  H  G  I  S  A  A  C  G  K  A  G  A  I  V  G  S  F
1381  GGGTTCCTGTATACTGCACAGAGCACAGATCCGGCAAAGGCTGATGCTGGCTACCCAACA
461    G  F  L  Y  T  A  Q  S  T  D  P  A  K  A  D  A  G  Y  P  T
1441  GGTATTGGAGTGAAAAATTCACTTATTGTGCTCGGTGTGATCAACTTCTTTGGAATCTTG
481    G  I  G  V  K  N  S  L  I  V  L  G  V  I  N  F  F  G  I  L
1501  TTCACTTTATTGGTTCCAGAATCGAAAGGAAAGTCCCTGGAAGAGCTTACAGGAGAAAAT
501    F  T  L  L  V  P  E  S  K  G  K  S  L  E  E  L  T  G  E  N
1561  GAAGATGAACCACAAGAAGCCTCTTGGAGTAGGACAGTGCCGGTGGCCGGATTTGGCTTC
521    E  D  E  P  Q  E  A  S  W  S  R  T  V  P  V  A  G  F  G  F
1621  ACAAAAAAACTTCACCAGATGAAATTATGTTGTAAGAAAATTTGTGAAATACAATATAAG
541    T  K  K  L  H  Q  M  K  L  C  C  K  K  I  C  E  I  Q  Y  K
1681  CATGGTTCTAAATTCTGGGGTAATAAATAATTATTGACAGTAAAAAAAAAAAAAAAAAA
561    H  G  S  K  F  W  G  N  K  *
1741  AAA
```

Figure 8 (Continued)

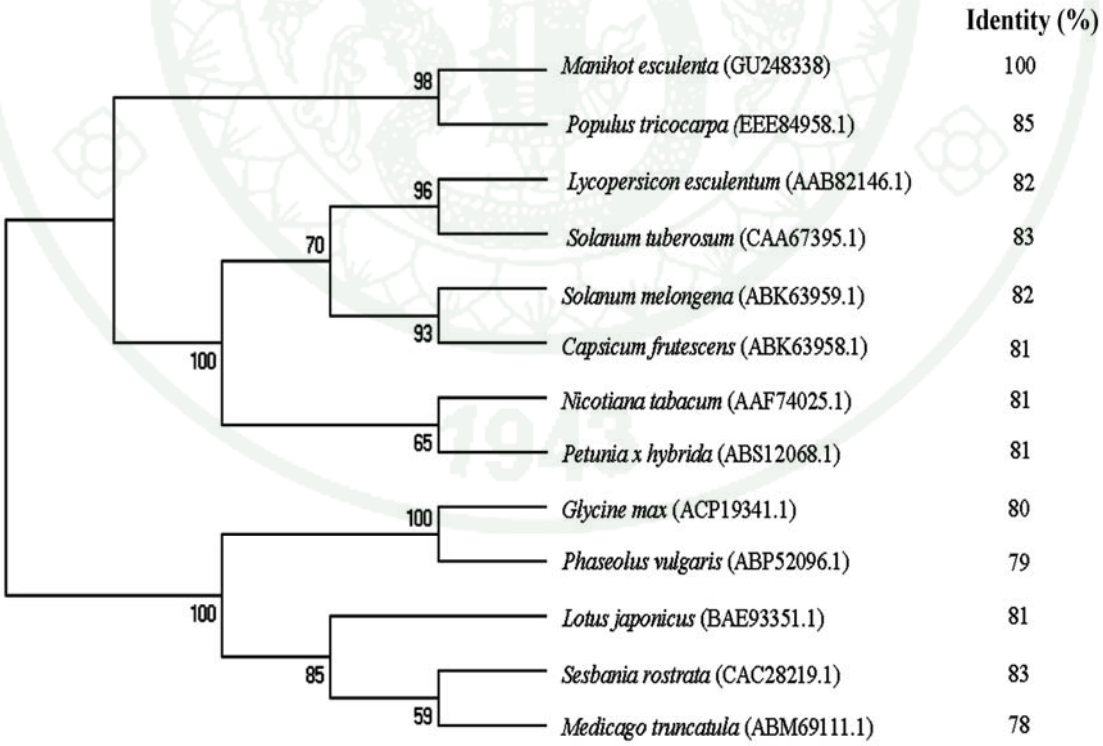

Figure 9 Phylogenetic analysis of aligned amino acid sequences deduced from *MePT1* cDNA and other *PT* genes.

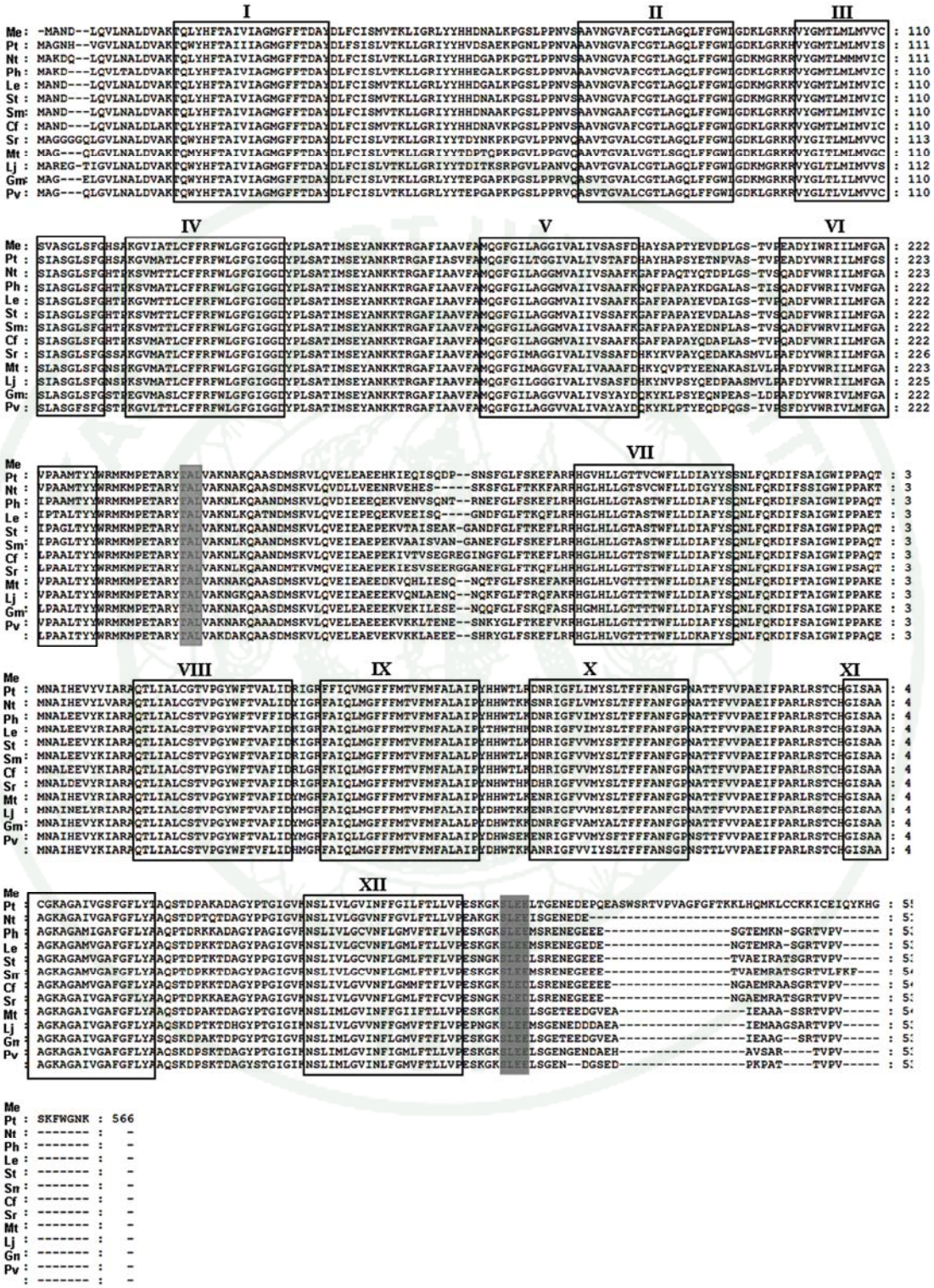

Figure 10 Alignment of *MePT1* with other plant *PT* amino acid sequences. Identical amino acids conserved substitutions are indicated by dots. The membrane-

spanning domains of *MePT1* as predicted by TopPred (Claros and von Heijne, 1994) are shaded and their numbering is indicated by Roman numerals (I-XII). The green boxed and shaded sequences are consensus sites for phosphorylation by casein kinase II, and blue boxed and shaded sequences are consensus sites for phosphorylation by protein kinase C. Abbreviations of the species of each sequence are: Me, *Manihot esculenta*; Pt, *Populus tricocarpa*; Nt, *Nicotiana tabacum*; Ph, *Petunia x hybrida*; Le, *Lycopersicon esculentum*; St, *Solanum tuberosum*; Sm, *Solanum melongena*; Cf, *Capsicum frutescens*; Sr, *Sesbania rostrata*; Mt, *Medicago truncatula*; Lj, *Lotus japonicus*; Gm, *Glycine max*; Pv, *Phaseolus vulgaris*.

1.4 Zinc transporter gene (*ZIP*)

Zinc transporter gene (*MeZIP*) was cloned from cassava fibrous root using primers designed to compass highly conserved regions of *ZIP*. Sequencing revealed that the partial *MeZIP* clone was 723 nucleotides in length and contained coding sequence that could encode a protein of 241 amino acids (Figure 11). The partial nucleotide sequences of *MeZIP* gene was deposited in the GenBank database under an accession number GU248339. The complete nucleotide of *ZIP* sequence was 1,062 bp in length based on *Fragaria x ananassa*. The sequence was blasted and aligned using the programs BLASTX and CLUSTALW. The deduced amino acid sequence of *MeZIP* was used to compare the amino acid composition of the polypeptide to the other plant *ZIP*. The analysis revealed a high degree of sequence identity of 67% to 73% when compared to other plants and highly conserved when compared with various plant species (Figure 12). The phylogenic tree indicated that the amino acid sequence of *MeZIP* was in separated clusters, which *MeZIP* appeared in the cluster with closer relationship to *Fragaria x ananassa ZIP* than to that of *Glycine max ZIP* (Figure 13).

```
1   TTGCCTGAAGGTTACGAGAGCTTGACATCCCCTTGTCTCAATGAGAATCCATGGGGAAAG
1    L  P  E  G  Y  E  S  L  T  S  P  C  L  N  E  N  P  W  G  K
61  TTTCCTTTCACTGGTTTTGTGGCCATGGTTTCTGCCATTGAGACTTTGATGGTTGATGCT
21   F  P  F  T  G  F  V  A  M  V  S  A  I  E  T  L  M  V  D  A
121 TTTGCGACTTCTTATTATACCAAGTCTCATGGACAAGTCAGAAATATAGCTGGAGATGAG
41   F  A  T  S  Y  Y  T  K  S  H  G  Q  V  R  N  I  A  G  D  E
181 GAGAAAACAGAAGAAGATGGAGGATTTCATACTCATGCAACTCATGATCATTCTCATTGT
61   E  K  T  E  E  D  G  G  F  H  T  H  A  T  H  D  H  S  H  C
241 TCAGGTTTGATTGAGAATTCTGCTTCACCTGAACTCCTTCGCCATCGAGTTATTTCTCAG
81   S  G  L  I  E  N  S  A  S  P  E  L  L  R  H  R  V  I  S  Q
301 GTTTTGGAGTTGGGAATTGTGGTTCACTCTGTGATAATAGGAATCTCTTTAGGTGCTTCT
101  V  L  E  L  G  I  V  V  H  S  V  I  I  G  I  S  L  G  A  S
361 CAAAGTCCTAAAACAATAAGGCCTCTAGTAGCTGCGCTCACCTTTCATCAGTTCTTTGAG
121  Q  S  P  K  T  I  R  P  L  V  A  A  L  T  F  H  Q  F  F  E
421 GGTATGGGACTTGGTGGTTGCATTTGTCAGGCAAAATTTAAGGGAAGAGTTATGGCGATT
141  G  M  G  L  G  G  C  I  C  Q  A  K  F  K  G  R  V  M  A  I
481 ATGGCACTTTTCTTCTCTCTGACAACACCAATTGGGATTGGGATTGGTATTGGGATATCA
161  M  A  L  F  F  S  L  T  T  P  I  G  I  G  I  G  I  G  I  S
541 AACGCGTACAATGAAAACAGCCCAACTGCCCTAATTGTTGAAGGGATTTTTAATTCAGCC
181  N  A  Y  N  E  N  S  P  T  A  L  I  V  E  G  I  F  N  S  A
601 TCAGCTGGAATTTTAATTTACATGGCATTGGTGGATCTTCTTGCTGCTGATTTCATGAAT
201  S  A  G  I  L  I  Y  M  A  L  V  D  L  L  A  A  D  F  M  N
661 CCCAAAGTACAGGTCAATGGAAAACTTCAAATTGGAGTTAATCTTTCTCTTCTTGTTGGA
221  P  K  V  Q  V  N  G  K  L  Q  I  G  V  N  L  S  L  L  V  G
721 GCA
241  A
```

Figure 11 Nucleotide and deduced amino acid sequences of cassava (*Manihot esculenta* Crantz.) cDNA fragment encoding full open reading frame of *ZIP* (*MeZIP*).

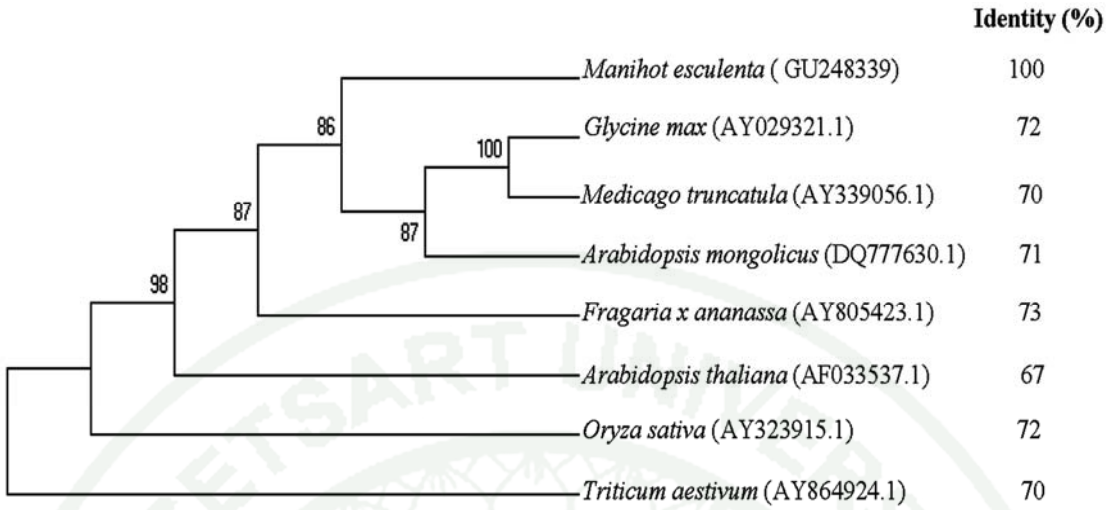

Figure 12 Phylogenetic analysis of aligned amino acid sequences deduced from *MeZIP* cDNA and other *ZIP* genes.

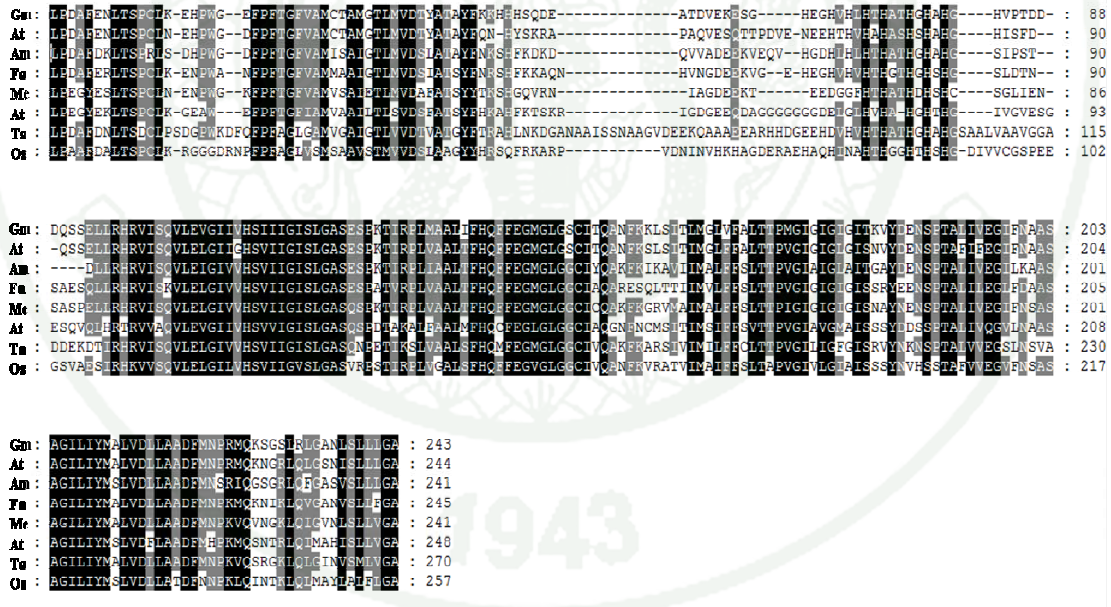

Figure 13 Alignment of amino acid sequences of *ZIP*. Abbreviations of the species of each sequence are: Gm, *Glycine max*; At, *Arabidopsis thaliana*; Am, *A. mongolicus*; Fa, *Fragaria x ananassa*; Me, *Manihot esculenta*; Mt, *Medicago truncatula*; Ta, *Triticum aestivum* and Os, *Oryza sativa*.

2. Different expression of transporter genes in cassava plant compared with agronomic characteristics during growth development

2.1 Response of cassava plants to ammonium, nitrate, Pi and zinc

The response of cassava to different NH_4NO_3, Pi and Zn supplies were studied; NH_4NO_3, Pi and Zn concentrations were doubly increased in each step of increment at concentrations of 0, 5, 10, 20 and 40 mM NH_4NO_3; 0, 0.3, 0.6, 1.2 and 1.8 mM KH_2PO_4; 0, 0.037, 0.075, 0.15 and 0.22 mM $ZnSO_4.7H_2O$, respectively in MS media. Cassava was grown under tissue culture system in order to investigate its visible phenotype.

2.1.1 Total fresh weight (FW) and dry weight (DW)

Statistical analysis showed highly significant difference in 3 variables; varieties, harvesting time and media treatment. Varietal effect was observed by measuring the total fresh weight (FW) and dry weight (DW) of young leaf, stem and fibrous root in all varieties along development. Per one plant, the average FW of R1 was higher than those of KU50 and R1 whereas the average DW of HB80 was the highest. The average FW and DW of HB80, KU50 and R1 varieties were maximum at eight weeks after harvesting and were also highest in +1E treatment when compared with the control (Figure 14 and 15). The +1E treatment demonstrated the highest FW and DW at 8 weeks and was greater than those obtained from E treatment (control) by 15% and 16%, respectively.

The partition of young leaf, stem and fibrous root DW of HB80 were 40%, 25% and 35%, of KU50 were 30%, 30% and 40% and of R1 were at 40%, 30% and 30%, respectively (Figure 16).

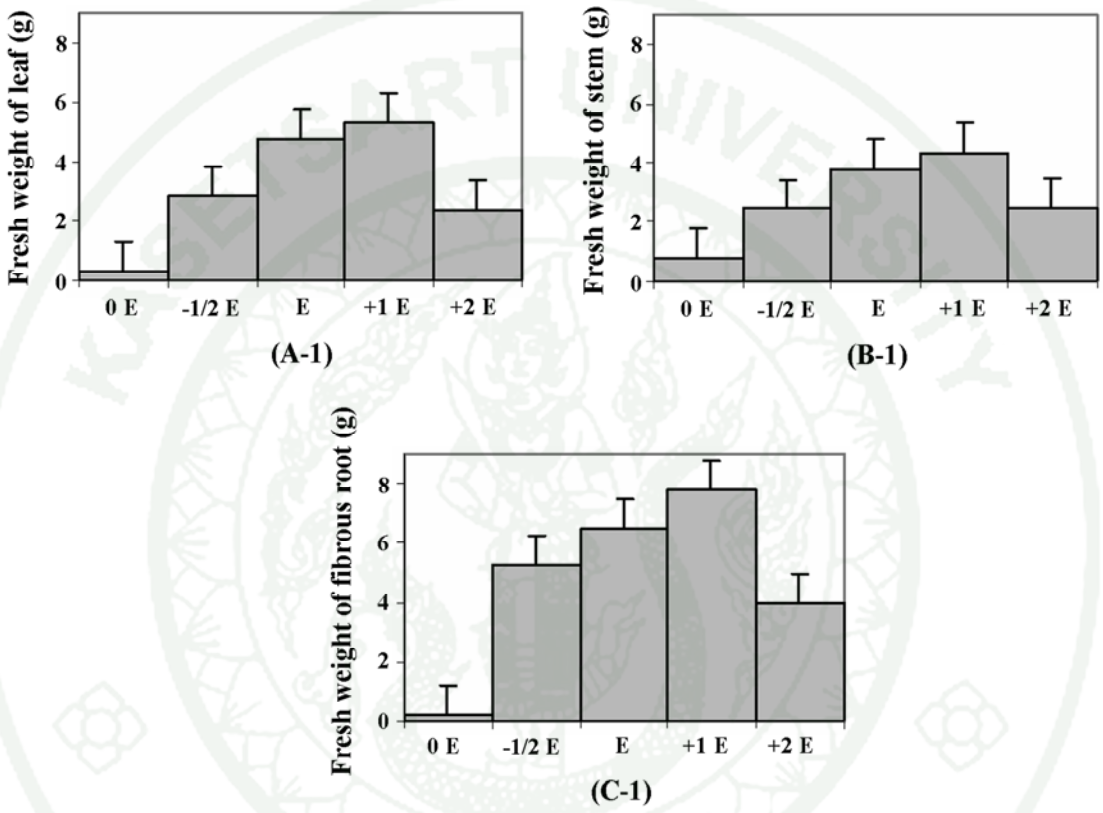

Figure 14 Fresh weight of young leaf, stem and fibrous root (g) of cassava grown under 5 different MS media as 0E, -1/2E, E, +1E and +2E treatment, fresh weight of young leaf (A-1), stem (B-1) and fibrous root (C-1).

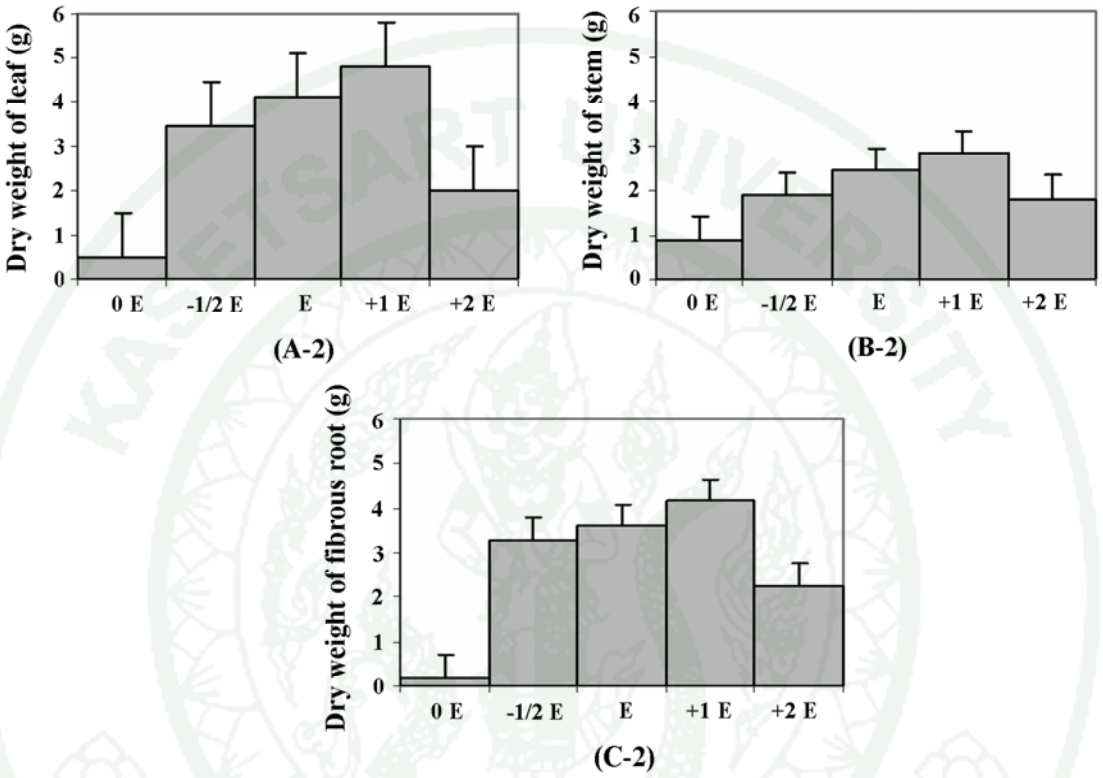

Figure 15 Dry weight of young leaf, stem and fibrous root (g) of cassava grown under 5 different MS media as 0E, -1/2E, E, +1E and +2E treatment, dry weight of young leaf (A-2), stem (B-2) and fibrous root (C-2).

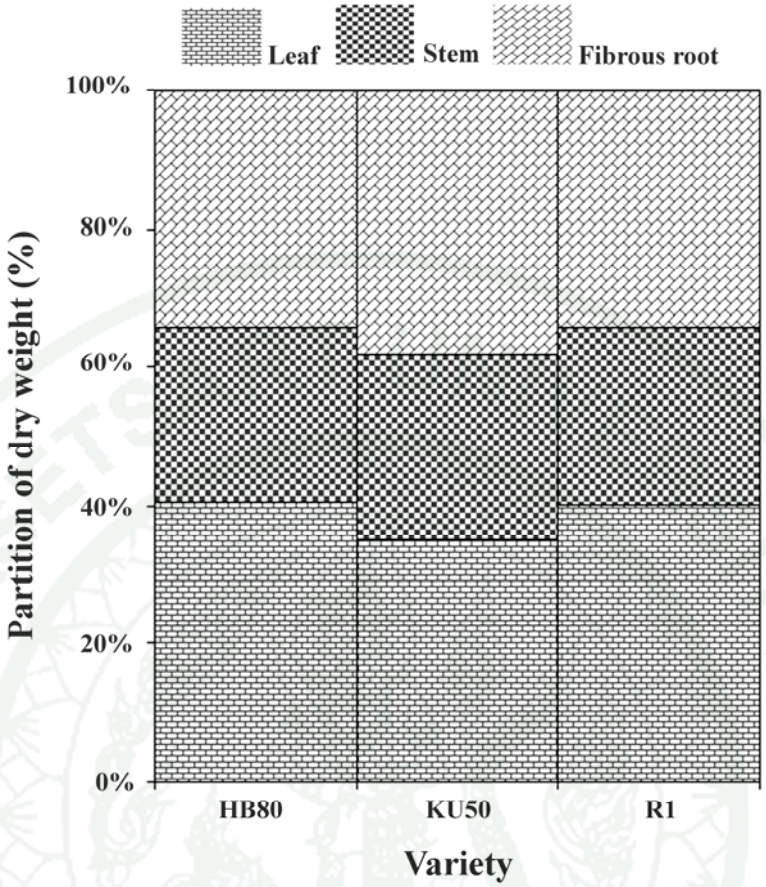

Figure 16 Partitioning percentages of dry weight in various plant parts of HB80, KU50 and R1.

2.1.2 Leaf, petiole, stem and fibrous root length

Statistical analysis showed highly significant differences in leaf, petiole, stem and fibrous root length for third varietal effect, harvesting time effect and different media effect. Per one plant, the average leaf, petiole, stem and fibrous root length of R1 were higher than those of KU50. The average leaf, petiole, stem and fibrous root length of HB80, KU50 and R1 were maximum at eight weeks after harvesting and were at the highest at +1E treatment when compared with the control (Figure 17).

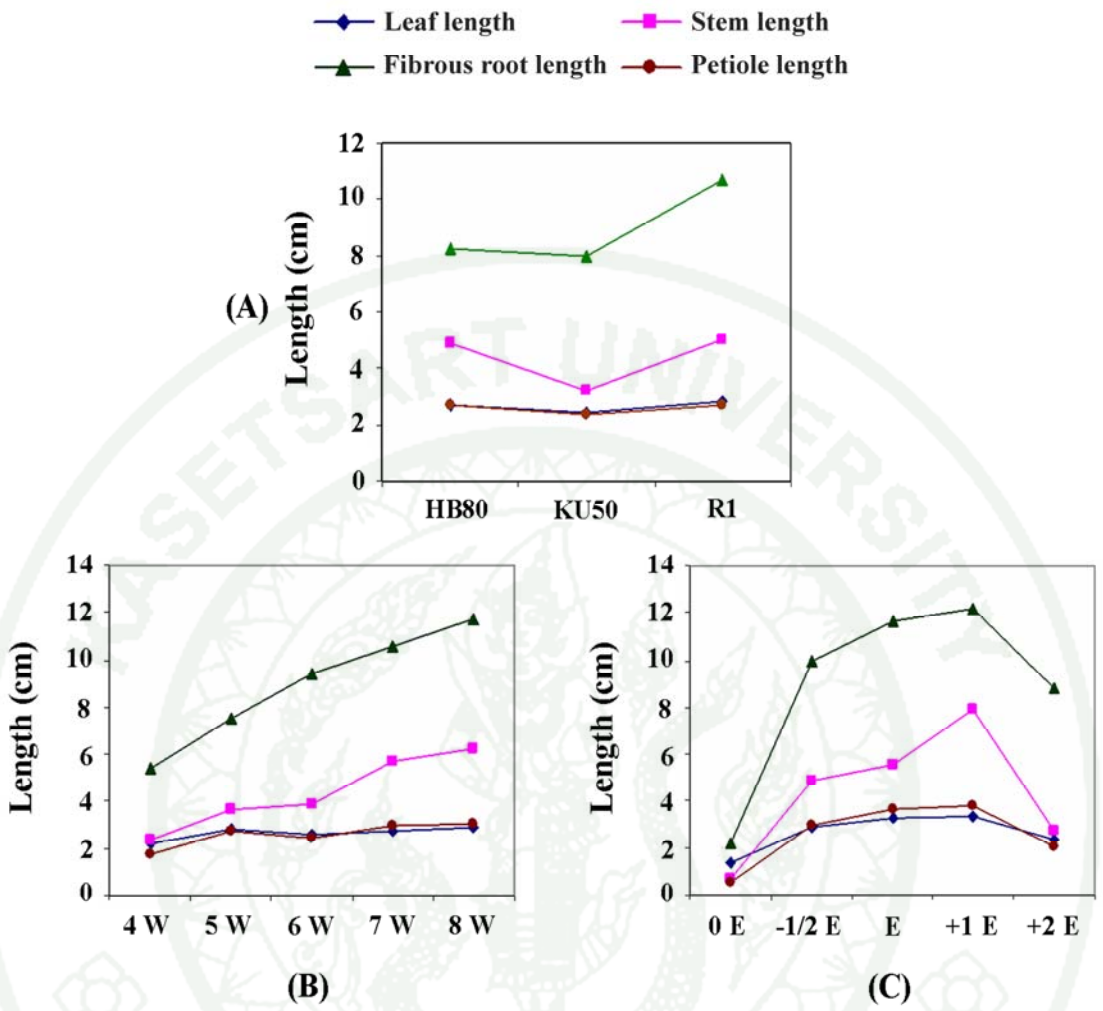

Figure 17 Average leaf, stem, fibrous root and petiole length comparing among 3 cassava varieties (A), at different harvesting times (B) and under different culture mediums (C).

2.1.3 Leaf width, leaf and fibrous root number

Overall plant size was smaller when plants were grown under NH_4^+, NO_3^-, Pi and Zn^{2+} deficiency (0E and -1/2E) and supra-level (+2E) treatment compared with sufficient (E and +1E) NH_4^+, NO_3^-, Pi and Zn^{2+} treatments (Figure 19). Per one plant, the average leaf width of R1 was greater than those of KU50 and were maximum at eight week after harvesting and was at the highest in +1E treatment when compared with the control (Figure 18-A).

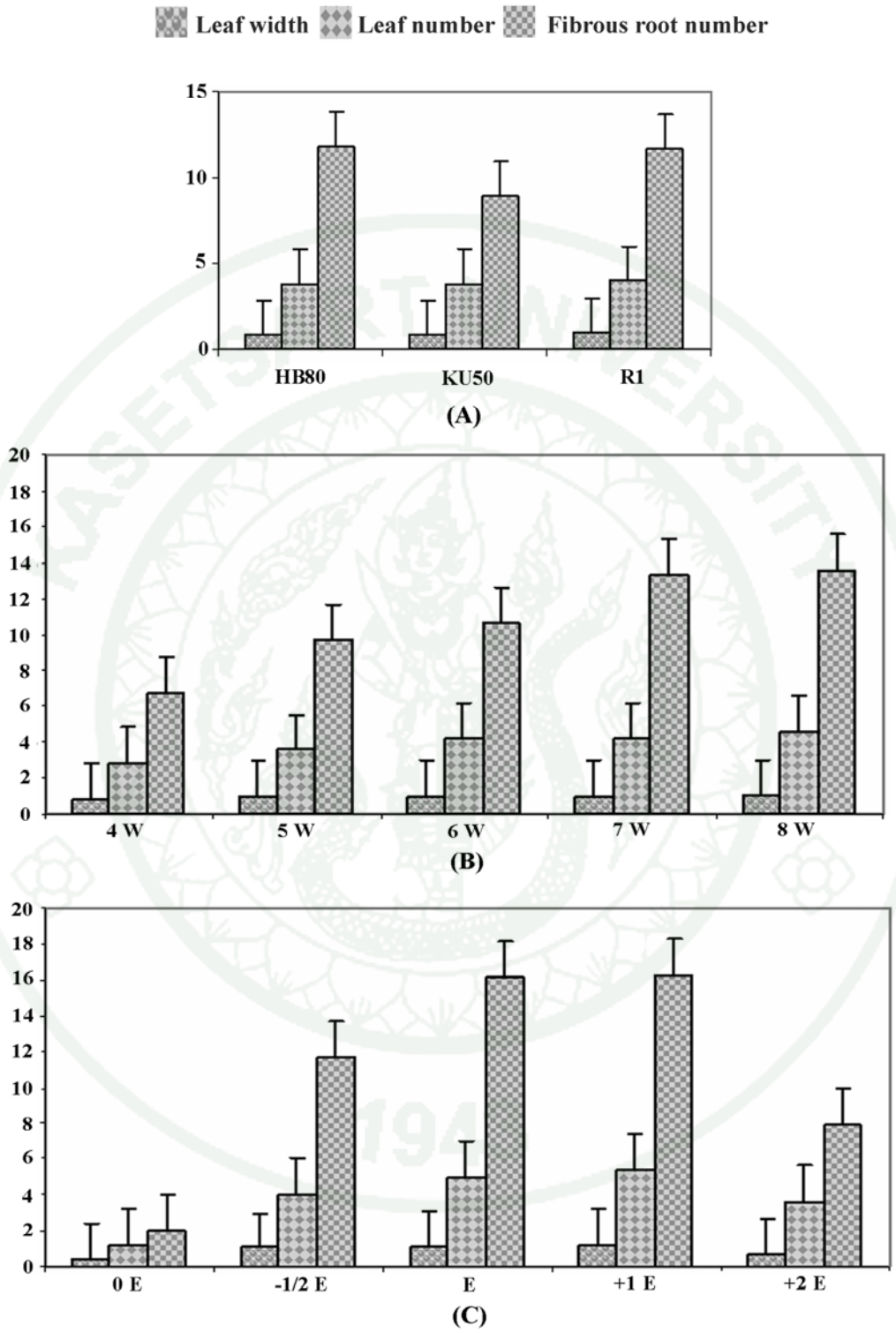

Figure 18 Average leaf width, leaf and fibrous root number comparing among 3 cassava varieties (A), at different harvesting times (B) and under different culture mediums (C).

Statistic analysis of leaf and fibrous number showed highly significant differences in leaf and fibrous root numbers for third varietal effect, harvesting time effect and different media effect. Per one plant, the average leaf and fibrous root numbers of R1 was greater than those of KU50. The average leaf and fibrous root numbers of HB80, KU50 and R1 were at the highest at eight week after harvesting time and was at the highest at +1E treatment when compared with the control (Figure 18-B, 18-C).

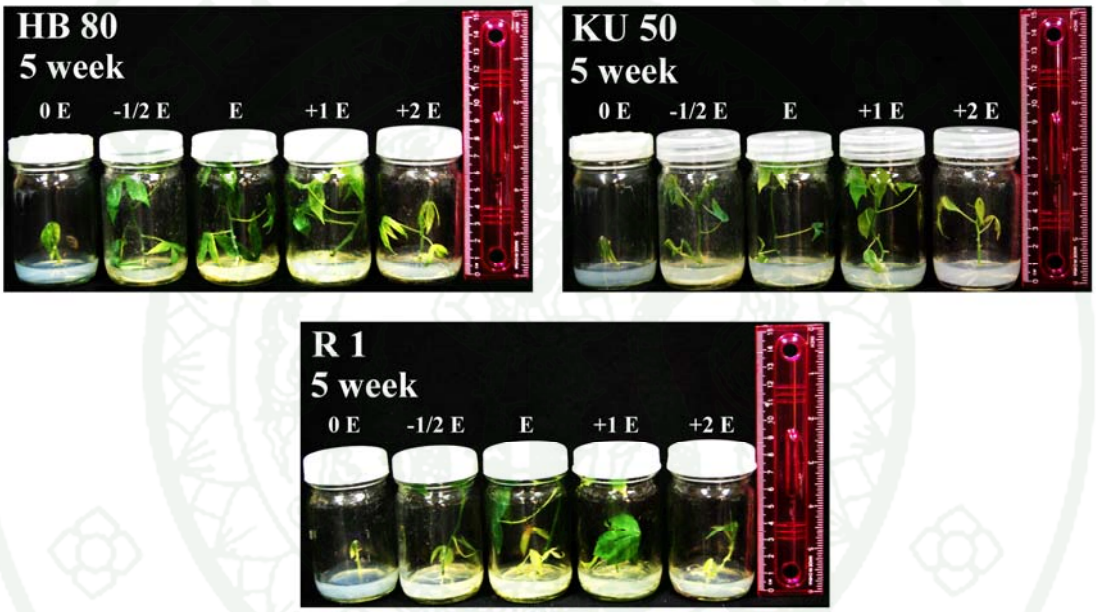

Figure 19 Phenotypic differences of cassava plants (cv. HB80, KU50 and R1) in response to deficient, sufficient and high NH_4^+, NO_3^-, Pi and Zn^{2+}. Cassava plants were grown tissue media under deficiency (0E and -1/2E treatment), sufficiency (E and +1E treatment) and high (+2E treatment) after 5 weeks.

Nitrate fed plants showed greater plant height, more leaf number, greater leaf area, higher dry weight, greater root length and higher moisture percent in stem and leaf. On the other hand, ammonium fed plants was greener due to higher chlorophyll concentration in leaves. Nitrate fed plants exhibited higher content of all essential elements such as N, P, K, Ca, Mg, Fe and Mn, while ammonium fed plants demonstrated higher Mg, Fe and P concentration (Sadiiu and Chattopadhyay, 2002) in

leaf which may be correlated to higher chlorophyll concentration. There was also a tendency of retention of Fe, Mn and P in roots of nitrate plants. Final harvest data indicated better yield under the influence of nitrate form of nitrogen due to larger size of panicle, higher spikelet number, grain number and grain weight (Sadiiu and Chattopadhyay, 2002).

Pi insufficiency affected both root and shoot growth, partitioning and assimilate levels in barley roots. In comparison with the Pi-sufficient treatment, moderate Pi insufficiency reduced the root dry mass of barley seedlings by about one-third when plants were grown under ambient CO_2. The impact of Pi deficiency was more severe on shoots than on roots, therefore, total shoot growth was reduced by almost one-half when the Pi supply was decreased from 1.0 to 0.05 mM. For all genotypes, high P supply increased the relative growth rate of shoot, shoot P concentration, and P-uptake rate of roots but decreased root-to-shoot ratio, root-hair length, and P-utilization efficiency. Simulation of P uptake revealed that no other P-mobilization mechanism was involved since predicted uptake approximated observed uptake indicating that the processes involved in P transport and morphological root characterstics affecting P uptake are well described. (Balemi and Schenk, 2009).

The Zn-deficiency phenotypes in cassava plants were studied in this research. In general, plants showed all the characteristics as previously described for Zn deficient plants in several other species: reduction of internodal growth with an enhanced rosette-like development, small and discoloured leaves, poor root formation and reduced seed and fruit production (Bergmann, 1992; Marschner 1995 and Broadley *et al*., 2007). The reduced height of Zn-deficient plants (0 mM Zn) compared with Zn-sufficient (0.15 mM Zn) plants could be due to a decrease of the growth hormone auxin correlating with decreased Zn levels in the plants. A previous study on the relationship of Zn and auxin has shown that a decrease in auxin precedes the appearance of visible symptoms of Zn deficiency in tomato and sunflower suggesting that Zn is required for auxin production. The effect of a reduction in auxin levels was only observed in Zn-deficient plants, whereas it was not observed in the Mn-deficient and Cu-deficient plants (Skoog, 1940). The ‘little leaf’ is typical for Zn-

deficiency, but it is also found in other plants. The increasing chlorosis in the leaf is assumed to be due to the effect of decrease in Zn in the photosynthetic enzymes. In Zn-deficient plants, it is also known to result in an impaired response to oxidative stress, likely due to a reduction in superoxide dismutase levels (Hacisalihoglu *et al*, 2003). Sharma *et al*., (1995) showed a requirement of Zn for stomatal opening in cauliflower. Zn-deficiency induced increased epicuticular wax deposits, lamina thickness, degree of succulence, water saturation deficit, diffusive resistance, and proline accumulation and decreases in carbonic anhydrase activity, water potential, stomatal aperture, and transpiration in the leaves of cauliflower plants (Sharma *et al*., 1995).

2.1.4 Chlorophyll content

Chlorophyll content was strongly affected by different varieties, media and harvesting times. In cassava, the Chl *a, b* and total chlorophyll content of R1 showed insignificant result when compared with KU50 and HB80 (Figure 20-A). At different harvesting times, chlorophyll content slightly increased at 7 weeks after culture in all varieties. After 7 weeks, all cassava varieties showed lower level of Chl *a*, *b* content and total chlorophyll (Figure 20-B). Under different media treatment, there was the highest Chl *a*, *b* contents and total chlorophyll content were observed when the plants treated with E and +1E treatment (0.66 and 0.64 g m^{-2}, respectively) and was the lowest when treated with 0E and +2E treatment (Figure 20-C) and chlorosis symptoms were observed in both treatments of all varieties.

In wheat, ammonium fed plants were greener due to higher chlorophyll concentration in leaves and ammonium fed plants had higher Mg, Fe and P concentration in leaves which may be correlated with higher chlorophyll concentration (Sadiiu and Chattopadhyay, 2002). This result is similar to chlorophyll concentrations of 0.52 g m^{-2} in leaves of nitrate supplied kohlrabi plants, while the ammonium supplied kohlrabi leaves synthesized 0.62 g chlorophyll m^{-2} (Lawlor, 1993).

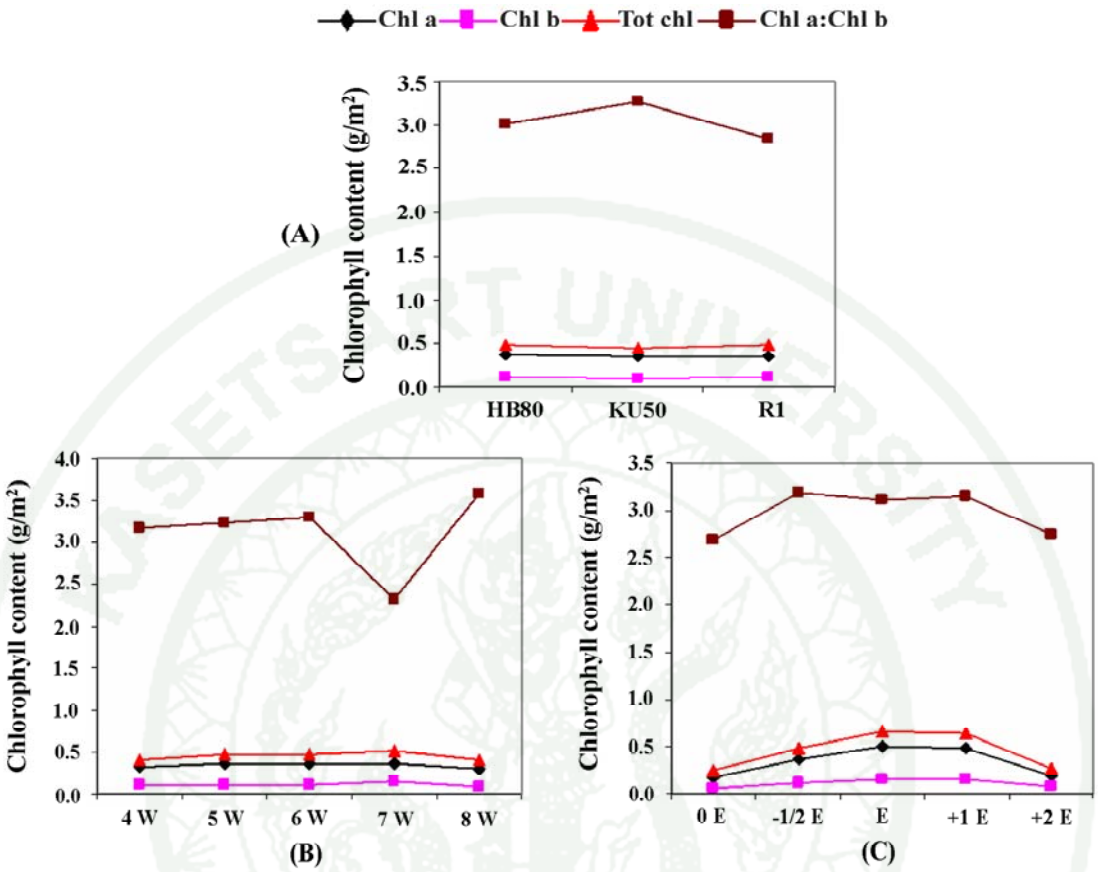

Figure 20 Average leaf pigment composition comparing among 3 cassava varieties (A), at different harvesting times (B) and under different culture mediums (C).

2.2 Expression of transporter genes in cassava plant

Various plant parts of developing cassava were harvested on a weekly basis, started at 4 weeks until 8 weeks after cultured in the different media and different parts of cassava tissues such as young leaves, stem and fibrous root were collected. Total RNA was extracted from each plant part using modified method by Dellaporta *et al*., 1983. Total RNA was obtained at high quality (Figure 21). By spectrophotometry, the calculated concentration of total RNA was approximately 2.0 µg/µl.

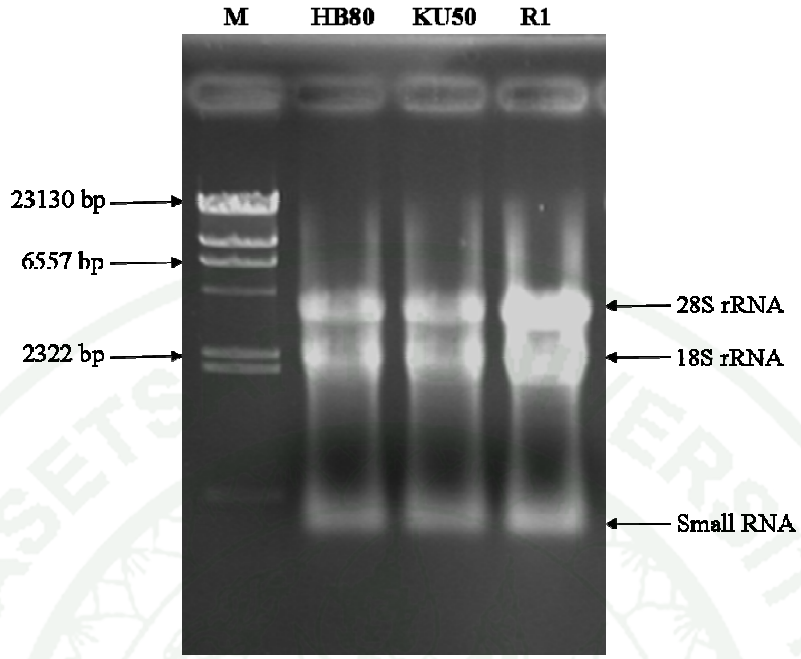

Figure 21 Ethidium bromides stained 1% agarose gel of total cassava RNA following an electrophoresis at 100 V for 20 min. Using modified Dellaporta RNA extraction method (1990), Molecular weight marker (M) is shown in the left lane.

2.2.1 *MeAMT2* expression towards the availability of NH_4^+ in the medium

To define the NH_4^+ concentration at which the ammonium transporter genes are expressed, cassava plants were grown in the presence of different concentrations MS media of NH_4^+ (NH_4NO_3). After 4-8 weeks of treatment, the young leaf, stems and fibrous roots were harvested for total RNA isolation. Real-time PCR analyses were carried out to determine the expression of the *MeAMT2* cDNA. Figure 22-A shows, the following results; R1 showed higher transcript levels of *MeAMT2* than those of KU50 and HB80. The expression pattern of *MeAMT2* in various tissues of cassava during vegetative stage was described as follows, *MeAMT2* gene highly expressed in stem when compared with fibrous root and young leaf. Thus, the expression pattern of *MeAMT2* demonstrated that this gene was differentially expressed in various tissues and at different growth stages. In *Arabidopsis thaliana*,

AtAMT2 was highly expressed in both roots and shoots (Sohlenkamp *et al*. 2000) and Sohlenkamp *et al*., (2002) also found that the highest *AtAMT21* transcription was observed in root, stems and rosette leaves while the lower levels of transcription were found in flowers and siliques.

As shown in Figure 22-C, the transcription level of the ammonium transporters was high in the young leaf, stem and fibrous root of plants grown at 0 (0E treatment) and 20 mM NH_4^+ (+1E treatment), and decreased with increasing NH_4^+ concentration. A comparison of the normalized *MeAMT2* transcription levels revealed that *MeAMT2* transcription were 1.03, 1.36 and 1.46-fold higher in the 20 mM treatment for young leaf, stem and fibrous root, respectively, when compared with the level in the 10 mM treatment (E treatment). *MeAMT2* is more sensitive to increasing ammonium levels and the transcript level is significantly lower in the 30 mM NH_4^+ treatment (+2E treatment). The regulation of *MeAMT2* expression by NH_4^+ availability was further examined by resupplying NH_4NO_3 to the plants that were NH_4^+ deficient and strongly expressing the genes. These observations suggested the existence of a fine coordination between gene expression, presumed to lead to the synthesis of more transporters and increased uptake, and the availability of NH_4^+ in the media.

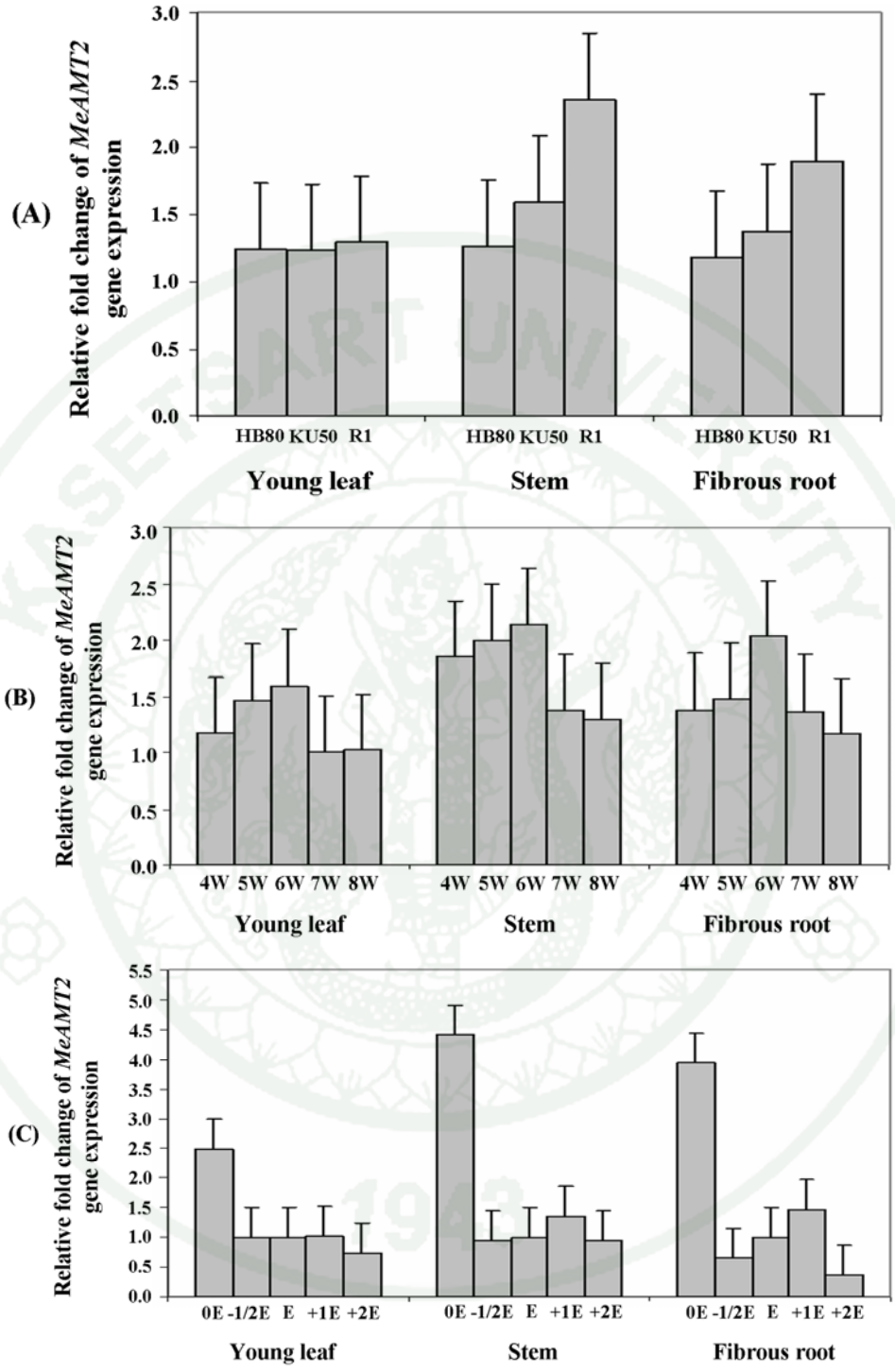

Figure 22 Relative fold change in *MeAMT2* expression in young leaves, stems and fibrous roots of three cassava varieties; HB80, KU50 and R1 (A). Cassava tissues were grown at different NH_4^+ concentration supplemented in MS media; 0, 5, 10, 20 and 30 mM (C) and harvested at 4, 5, 6, 7 and 8 weeks after subculture (B).

During growth development, the expression pattern of *MeAMT*2 gene in young leaf, stem and fibrous root showed that the maximal transcription levels of *MeAMT*2 could be observed between 5 and 6 weeks after subculture, and it gradually decreased afterward (Figure 22-B). Similar results were also observed in *Arabidopsis* as *AtAMT2* in roots when subjected to N supply regulation. Thus, the amount of *AtAMT2* transcription increased in roots after 3 days of N deprivation (Sohlenkamp *et al*., 2002). It has been shown for *AtAMT1;1* that its transcription levels respond to the internal N status of the plant (Rawat *et al*., 1999). Given the similarities in the responses of *AtAMT2* and *AtAMT1;1* to altered N supply, it is likely that transcription of *AtAMT2* in *Arabidopsis* roots responds to the same N cues. The lack of response of *AtAMT2* expression in shoots of plants exposed to different N regimes may reflect real differences in the regulation of the gene in shoots and roots.

2.2.2 *MeNRT2* expression towards the availability of NO_3^- in the medium

To define the NO_3^- concentration at which the nitrate transporter genes are expressed, cassava was grown in the presence of different concentrations of NO_3^- (NH_4NO_3) in MS media. After 4-8 weeks of treatment the young leaves, stems and fibrous roots were harvested for total RNA isolation. Real-time PCR analyses were carried out to determine the expression of the *MeNRT2* cDNA. The results which summarized in Figure 23-A demonstrated that R1 showed higher transcription levels of *MeNRT2* than those of KU50 and HB80. The expression patterns of *MeNRT2* in various tissues of cassava during vegetative stage showed that, *MeNRT2* gene had the highest expression in fibrous root when compared with young leaves and stem. Thus, the expression pattern of *MeNRT2* demonstrated that this gene was differentially expressed in various tissues and at different growth stages.

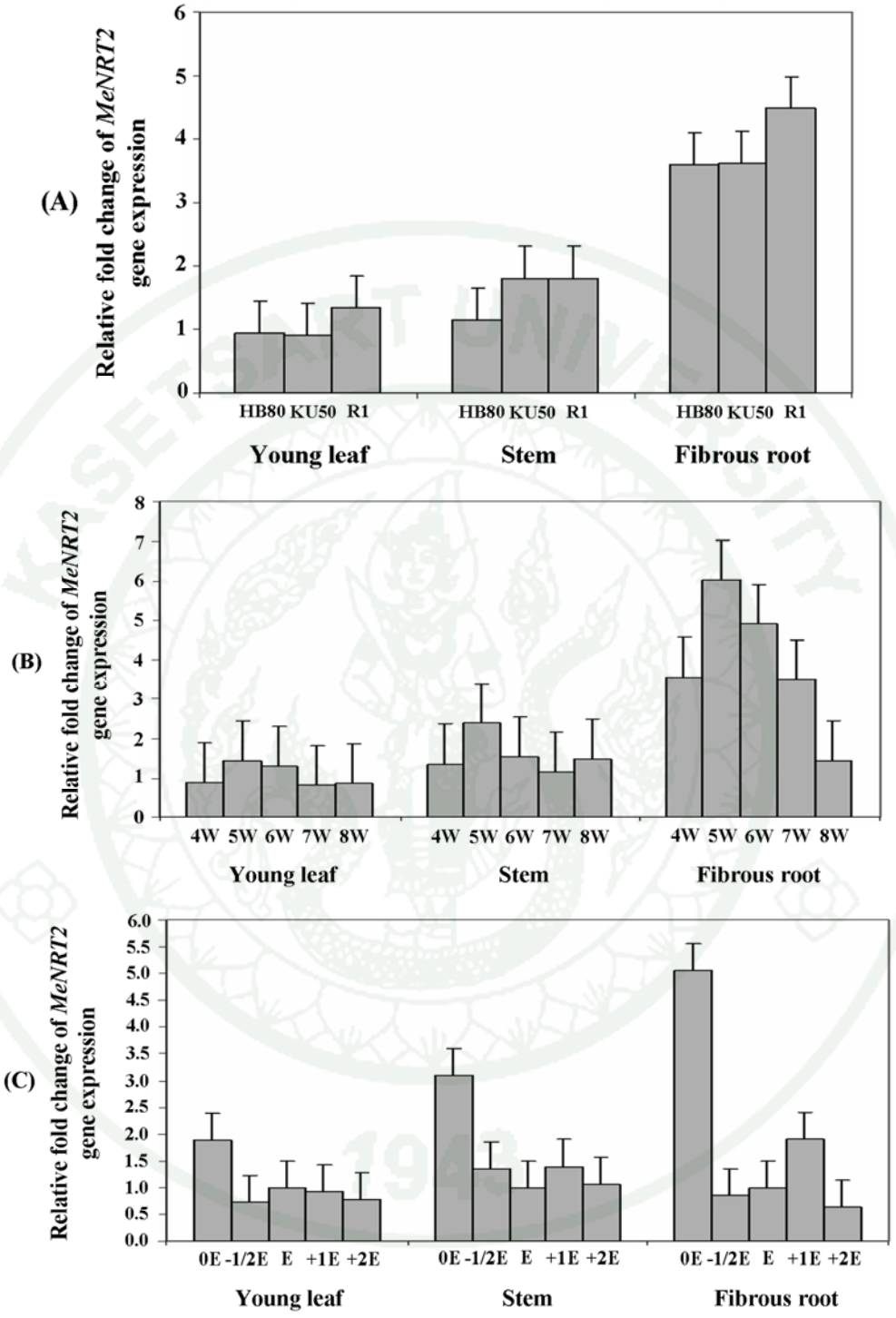

Figure 23 Relative fold changes in *MeNRT2* expression in young leaves, stems and fibrous roots of three cassava varieties; HB80, KU50 and R1 (A). Cassava tissues were grown at different NO_3^- concentration supplemented in MS media; 0, 5, 10, 20 and 30 mM (C) and harvested at 4, 5, 6, 7 and 8 weeks after subculture (B).

As shown in Figure 23-C, the transcription level of the nitrate transporters was high in the young leaf, stem and fibrous root of plants grown at 0 (0E treatment) and 20 mM NO_3^- (+1E treatment), and decreased with increasing NO_3^- concentration. *MeNRT2* was more sensitive to decreasing nitrate levels and the transcription level was significantly lower in the 30 mM treatment (+2E treatment). The regulation of *MeNRT2* expression by NO_3^- availability was further examined by resupplying NH_4NO_3 to the plants that were NO_3^- deficient and strongly expressing the genes. When 20 mM NO_3^- was then resupplied to these plants; transcription levels of gene increased when compared with 10 mM (E treatment). These observations suggest the existence of a fine coordination between gene expression, presumed to lead to the synthesis of more transporters and increased uptake, and the availability of NO_3^- in the media. During growth development, the expression pattern of *MeNRT2* gene in young leaf, stem and fibrous root showed that the maximal transcript levels of *MeNRT2* could be observed between 5 and 6 weeks, then it gradually decreased (Figure 23-B).

In this study, we observed the different expression of *NRT2* gene in several plant parts of cassava. Among three parts of the plant; young leaves, stems and fibrous roots, *MeNRT2* expression was high in fibrous roots (Figure 23). This result is contrasting to the study in tomato, no *LeNRT2* expression was observed in whole shoots or leaves (Ono *et al*., 2000) while in *N. plumbaginifolia*, *NpNRT2* transcriptions were also detected at low levels in leaves, petioles, buds, flowers, and seeds (Quesada *et al*., 1997). The expression of the *NRT2* genes identified so far has been demonstrated to be NO_3^- responsive, showing a rapid accumulation of mRNA following NO_3^- induction. The *NRT2* genes in *N. plumbaginifolia* and Arabidopsis were shown to be induced by very low levels of NO_3^- (10–50 mM) (Krapp *et al*., 1998; Filleur and Daniel- Vedele, 1999) to a transient maximum, while in barley, *NRT2* mRNAs accumulated to the highest levels when the NO_3^- is maintained at 50 mM under quasi steady state conditions (Vidmar *et al*., 2000). NO_3^- influx shows the same transient induction followed by a down-regulation when plants are starved of nitrogen (Lejay *et al*., 1999).

2.2.3 *MePT1* expression is towards the availability of Pi in the medium

To define the Pi concentration at which the phosphate transporter genes are expressed, cassava plants were grown in the presence of different concentrations MS media of Pi (KH_2PO_4). After 4-8 weeks of treatment, the young leaves, stems and fibrous roots were harvested for total RNA isolation. Real-time PCR analyses were carried out to determine the expression of the *MePT1* cDNA. The results were summarized in Figure 24; R1 showed higher transcription levels of *MePT1* than those of KU50 and HB80 (Figure24-A). The expression pattern of *MePT1* in various tissues of cassava during vegetative stage was also studied and it was found that *MePT1* gene was highly expressed in stem when compared with fibrous root and young leaves. Thus, the expression pattern of *MePT1* demonstrated that this gene was differentially expressed in various tissues and at different growth stages. In *Medicago truncatula*, *MtPT1* highly expressed in root, but did not expressed in leaves (Liu *et al*. 1998). In *Arabidopsis thaliana*, the highest *AtPT1* transcription was observed in root, but undetectable in leaves (Muchhal *et al*., 1996), whereas the transcription of *LePT1* accumulated at high levels in tomato roots, with a small amount of the message was also detectable in leaves (Liu *et al*. 1998).

As shown in Figure 24-C, the transcription level of the inorganic phosphate transporters was high in the young leave, stem and fibrous root of plants grown at concentration of 0, 0.3, and 0.6 mM Pi (0E, -1/2E and E treatments), and decreased with increasing Pi. A comparison of the normalized *MePT1* transcription levels revealed that *MePT1* transcriptions were 8.8, 6.2 and 2.3-fold higher in the 0 mM treatment for young leaf, stem and fibrous root, and were respectively when compared with the level in the 0.6 mM treatment (control). *MePT1* was more sensitive to increasing phosphate levels and the transcription level was significantly lower in the 1.8 mM Pi treatment (+2E treatment). The regulation of *MePT1* expression by phosphorus availability was further examined by resupplying KH_2PO_4 to the plants that were Pi deficient and strongly expressing the genes. When 1.2 mM (+1E treatment) and 1.8 mM Pi was resupplied to these plants, transcript levels of gene decreased and reached a significantly low level. These observations suggest the

existence of a fine coordination between gene expression, presumed to lead to the synthesis of more transporters and increased uptake, and the availability of Pi in the media.

During growth and development, the expression pattern of *MePT1* gene in young leaf, stem and fibrous root showed that the maximal transcript levels of *MePT1* could be observed between 5th and 6th week, then it gradually decreased (Figure 24-B). Similar results were observed in tomato in which the transcripted Pi transporter (*LePT1*) was highly induced under Pi-deficient conditions (Liu *et al*., 1998). The high-affinity Pi transport is considered the primary mode of Pi uptake in plants. This transport system is composed of the high-affinity Pi transporters that are inducible during Pi starvation (Raghotman *et al*., 1998; Muchhal *et al*., 1996). Inducible expression of Pi transporters is vital for growth of plants under varying concentrations of Pi. The induction of *LePT1* protein in response to Pi starvation correlates with the observed increase in Pi uptake rate of roots and cell cultures (Clarkson and Scattergood, 1982; Goldstein *et al*., 1989). Addition of protein synthesis inhibitors suppressed Pi starvation-induced Pi uptake in tobacco cell cultures, indicating that *de novo* synthesis of Pi transporters is required for the transport process (Shimogawara and Usuda, 1995).

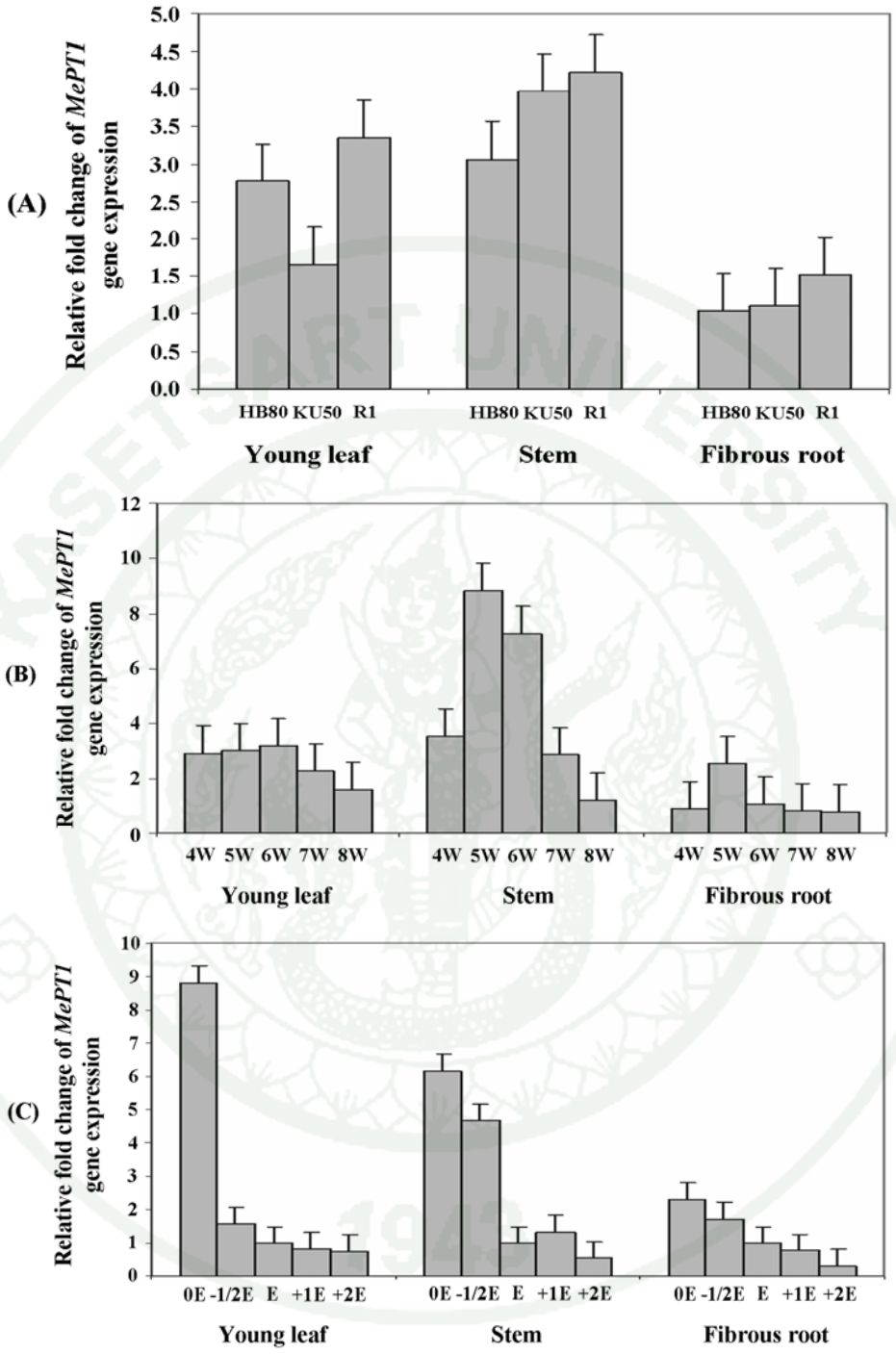

Figure 24 Relative fold changes in *MePT1* expression in young leaves, stems and fibrous roots of three cassava varieties; HB80, KU50 and R1 (A). Cassava tissues were grown at different Pi concentration in MS media; 0, 0.3, 0.6, 1.2 and 1.8 mM (C) and harvested at 4, 5, 6, 7 and 8 weeks after subculture (B).

2.2.4 *MeZIP* expression towards the availability of Zn^{2+} in the medium

To define the Zn^{2+} concentration at which the zinc transporter genes are expressed, cassava was grown in the presence of different concentrations of Zn^{2+} ($ZnSO_4.7H_2O$) in MS media. After 4-8 weeks of treatment the young leaves, stems and fibrous roots were harvested for total RNA isolation. Real-time PCR analyses were carried out to determine the expression of the *MeZIP* cDNA. The results were summarized in Figure 25-A and it was found that R1 showed higher transcript levels of *MeZIP* than those of KU50 and HB80. The expression pattern of *MeZIP* in various cassava tissues during vegetative stage, *MeZIP* gene highly expressed in young leaf and stem when compared with fibrous root. Thus, the expression pattern of *MeZIP* demonstrated that this gene was differentially expressed in various tissues and at different growth stages.

As shown in Figure 25-C, the transcription level of the zinc transporters was high in the young leaf, stem and fibrous root of plants grown at 0 (0E treatment) and 0.15 (+1E treatment) mM Zn^{2+}, and decreases with increasing Zn^{2+}. *MeZIP* was more sensitive to decreasing zinc levels and the transcription level was significantly lower in the 0.22 mM Zn^{2+} treatment (+2E treatment). The regulation of *MeZIP* expression by Zn^{2+} availability was further examined by resupplying $ZnSO_4.7H_2O$ to the plants that were Zn^{2+} deficient and strongly expressing the genes. When 0.15 mM $Zn^{2+}$was resupplied to these plants; transcription levels of gene increased when compared with 0.075 mM (E treatment). These observations suggest the existence of a fine coordination between gene expression, presumed to lead to the synthesis of more transporters and increased uptake, and the availability of Zn^{2+} in the media. During growth development, the expression pattern of *MeZIP* gene in young leaf, stem and fibrous root showed that the maximal transcription levels of *MeZIP* could be observed between 5^{th} and 6^{th} week, before it gradually decreased (Figure 25-B).

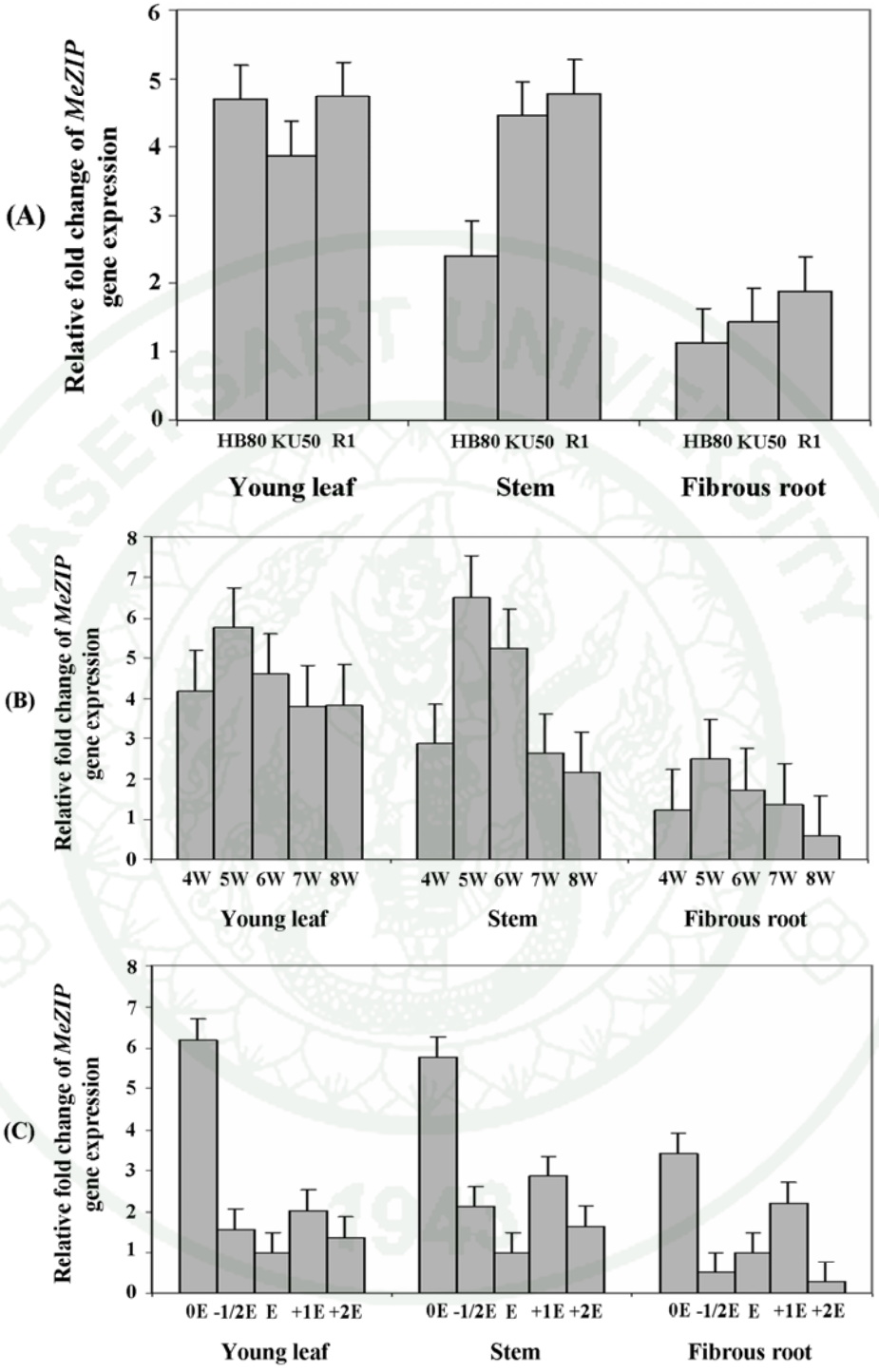

Figure 25 Relative fold changes in *MeZIP* expression in young leaves, stems and fibrous roots of three cassava varieties; HB80, KU50 and R1 (A). Cassava tissues were grown at different Zn^{2+} in MS media; 0, 0.037, 0.075, 0.15 and 0.22 mM (C) and harvested at 4, 5, 6, 7 and 8 weeks after subculture (B).

In this study, the different expressions of *ZIP* gene were observed in several plant parts of cassava. Among three parts of the plant; young leaves, stems and fibrous roots, *MeZIP* expression was high in young leaves. This result is contrasting to that of study in Arabidopsis (Grotz *et al*., 1998) where the highest level of *ZIP1* and *ZIP3* genes was found in the roots and *ZIP4* was high in shoots and roots. Whereas the result of the different expression of *MeZIP* in all parts of the plant correlated with the physiology of cassava growth in which the fresh and dry mass of young leaf, stem and fibrous root were used to support vegetative growth of the cassava. The results suggested that *MeZIP* might be involved in Zn^{2+} uptake of cassava at different developmental stages and, as well, in various processes.

3. Determination of transporter genes copy number

Genomic DNA was extracted from cassava leaf. The concentration and purity of DNA was determined using spectrophotometer at the wavelength of 260 and 280 nm. The isolated genomic DNA migrated as a single band with the size of approximately greater than 10 kb (Figure 26). The restriction endonucleases *Bam*HI, *Eco*RV, *Hind*III, *Pst*I, *Sac*I and *Sal*I were separately added to digest cassava genomic DNA. As shown in Figure 27, the cassava genomic DNA was completely digested by each endonuclease. The presences of related *AMT2 NRT2 PT1* and *ZIP* genes in cassava genome were determined by Southern analysis.

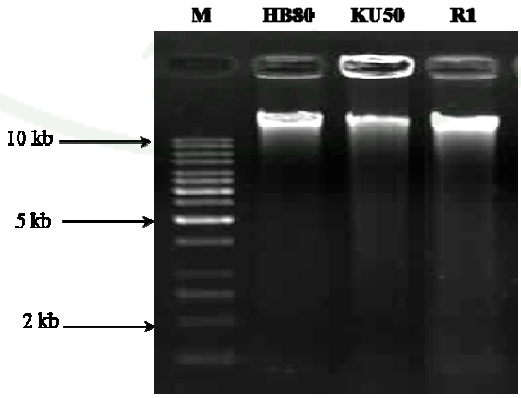

Figure 26 Ethidium bromide stained 1% agarose gel of cassava genomic DNA

following the electrophoresis at 50 V for 1 hr. Molecular weight marker (M) is shown on the left.

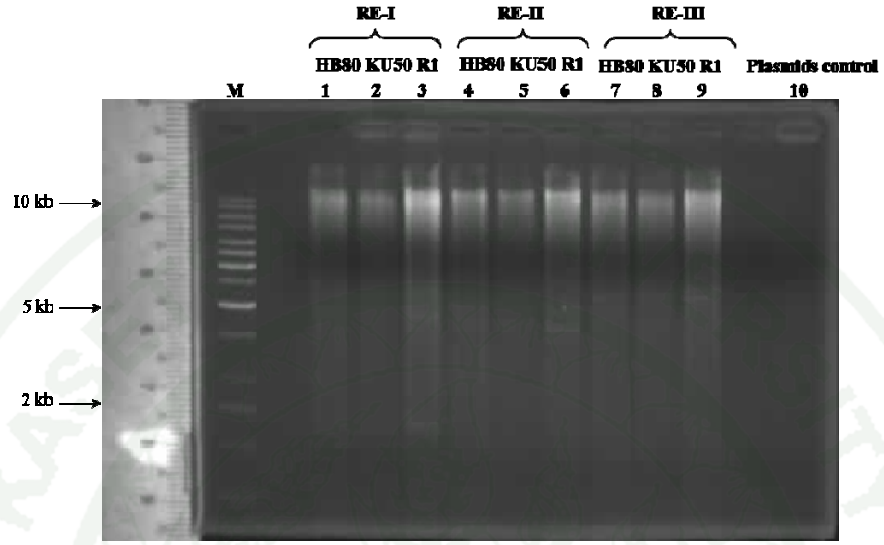

Figure 27 Ethidium bromide stained 1% agarose gel of cassava genomic DNA following the electrophoresis at 50 V for 1.30 hrs. M = DNA molecular weight marker, lane 1,2 and 3 (HB80, KU50 and R1) cassava DNA digested with restriction enzyme-I, lane 4,5 and 6 cassava DNA digested with restriction enzyme-II and lane 7,8 and 9 cassava DNA digested with restriction enzyme-III, repectively. Lane 10 plasmid control digested with *Eco*RI.

3.1 *MeAMT2* copy number

The number of *AMT2* gene in the cassava genome was estimated by DNA gel blot analysis. Genomic DNA isolated from cassava young leaves (5 weeks old) was digested with *Bam*HI, *Eco*RV, *Pst*I and probed with the 730 bp internal fragment from *MeAMT2* gene. A result showed a single band of approximately 10 kb when cut with *Eco*RV and *Pst*I whereas the *Bam*HI lane showed two bands of 9.0 and 6.5 kb (Figure 28). The analysis of the nucleotide sequences of *AMT2* revealed no cleavage sites for all enzymes used. The results suggested that at least two copies of *MeAMT*2 gene are present in cassava genome. In *A. thaliana* and rice; DNA blot analysis

suggested that there were multi copies of *AtAMT2* and *OsAMT2* gene in the genome (Suenaga *et al*. 2003).

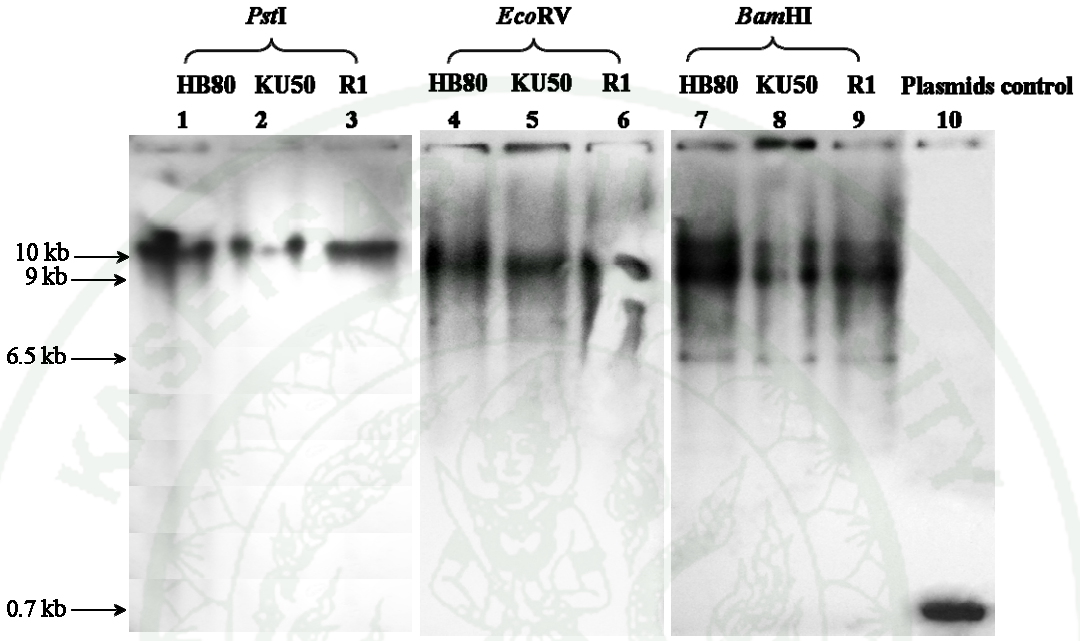

Figure 28 Southern hybridization of cassava genomic DNA was used *AMT2* probe. Lane 1, 2, 3 cassava (HB80, KU50 and R1) DNA digested with *Pst*I, lane 4, 5, 6 digested with *Eco*RV, lane 7, 8, 9 digested with *Bam*HI, respectively. Lane 10 plasmids control digested with *Eco*RI.

3.2 *MeNRT2* copy number

The number of *NRT2* gene in the cassava genome was estimated by DNA gel blot analysis. Genomic DNA isolated from cassava young leaves (5 weeks old) was digested with *Bam*HI, *Eco*RV, *Pst*I and probed with the 707 bp internal fragment from *MeNRT*2 gene. A result showed a single band of approximately 10 kb when cut with *Pst*I. And the *Bam*HI lane showed two bands of 8.0 and 5.5 kb whereas two bands of 9.0 and 5.5 kb were detected in *Eco*RV lane (Figure 29). The Analysis of the nucleotide sequences of *NRT2* revealed no cleavage sites for all enzymes used. The results suggested that at least two copies of *MeNRT*2 gene are present in cassava

genome. In wheat, genomic Southern analysis illustrated that *TaNRT2.1* was present as a single copy in genome (Yin *et al*., 2007).

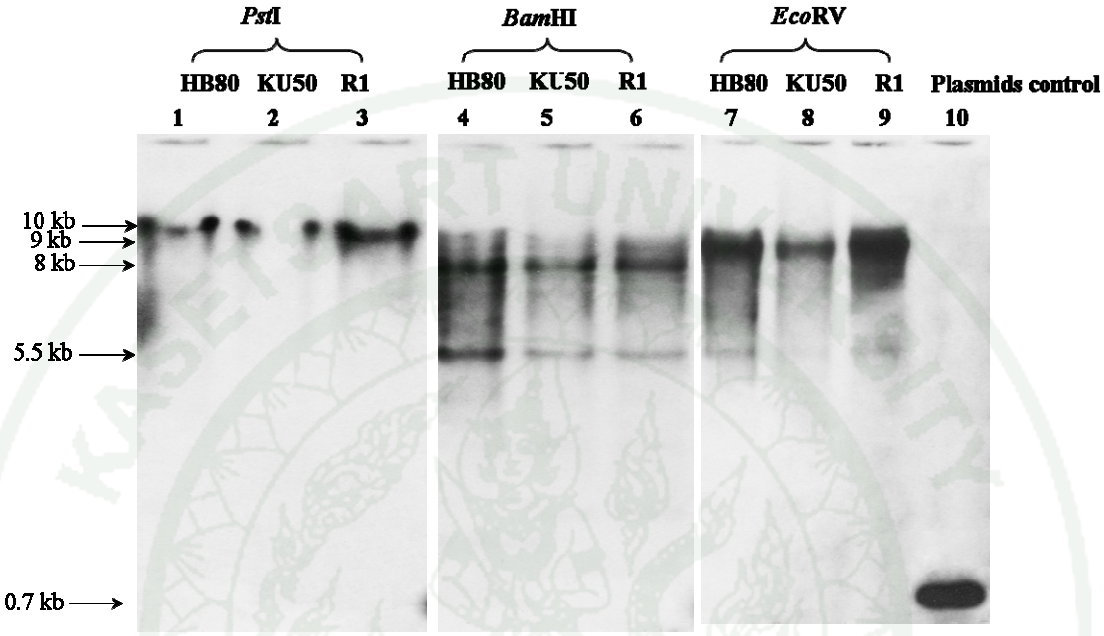

Figure 29 Southern hybridization of cassava genomic DNA was used *NRT2* probe. Lane 1, 2, 3 cassava (HB80, KU50 and R1) DNA digested with *Pst*I, lane 4, 5, 6 digested with *Bam*HI, lane 7, 8, 9 digested with *Eco*RV, respectively. Lane 10 plasmids control digested with *Eco*RI.

3.3 *MePT1* copy number

The number of *PT1* gene in the cassava genome was estimated by DNA gel blot analysis. Genomic DNA isolated from cassava young leaves (5 weeks old) was digested with *Hind*III, *Eco*RV, *Sac*I and probed with the 763 bp internal fragment from *MePT*1 gene. A result showed a single band of approximately 10 kb when cut with *Hind*III. The *Eco*RV lanes showed three bands of 9.5, 2.5 and 1.2 kb whereas three bands of 8.8, 7.5 and 1.2 kb were detected in *Sac*I lane (Figure 30). The analysis of the nucleotide sequences of *PT*1 revealed no cleavage sites for all enzymes used. The results suggested that at least three copies of *MePT1* gene presented in cassava genome. In *Arabidopsis thaliana*, DNA blot analysis suggested that there were at least

two to three copies of *AtPT1* gene in the genome (Muchhal *et al*., 1996); *Medicago truncatula* (Liu *et al*., 1998) and tomato (Liu *et al*., 1998).

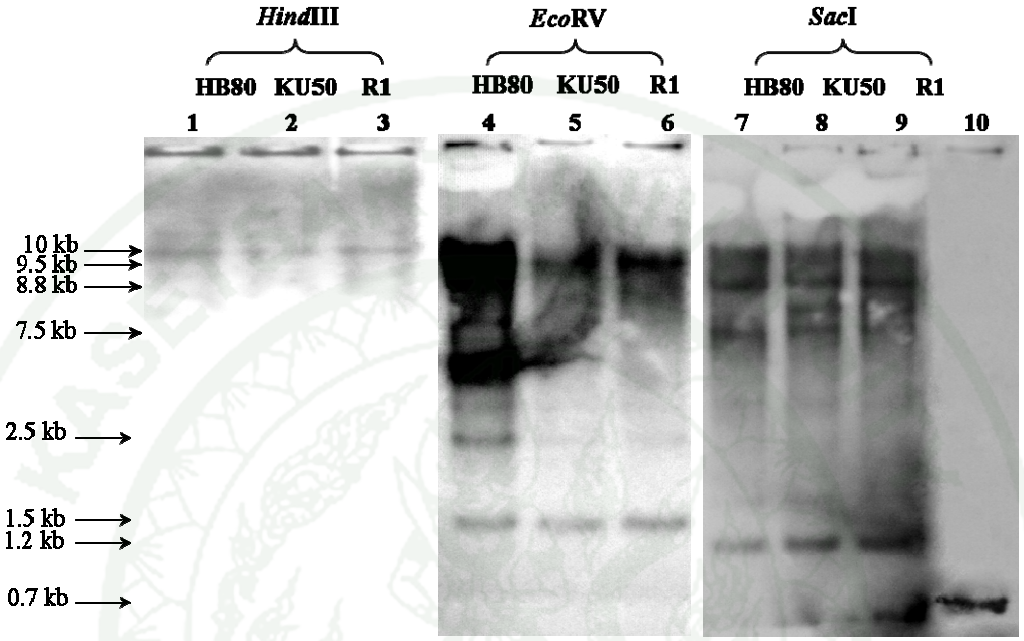

Figure 30 Southern hybridization of cassava genomic DNA was used *PT1* probe. Lane 1, 2, 3 cassava (HB80, KU50 and R1) DNA digested with *Hind*III, lane 4, 5, 6 digested with *Eco*RV, lane 7, 8, 9 digested with *Sac*I, respectively. Lane 10 plasmids control digested with *Eco*RI.

3.4 *MeZIP* copy number

The number of *ZIP* gene in the cassava genome was estimated by DNA gel blot analysis. Genomic DNA isolated from cassava young leaves (5 weeks old) was digested with *Sac*I, *Sal*I, *Pst*I and probed with the 723 bp internal fragment from *MeZIP* gene. A result showed a single band of approximately 10 kb when cut with *Pst*I. And the *Sac*I lane showed two bands of 8.0 and 6.5 kb whereas two bands of 9.0 and 0.9 kb were detected in *Sal*I lane (Figure 31). The analysis of the nucleotide sequences of *ZIP* revealed no cleavage sites for all enzymes used. The results suggested that at least two copies of *MeZIP* gene presented in cassava genome. In *A. halleri* and *A. thaliana* genome, DNA blot analysis suggested that there are more than

one gene copy of *ZIP3*, *ZIP6*, *ZIP9* gene in the genome (Talke *et al*., 2006) and in strawberry indicated that there is multi copy number of *FaZIP1* (Shi and Shih, 2006).

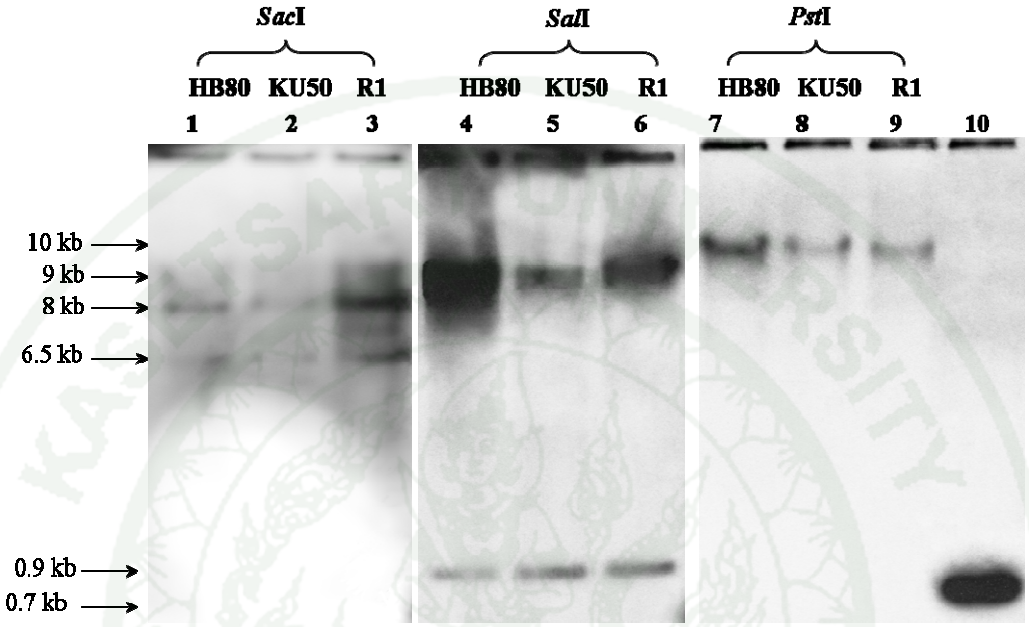

Figure 31 Southern hybridization of cassava genomic DNA was used *ZIP* probe. Lane 1, 2, 3 cassava (HB80, KU50 and R1) DNA digested with *Sac*I, lane 4, 5, 6 digested with *Sal*I, lane 7, 8, 9 digested with *Pst*I, respectively. Lane 10 plasmids control digested with *Eco*RI.

CONCLUSIONS AND RECOMMENDATION

Conclusions

In this study, isolation, characterization and expression analysis of *AMT2*, *NRT2*, *PT1* and *ZIP* genes in cassava (HB80, KU50 and R1) were conducted. Results from this study provide information, knowledge and application that can be concluded as shown below.

The complete coding sequence of *MeAMT2, MeNRT2, MePT1* and partial sequence of cassava *ZIP* genes were identified and characterized from developing fibrous roots.

In this study, R1 showed higher total fresh weight of whole plant than HB80 and KU50. Total fresh weight, total dry weight, leaf number, root number, leaf length, petiole length, stem length and fibrous root length were found to reach highest levels at 8 weeks after subculture and the arrange highest fresh weight was observed in +1E treatment of all three varieties.

Using real time PCR analysis, *MeAMT2, MeNRT2, MePT1* and *MeZIP* genes were expressed in different plant parts during growth and development. The spatial expression of these genes also showed organ specific. *MeAMT2* and *MePT1* transcriptions demonstrated the highest expression level in stem; while *MeNRT2* showed the highest expression level in fibrous roots. Whereas, the highest *MeZIP* transcription was detected in young leaves.

The expression levels of *MeAMT2*, *MeNRT2*, *MePT1* and *MeZIP* genes in R1 showed higher level of expression than those observed from KU50 and HB80 and the levels of *AMT NRT* and *ZIP* gene expression were high in +1E treatment, reflecting the highest fresh weight, dry weight, leaf number, fibrous root number, leaf length, stem length, petioles length and fibrous root length. The up and down regulation of

MeAMT2, MeNRT2, MePT1 and *MeZIP* in different stages of growth and development in the field should be further investigated.

According to the determination of *MeAMT2, MeNRT2, MePT1* and *MeZIP* gene copy number by Southern blot analysis showed that at least 3 copies of *MePT1* gene were found while only 2 copies of *MeAMT2, MeNRT2* and *MeZIP* were detected in the cassava genome.

Since results from this study indicate the importance of *MeAMT2, MeNRT2, MePT1* and *MeZIP* in growth and development of cassava as these genes show effects on some physiological parameters. Increase potential of these genes by using up-regulation gene technology should result in the knowledge obtained which could be applied as follows (a) to produce transgenic plants with increased growth rate via up-regulation gene technology and (b) to facilitate breeding program in screening cassava varieties with effective nutrient uptake ability and consequently giving improved yield.

Recommendation

The results from this study provided the basic information of cassava *AMT2*, *NRT2*, *PT1* and *ZIP* molecular biology which is useful for understanding the key roles of these genes involving in the regulatory of nutrients uptake as it has an affect on growth and development. It can also be used as a guideline for elucidating the answers of how and where to focus either up- and down- stream of the pathway that will eventually accomplish the goal of maximizing nutrient utilization for improving yield in cassava.

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APPENDIX

Appendix Table 1 Mean comparisons of leaf, stem, fibrous root and whole plant fresh weight (g) comparing among 3 cassava varieties (V), harvest time (H) and culture mediums (M).

	Leaf (g)	Stems (g)	Fibrous Roots (g)	Whole Plant (g)
Variety (V)				
HB 80	0.1720 A	0.1333 B	0.2344 B	0.5397 B
KU 50	0.1159 B	0.1234 B	0.2173 B	0.4566 C
R 1	0.1822 A	0.1589 A	0.2602 A	0.6013 A
F-Test	**	**	**	**
Harvest (H)				
4 week	0.0677 D	0.0674 D	0.0916 D	0.2267 D
5 week	0.1625 B	0.1190 C	0.1862 C	0.4677 C
6 week	0.1331 C	0.1138 C	0.1860 C	0.4329 C
7 week	0.1614 B	0.1844 B	0.3302 B	0.6760 B
8 week	0.2588 A	0.2080 A	0.3924 A	0.8592 A
F-Test	**	**	**	**
V x H	**	**	**	**
Medium (M)				
0E	0.0170 E	0.0389 D	0.0120 E	0.0679 E
-1/2E	0.1440 C	0.1224 C	0.2644 C	0.5308 C
E	0.2397 B	0.1920 B	0.3229 B	0.7546 B
+1E	0.2639 A	0.2165 A	0.3893 A	0.8697 A
+2E	0.1189 D	0.1229 C	0.1979 D	0.4397 D
F-Test	**	**	**	**
V x M	**	**	**	**
H x M	**	**	**	**
V x H x M	**	**	**	**
CV	32.12 %	29.58 %	32.69 %	31.46 %

Mean followed by a common letter are not significantly different at the 95% level by DMRT

** = significantly different at the 99% level

* = significantly different at the 95% level

ns = not significantly different

Appendix Table 2 Mean comparisons of leaf, stem, fibrous root and whole plant dry weight (g) comparing among 3 cassava varieties (V), harvest time (H) and culture mediums (M).

	Leaf (g)	Stems (g)	Fibrous Roots (g)	Whole Plant (g)
Variety (V)				
HB 80	0.0351 A	0.0220 A	0.0298 A	0.0869 A
KU 50	0.0223 C	0.0173 C	0.0244 B	0.0640 C
R 1	0.0313 B	0.0200 B	0.0267 B	0.0780 B
F-Test	**	**	**	**
Harvest (H)				
4 week	0.0148 D	0.0115 D	0.0107 D	0.0370 D
5 week	0.0287 C	0.0159 C	0.0211 C	0.0657 C
6 week	0.0255 C	0.0172 C	0.0232 C	0.0659 C
7 week	0.0343 B	0.0254 B	0.0377 B	0.0974 B
8 week	0.0446 A	0.0288 A	0.0422 A	0.1156 A
F-Test	**	**	**	**
V x H	**	**	**	**
Medium (M)				
0E	0.0050 E	0.0090 D	0.0020 D	0.0160 E
-1/2E	0.0343 C	0.0191 C	0.0329 B	0.0863 C
E	0.0408 B	0.0243 B	0.0359 B	0.1010 B
+1E	0.0479 A	0.0282 A	0.0416 A	0.1177 A
+2E	0.0198 D	0.0182 C	0.0226 C	0.0606 D
F-Test	**	**	**	**
V x M	**	**	**	**
H x M	**	**	**	**
V x H x M	**	**	**	**
CV	25.93 %	27.36 %	29.76 %	27.68 %

Mean followed by a common letter are not significantly different at the 95% level by DMRT

** = significantly different at the 99% level

* = significantly different at the 95% level

ns = not significantly different

Appendix Table 3 Mean comparisons of leaf, stem, fibrous root and petiole length (cm) comparing among 3 cassava varieties (V), harvest time (H) and culture mediums (M).

	Leaf (cm)	Stem (cm)	Fibrous Roots (cm)	Petioles (cm)
Variety (V)				
HB 80	2.69 B	4.88 A	8.23 B	2.70 A
KU 50	2.43 C	3.24 B	7.96 B	2.36 B
R 1	2.82 A	5.02 A	10.66 A	2.71 A
F-Test	**	**	**	**
Harvest (H)				
4 week	2.22 D	2.39 D	5.44 E	1.74 D
5 week	2.80 AB	3.69 C	7.57 D	2.74 B
6 week	2.59 C	3.90 C	9.42 C	2.46 C
7 week	2.73 B	5.71 B	10.57 B	2.97 A
8 week	2.90 A	6.22 A	11.74 A	3.03 A
F-Test	**	**	**	**
V x H	**	**	**	**
Medium (M)				
0E	1.40 D	0.72 E	2.24 D	0.53 D
-1/2E	2.86 B	4.86 C	9.93 B	2.94 B
E	3.24 A	5.57 B	11.62 A	3.66 A
+1E	3.36 A	7.92 A	12.14 A	3.80 A
+2E	2.39 C	2.73 D	8.82 C	2.02 C
F-Test	**	**	**	**
V x M	**	**	**	**
H x M	**	**	**	**
V x H x M	**	**	**	**
CV	12.06 %	20.70 %	15.20 %	16.48 %

Mean followed by a common letter are not significantly different at the 95% level by DMRT

** = significantly different at the 99% level

* = significantly different at the 95% level

ns = not significantly different

Appendix Table 4 Mean comparisons of leaf width (cm), leaf and fibrous root mumber comparing among 3 cassava varieties (V), harvest time (H) and culture mediums (M).

	Leaf Width (cm)	Leaf Number	Fibrous Roots Number
Variety (V)			
HB 80	0.87 B	3.75 B	11.73 A
KU 50	0.81 C	3.83 B	8.96 B
R 1	1.01 A	4.00 A	11.65 A
F-Test	**	**	**
Harvest (H)			
4 week	0.78 C	2.80 D	6.76 D
5 week	0.91 B	3.56 C	9.67 C
6 week	0.89 B	4.18 B	10.62 B
7 week	0.90 B	4.16 B	13.33 A
8 week	1.01 A	4.60 A	13.53 A
F-Test	**	**	**
V x H	**	**	**
Medium (M)			
0E	0.45 E	1.22 E	2.00 D
-1/2E	1.02 C	4.02 C	11.64 B
E	1.11 B	5.02 B	16.13 A
+1E	1.19 A	5.42 A	16.24 A
+2E	0.71 D	3.60 D	7.89 C
F-Test	**	**	**
V x M	**	**	**
H x M	**	**	**
V x H x M	**	**	**
CV	11.40 %	11.20 %	19.34 %

Mean followed by a common letter are not significantly different at the 95% level by DMRT

** = significantly different at the 99% level

* = significantly different at the 95% level

ns = not significantly different

Appendix Table 5 Mean comparisons of chlorophyll a, b and total (g/m^2) comparing among 3 cassava varieties (V), harvest time (H) and culture mediums (M).

	Chlorophyll A	Chlorophyll B	Total Chlorophyll
Variety (V)			
HB 80	0.3558	0.1176 A	0.4731 A
KU 50	0.3386	0.1033 B	0.4430 B
R 1	0.3428	0.1206 A	0.4782 A
F-Test	ns	**	**
Harvest (H)			
4 week	0.3258 B	0.1029 C	0.4212 C
5 week	0.3653 A	0.1129 B	0.4816 B
6 week	0.3617 A	0.1093 BC	0.4767 B
7 week	0.3693 A	0.1585 A	0.5236 A
8 week	0.3065 B	0.0856 D	0.4207 C
F-Test	**	**	**
V x H	**	**	**
Medium (M)			
0E	0.1725 E	0.0644 E	0.2496 D
-1/2E	0.3719 C	0.1166 C	0.4947 B
E	0.5039 A	0.1621 A	0.6636 A
+1E	0.4777 B	0.1521 B	0.6353 A
+2E	0.2026 D	0.0740 D	0.2806 C
F-Test	**	**	**
V x M	**	**	**
H x M	**	**	**
V x H x M	**	**	**
CV	15.08 %	15.42 %	15.62 %

Mean followed by a common letter are not significantly different at the 95% level by DMRT

** = significantly different at the 99% level

* = significantly different at the 95% level

ns = not significantly different

Appendix Table 6 Mean comparisons of relative fold change in *MeAMT2* expression in leaf, stems and fibrous roots comparing among 3 cassava varieties (V), harvest time (H) and culture mediums (M).

	Leaf	Stems	Fibrous Roots
Variety (V)			
HB 80	1.2423	1.2583 B	1.1791
KU 50	1.2301	1.5862 B	1.3774
R 1	1.2902	2.3510 A	1.9034
F-Test	ns	**	ns
Harvest (H)			
4 week	1.1746	1.8542	1.3802
5 week	1.4707	2.0010	1.4813
6 week	1.5933	2.1372	2.0377
7 week	1.0082	1.3743	1.3704
8 week	1.0242	1.2924	1.1636
F-Test	ns	ns	ns
V x H	ns	ns	ns
Medium (M)			
0E	2.4959 A	4.4133 A	3.9413 A
-1/2E	1.0050 B	0.9499 B	0.6611 B
E	1.0000 B	1.0000 B	1.0000 B
+1E	1.0263 B	1.3553 B	1.4648 B
+2E	0.7438 B	0.9406 B	0.3659 B
F-Test	**	**	**
V x M	ns	**	ns
H x M	*	ns	ns
V x H x M	ns	ns	ns
CV	27.08 %	28.16 %	23.00 %

Mean followed by a common letter are not significantly different at the 95% level by DMRT

** = significantly different at the 99% level

* = significantly different at the 95% level

ns = not significantly different

Appendix Table 7 Mean comparisons of relative fold change in *MeNRT2* expression in leaf, stems and fibrous roots comparing among 3 cassava varieties (V), harvest time (H) and culture mediums (M).

	Leaf	Stems	Fibrous Roots
Variety (V)			
HB 80	0.9384 B	1.1444	3.5860
KU 50	0.8956 B	1.8035	3.6113
R 1	1.3518 A	1.8049	4.4766
F-Test	*	ns	ns
Harvest (H)			
4 week	0.8928 B	1.3556	3.5582
5 week	1.4386 A	2.3954	6.0342
6 week	1.2925 AB	1.5287	4.9103
7 week	0.8204 B	1.1516	3.5057
8 week	0.8655 B	1.4900	1.4484
F-Test	*	ns	ns
V x H	ns	*	ns
Medium (M)			
0E	1.8843 A	3.1004 A	5.0581 A
-1/2E	0.7288 B	1.3591 B	0.8546 B
E	1.0000 B	1.0000 B	1.0000 B
+1E	0.9174 B	1.3974 B	1.9075 B
+2E	0.7794 B	1.0644 B	0.6368 B
F-Test	**	**	**
V x M	ns	ns	ns
H x M	*	ns	ns
V x H x M	ns	ns	ns
CV	28.92 %	21.38 %	30.43 %

Mean followed by a common letter are not significantly different at the 95% level by DMRT

** = significantly different at the 99% level

* = significantly different at the 95% level

ns = not significantly different

Appendix Table 8 Mean comparisons of relative fold change in *MePT1* expression in leaf, stems and fibrous roots comparing among 3 cassava varieties (V), harvest time (H) and culture mediums (M).

	Leaf	Stems	Fibrous Roots
Variety (V)			
HB 80	2.7687 AB	3.0554 B	1.0377
KU 50	1.6586 B	3.9571 B	1.0976
R 1	3.3481 A	4.2175 A	1.5176
F-Test	*	**	ns
Harvest (H)			
4 week	2.9286	3.5471 B	0.8797 B
5 week	3.0098	8.8273 A	2.5662 A
6 week	3.1823	7.2679 A	1.0678 B
7 week	2.2555	2.8687 B	0.7993 B
8 week	1.5827	1.2072 B	0.7752 B
F-Test	ns	**	**
V x H	ns	ns	*
Medium (M)			
0E	8.8095 A	6.1763 A	2.3001 A
-1/2E	1.5794 B	4.6719 B	1.7137 AB
E	1.0000 B	1.0000 C	1.0000 BC
+1E	0.8179 B	1.3307 BC	0.7714 C
+2E	0.7522 B	0.5405 C	0.3029 C
F-Test	**	**	**
V x M	**	**	ns
H x M	ns	**	**
V x H x M	ns	ns	ns
CV	29.68 %	28.41 %	24.68 %

Mean followed by a common letter are not significantly different at the 95% level by DMRT

** = significantly different at the 99% level

* = significantly different at the 95% level

ns = not significantly different

Appendix Table 9 Mean comparisons of relative fold change in *MeZIP* expression in leaf, stems and fibrous roots comparing among 3 cassava varieties (V), harvest time (H) and culture mediums (M).

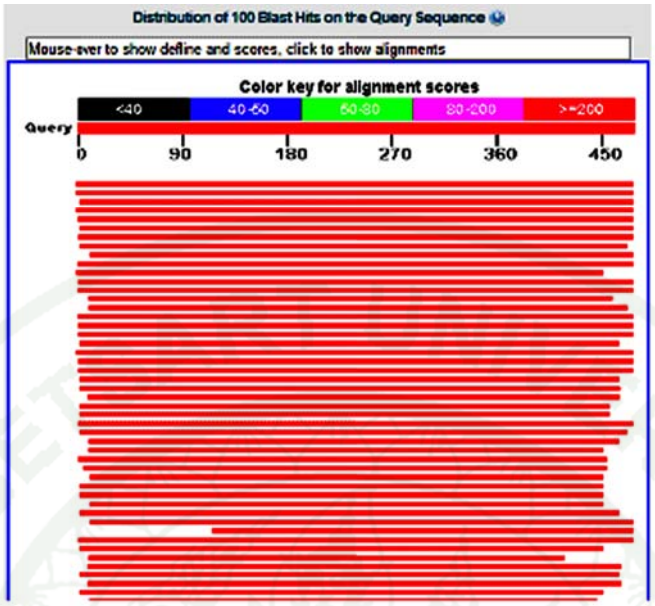
	Leaf	Stems	Fibrous Roots
Variety (V)			
HB 80	4.6939	2.4014 B	1.1336
KU 50	3.8775	4.4521 A	1.4314
R 1	4.7382	4.7751 A	1.8801
F-Test	ns	*	ns
Harvest (H)			
4 week	4.1812	2.8524 BC	1.2339
5 week	5.7494	6.5162 A	2.4903
6 week	4.5971	5.2284 AB	1.7348
7 week	3.8132	2.6301 BC	1.3697
8 week	3.8384	2.1564 C	0.5797
F-Test	ns	**	ns
V x H	ns	**	ns
Medium (M)			
0E	6.2052 A	5.7594 A	3.4336 A
-1/2E	1.5703 B	2.1082 B	0.5034 C
E	1.0000 B	1.0000 B	1.0000 BC
+1E	2.0231 B	2.8571 B	2.1998 AB
+2E	1.3804 B	1.6403 B	0.2716 C
F-Test	**	**	**
V x M	ns	*	ns
H x M	ns	**	*
V x H x M	ns	ns	ns
CV	26.69 %	29.82 %	26.81 %

Mean followed by a common letter are not significantly different at the 95% level by DMRT

** = significantly different at the 99% level

* = significantly different at the 95% level

ns = not significantly different



Sequences producing significant alignments:	Score (Bits)	E Value	
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ref|XP_002530488.1| ammonium transporter, putative [Ricinus c...	721	0.0	G
ref|XP_002323600.1| ammonium transporter [Populus trichocarpa...	711	0.0	UG
gb|AAL08212.1| putative ammonium transporter AMT2 [Lotus japo...	695	0.0	G
emb|CAN66309.1| hypothetical protein [Vitis vinifera]	691	0.0	
gb|AAN06953.1| amt2-like protein [Medicago truncatula]	686	0.0	
ref|XP_002268830.1| PREDICTED: hypothetical protein [Vitis vi...	655	0.0	UG
gb|ABR17836.1| unknown [Picea sitchensis]	629	3e-178	
dbj|BAF32139.1| putative ammonium transporter [Taxodium disti...	625	5e-177	
dbj|BAF31946.1| putative ammonium transporter AMT2 [Cryptomer...	624	1e-176	
ref|NP_001147783.1| ammonium transporter 2 [Zea mays] >gb|ACG...	623	2e-176	UG
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ref|XP_002300222.1| ammonium transporter [Populus trichocarpa...	601	9e-170	UG
ref|XP_002272735.1| PREDICTED: hypothetical protein [Vitis vi...	599	2e-169	UG
ref|XP_002272503.1| PREDICTED: hypothetical protein [Vitis vi...	593	1e-167	UG
ref|XP_002458715.1| hypothetical protein SORBIDRAFT_03g038840...	590	1e-166	UG
gb|AAR87397.1| ammonium transporter AMT2.1 [Triticum aestivum]	589	3e-166	G
gb|EAY92501.1| hypothetical protein OsI_14239 [Oryza sativa I...	588	7e-166	

Appendix Figure 1 The nucleotide blast of *AMT2* gene for cassava using BLASTP program.

```
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ACCESSION   GU248340
VERSION     GU248340.1  GI:282721273
KEYWORDS    .
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REFERENCE   1  (bases 1 to 1578)
  AUTHORS   Bamrungsetthapong,T., Nakasathien,S. and Vichukit,V.
  TITLE     Cloning and molecular characterization of transporter genes in Thai
            cassava during growth development under tissue culture
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1578)
  AUTHORS   Bamrungsetthapong,T., Nakasathien,S. and Vichukit,V.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-NOV-2009) Center for Agricultural Biotechnology,
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      301 aagggacaag caaagatccc aaatagtaat gtggcggcgc cgtggtatcc gatggcgacg
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      481 tacacagtng gagcttatag tatatgggga ggtgggtttc tgtatcagtg gggagttatt
      541 gattattccg gcggttatgt tattcatctc tcctcaggag ttgccggttt cgtcgctgct
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      661 ctgatgcttg ccggtgctgg gctgctgtgg atgggctggt ctggcttcaa cggaggagca
      721 ccgtatgcag ctaatctaaa tgcttcgatt gcgatattaa acaccaacat aagtgcagca
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      901 caatcgtggg cggctattgt gatgggagct ctttctggaa gcattccatg ggtgtctatg
      961 atggtgcttc acaaaaagtc ttcgctgcta cagcaggtgg acgacacact aggcgtgttt
     1021 cacactcacg cggtggctgg gctattaggt ggcctcctca cagggcttct agcagagcca
     1081 gatctttgcg accttattct accgaagaaa acacgaggcg cattttacgg cggaaatggt
     1141 ggacggcaat tcttgaagca attggttgct gcttgcttta ttatagtttg gaacatagtc
     1201 tccaccacca taatcctttt agctataaga ttgttcatac ccttgagaat gccggaagag
     1261 caactggtaa tcggagacga tgccgttcat ggagaggaag cttatgctct ttggggagat
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     1381 cagtctcctt atgttcatgg tgctagaggt gtcaccatca atctatgatt tttcttcttc
     1441 tttttcttat ttatttttt gtgtattgat ttccttatga cattagagat gtgaatgagt
     1501 tatttgtgtt ctgtataggt ggtaaaactt cacttttcat atttttggt gtgtatttgg
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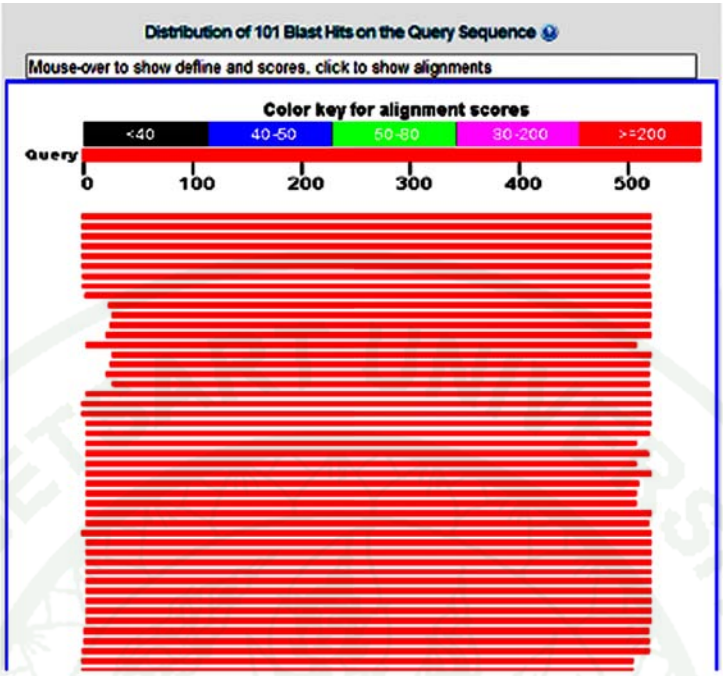
Appendix Figure 2 cDNA *AMT2* gene of cassava deposited in GenBank data base.



Sequences producing significant alignments:	Score (Bits)	E Value	
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gb|AAV73917.1| putative high affinity nitrate transporter [Ze...	1014	0.0	
ref|NP_001105195.1| nrt2.1 protein [Zea mays] >gb|AAN05088.1|...	997	0.0	U G
ref|XP_002451375.1| hypothetical protein SORBIDRAFT_04g000990...	979	0.0	U G
ref|XP_002453159.1| hypothetical protein SORBIDRAFT_04g001000...	978	0.0	U G
ref|NP_001045658.1| Os02g0112100 [Oryza sativa (japonica cult...	925	0.0	U G
gb|EAY84173.1| hypothetical protein OsI_05552 [Oryza sativa I...	923	0.0	
dbj|BAC76606.1| putative high affinity nitrate transporter [P...	909	0.0	
gb|AAK19519.1|AF332214_1 high affinity nitrate transporter [T...	865	0.0	G
gb|AAG01172.1| high affinity nitrate transporter TaNRT2 [Trit...	861	0.0	G
gb|ABG20829.1| high affinity nitrate transporter NRT2.6 [Hord...	860	0.0	
gb|AAL11016.1| high-affinity nitrate transporter [Triticum ae...	860	0.0	G
ref|XP_002523687.1| nitrate transporter, putative [Ricinus co...	859	0.0	G
gb|AAC49531.1| BCH1	859	0.0	
gb|AAD28364.1|AF091116_1 putative high affinity nitrate trans...	853	0.0	
gb|AAC49532.1| BCH2	852	0.0	
gb|AAD28363.1|AF091115_1 putative high affinity nitrate trans...	851	0.0	
gb|AAC09320.1| putative high affinity nitrate transporter; Gm...	850	0.0	G
dbj|BAD02939.1| high-affinity nitrate transporter [Prunus per...	850	0.0	
dbj|BAD04063.1| high-affinity nitrate transporter [Prunus per...	849	0.0	
gb|ABQ57241.1| nitrate transporter [Citrus sinensis x Ponciru...	847	0.0	
gb|AAS93686.2| high-affinity nitrate transporter [Cucumis sat...	845	0.0	
ref|XP_002313529.1| high affinity nitrate transporter [Populu...	842	0.0	U G
ref|XP_002519504.1| nitrate transporter, putative [Ricinus co...	840	0.0	G
emb|CAG26716.1| nitrate transporter [Populus tremula x Populu...	840	0.0	

Appendix Figure 3 The nucleotide blast of *NRT2* gene for cassava using BLASTP program.

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LOCUS       GU248341               1710 bp    mRNA    linear   PLN 05-JAN-2010
DEFINITION  Manihot esculenta NRT2 mRNA, complete cds.
ACCESSION   GU248341
VERSION     GU248341.1  GI:282721275
KEYWORDS    .
SOURCE      Manihot esculenta (cassava)
  ORGANISM  Manihot esculenta
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
            rosids; fabids; Malpighiales; Euphorbiaceae; Crotonoideae;
            Manihoteae; Manihot.
REFERENCE   1  (bases 1 to 1710)
  AUTHORS   Bamrungsetthapong,T., Nakasathien,S. and Vichukit,V.
  TITLE     Cloning and molecular characterization of transporter genes in Thai
            cassava during growth development under tissue culture
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1710)
  AUTHORS   Bamrungsetthapong,T., Nakasathien,S. and Vichukit,V.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-NOV-2009) Center for Agricultural Biotechnology,
            Kasetsart University, Kamphaeng Saen, Nakhon Pathom 73140, Thailand
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ORIGIN
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      601 gcaactcagc tgataatgcc tttggtctat gatgtgattc agcgagctgg tgcaactcca
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      781 ggtgatgtcg ccagggataa gttctccaag gttctttggt atgccatcac aaattacagg
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     1561 gccacgccgg agcatgtcta agtctactac taagatggat cgatcgacga tcacctatac
     1621 ctctttgtat gtacgaatat gccttgttat tactgcgcgc gcgcatatac aatacacgtg
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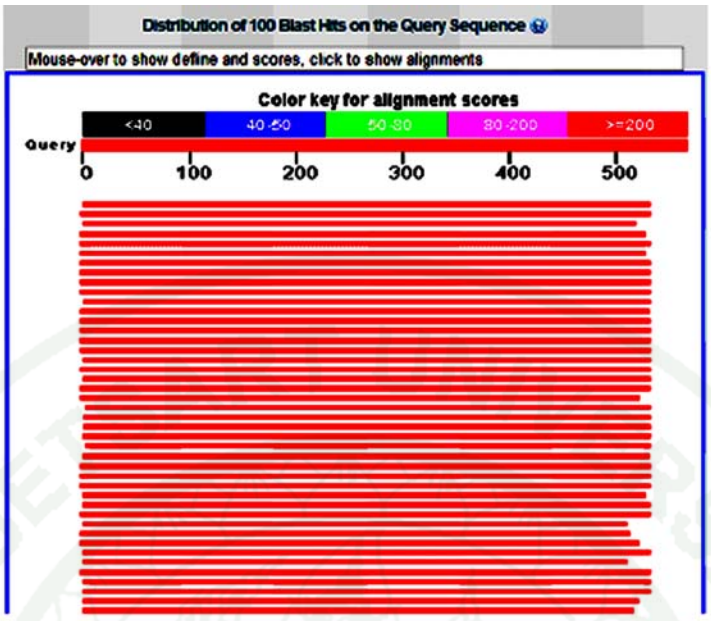
Appendix Figure 4 cDNA *NRT2* gene of cassava deposited in GenBank data base.



Sequences producing significant alignments:	Score (Bits)	E Value	
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ref|XP_002300153.1| high affinity inorganic phosphate transpo...	932	0.0	UG
emb|CAA67395.1| inorganic phosphate transporter 1 [Solanum tu...	931	0.0	
emb|CAA76075.1| putative phosphate transporter [Solanum lycop...	928	0.0	G
gb|AAD38859.1|AF156695_1 inorganic phosphate transporter [Sol...	928	0.0	
gb|AAF74025.1|AF156696_1 inorganic phosphate transporter [Nic...	924	0.0	
dbj|BAB21545.1| phosphate transporter [Nicotiana tabacum]	922	0.0	
gb|ABK63959.1| PT1 [Solanum melongena]	919	0.0	
ref|XP_002524622.1| inorganic phosphate transporter, putative...	918	0.0	G
emb|CAC28219.1| phosphate transporter [Sesbania rostrata]	917	0.0	
ref|XP_002306845.1| high affinity inorganic phosphate transpo...	916	0.0	UG
gb|ABS12068.1| high affinity phosphate transporter [Petunia x...	913	0.0	
ref|XP_002306844.1| high affinity inorganic phosphate transpo...	912	0.0	UG
gb|ABK63958.1| PT1 [Capsicum frutescens]	911	0.0	
dbj|BAA86070.1| Phospate transpoter [Nicotiana tabacum]	911	0.0	
ref|XP_002281264.1| PREDICTED: hypothetical protein [Vitis vi...	907	0.0	UG
emb|CAA74607.1| phosphate transporter [Solanum lycopersicum]	903	0.0	
ref|XP_002285145.1| PREDICTED: hypothetical protein [Vitis vi...	901	0.0	UG
gb|ACP19343.1| phosphate transporter [Glycine max]	900	0.0	
ref|XP_002524621.1| inorganic phosphate transporter, putative...	899	0.0	G
ref|XP_002531532.1| inorganic phosphate transporter, putative...	896	0.0	G
gb|ACN80147.1| phosphate transporter 2 [Glycine max]	892	0.0	
gb|ACP19341.1| phosphate transporter [Glycine max]	892	0.0	
emb|CAC87043.1| transmembrane protein [Solanum tuberosum]	891	0.0	
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gb|ACP19342.1| phosphate transporter [Glycine max]	889	0.0	
gb|AAV97729.1| phosphate transporter 3 [Solanum lycopersicum]	885	0.0	
gb|ABM69111.1| phosphate transporter 5 [Medicago truncatula] ...	885	0.0	
gb|ABP52096.1| phosphate transporter [Phaseolus vulgaris]	884	0.0	

Appendix Figure 5 The nucleotide blast of *PT1* gene for cassava using BLASTP program.

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            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
            rosids; fabids; Malpighiales; Euphorbiaceae; Crotonoideae;
            Manihoteae; Manihot.
REFERENCE   1  (bases 1 to 1743)
  AUTHORS   Bamrungsetthapong,T., Nakasathien,S. and Vichukit,V.
  TITLE     Cloning and molecular characterization of transporter genes in Thai
            cassava during growth development under tissue culture
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1743)
  AUTHORS   Bamrungsetthapong,T., Nakasathien,S. and Vichukit,V.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-NOV-2009) Center for Agricultural Biotechnology,
            Kasetsart University, Kamphaeng Saen, Nakhon Pathom 73140, Thailand
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      901 cttggaacca ccgtgtgctg gttcttacta gacatagctt attacagttc aaatcttttc
      961 cagaaggata tctttagcgc aatcggttgg attccacctg cacagaccat gaatgcaatt
     1021 catgaagtat atgtgattgc cagagcacaa acacttatcg ccttgtgtgg cacagttcct
     1081 ggatattggt tcacagtggc tctcattgat cgtataggga gatttttcat ccaagtgatg
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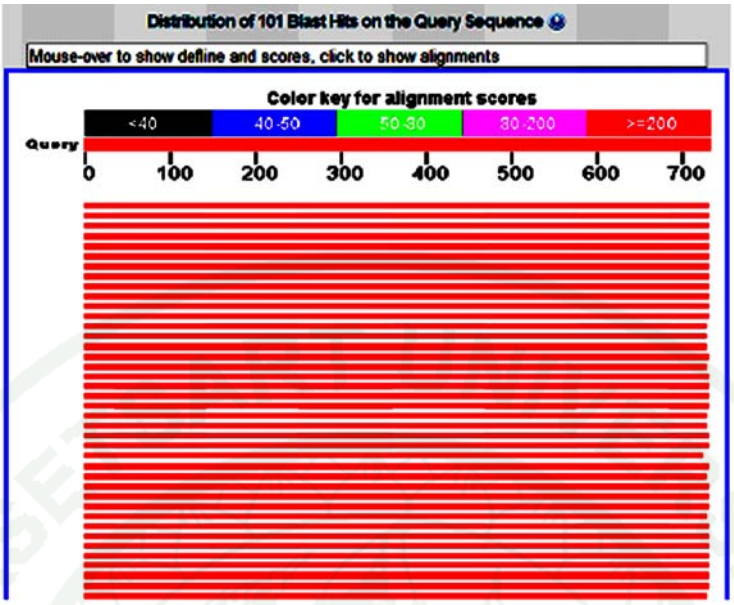
Appendix Figure 6 cDNA *PT1* gene of cassava deposited in GenBank data base.



Sequences producing significant alignments:	Score (Bits)	E Value	
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emb|CAN74488.1| hypothetical protein [Vitis vinifera]	308	3e-82	
ref|XP_002307860.1| ZIP transporter [Populus trichocarpa] >gb...	304	6e-81	U G
ref|XP_002307858.1| ZIP transporter [Populus trichocarpa] >gb...	295	3e-78	G
dbj|BAH66920.1| metal transporter [Nicotiana tabacum]	290	1e-76	
ref|XP_002264707.1| PREDICTED: hypothetical protein isoform 2...	286	1e-75	U G
gb|AAX28838.1| zinc transporter protein ZIP2 [Fragaria x anan...	285	4e-75	
emb|CAN67594.1| hypothetical protein [Vitis vinifera]	284	7e-75	
ref|XP_002265102.1| PREDICTED: hypothetical protein [Vitis vi...	283	2e-74	G
gb|AAR08414.1| metal transport protein [Medicago truncatula]	282	3e-74	
gb|ABH01187.1| zinc transporter protein [Ammopiptanthus mongo...	280	8e-74	
ref|XP_002532140.1| zinc/iron transporter, putative [Ricinus ...	280	1e-73	G
emb|CAN72265.1| hypothetical protein [Vitis vinifera]	279	2e-73	
ref|XP_002264603.1| PREDICTED: hypothetical protein [Vitis vi...	278	3e-73	U G
gb|AAX28837.1| zinc transporter protein ZIP1 [Fragaria x anan...	278	3e-73	
emb|CAN72264.1| hypothetical protein [Vitis vinifera]	278	4e-73	
gb|ACU24645.1| unknown [Glycine max]	276	2e-72	
gb|AAK37761.1| zinc transporter protein ZIP1 [Glycine max]	276	2e-72	G
gb|AAR08412.1| metal transport protein [Medicago truncatula]	276	2e-72	
ref|XP_002315075.1| ZIP transporter [Populus trichocarpa] >gb...	272	2e-71	U G
ref|XP_002275820.1| PREDICTED: hypothetical protein [Vitis vi...	271	3e-71	G
emb|CAN83129.1| hypothetical protein [Vitis vinifera]	271	3e-71	
ref|XP_002312231.1| ZIP transporter [Populus trichocarpa] >gb...	268	5e-70	G
gb|ACJ84426.1| unknown [Medicago truncatula]	266	1e-69	
gb|AAR08413.1| metal transport protein [Medicago truncatula]	265	3e-69	
ref|XP_002274548.1| PREDICTED: hypothetical protein isoform 1...	264	6e-69	U G
emb|CAO41938.1| unnamed protein product [Vitis vinifera]	252	2e-65	
gb|ACN38063.1| zinc transporter protein [Sedum alfredii]	245	4e-63	
gb|ABF55690.1| putative zinc transporter [Triticum aestivum]	245	4e-63	G

Appendix Figure 7 The nucleotide blast of *ZIP* gene for cassava using BLASTX program.

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LOCUS       GU248339                723 bp    mRNA    linear   PLN 05-JAN-2010
DEFINITION  Manihot esculenta ZIP mRNA, partial cds.
ACCESSION   GU248339
VERSION     GU248339.1  GI:282721271
KEYWORDS    .
SOURCE      Manihot esculenta (cassava)
  ORGANISM  Manihot esculenta
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            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
            rosids; fabids; Malpighiales; Euphorbiaceae; Crotonoideae;
            Manihoteae; Manihot.
REFERENCE   1  (bases 1 to 723)
  AUTHORS   Bamrungsetthapong,T., Nakasathien,S. and Vichukit,V.
  TITLE     Cloning and molecular characterization of transporter genes in Thai
            cassava during growth development under tissue culture
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 723)
  AUTHORS   Bamrungsetthapong,T., Nakasathien,S. and Vichukit,V.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-NOV-2009) Center for Agricultural Biotechnology,
            Kasetsart University, Kamphaeng Saen, Nakhon Pathom 73140, Thailand
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Appendix Figure 8 Partial *ZIP* gene of cassava deposited in GenBank data base.

CIRRICULUM VITAE

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2002	Kasetsart Univ.	B.Sc.(Agriculture, First Class Honors)
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