

# **Molecular Basis of Catecholamine Biosynthesis in Banana Fruit**

**Thesis submitted to the R.H. Smith Faculty of Agriculture,  
Food and Environmental Quality Sciences of the Hebrew  
University of Jerusalem for the degree of Master of Science in  
Agriculture**

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**March, 2009**

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## **Declaration**

This study was conducted under the supervision of Dr. Haya Freidman, Dr. Victor Rodov and Prof. Eliezer Goldschmidt at the Agricultural Research Organization (Volcani institute) and R.H. Smith Faculty of Agriculture, Food and Environmental Quality Sciences of The Hebrew University of Jerusalem.

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## **Acknowledgment**

I thank the Lord God for His mercy and grace which has been abundant and seen me through all the years of my studies. My thanks also go to my supervisors; Dr. Haya Freidman who through her constant criticism and corrections made me more independent person and helped me to write this paper. Profound gratitude goes to Dr. Victor Rodov and Prof. Eliezer Goldschmidt for their advice and corrections.

I say a big thank you to Dr. Yakov Vinokur for helping me with the HPLC analysis.

I also thank Tomer Elitur and Shilo Rosenvasser (PhD. Students) who took time out of their busy schedule in the lab to teach me all the techniques that enabled me to complete this work. You guys have been wonderful and I cannot thank you enough. Next thanks go to Elona Rot for her motherly advice during the work and encouragement during the writing. My gratitude also goes to Prof. Moshe Coll (head, International M.Sc. Plant Science program) for always encouraging me during the thesis research.

I also thank the Pears foundation for sponsoring the first and part of my second year studies, God richly bless you. I am also grateful to all the hard working staff of the Division for External Studies especially Nina Rosenthal for her tireless effort making sure that all paper work get done on time and making my stay here comfortable.

I wish to thank all my friends especially Orit Agami words cannot express how much your friendship, encouragement and love helped me during my stay at the lab. I love you very much.

My greatest gratitude goes to Dr. and Mrs V.A. Clottey who have been my guardian angel and also encouraging me to apply for the program.

Last but not the least my thanks goes to my family; My Mum and my siblings and also to my Aunt Charlotte and her family for their support and prayers.

### **Dedication**

I dedicate this work to my guardian angels Dr. and Mrs. V.A. Clottey for believing in me and being a strong pillar during my studies. I appreciate your love and investment in me. Also to my Mum Grace Donkoh, siblings Mavis and Dominic Quansah for their continues love, prayer and support though far away but kept me and when times were hard I always remembered you and that kept me going. I love you all.

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## Abbreviations

|          |  |
|----------|--|
| BBB      | Blood brain barrier  |
| CNS      | Central nervous system                                     |
| COMT     | Catechol-O-methyl transferase                              |
| DAH      | Day after harvest  |
| DDW      | Double distil water  |
| HPLC     | High performance liquid chromatography                     |
| IPTG     | Isopropyl $\beta$ -D-1-thiogalactopyranoside               |
| MAO      | Monoamine oxidase  |
| MAOIs    | Monoamine oxidase inhibitors                               |
| PPO      | Polyphenol oxidase   |
| PCR      | Polymerase chain reaction                                  |
| RH       | Relative humidity  |
| RT       | Room temperature   |
| RT-PCR   | Real time- polymerase chain reaction                       |
| SDS-PAGE | Sodium dodecyl sulfate- polyacrylamide gel electrophoresis |
| SL       | Shelf life   |
| TH       | Tyrosine hydroxylase                                       |
| Tyrdec   | Tyrosine decarboxylase                                     |

## 1.0. Abstract

Catecholamines (dopamine, norepinephrine and epinephrine) are a group of biogenic amines involved in impulse transmission in animal nervous systems. Tyrosine hydroxylase (TH) is a rate limiting enzyme of catecholamine biosynthesis in mammals, resulting in L-dopa biosynthesis which leads to dopamine and the other catecholamines. Dopamine has been found in high amount in banana peel and in lower quantity in the pulp. Neither function nor biosynthesis of dopamine in plants is fully understood. Two possible pathways have been postulated; one through L-dopa, like in animals, and the second through tyramine produced by decarboxylation of tyrosine and further hydroxylated by an oxidase presumably belonging to polyphenol oxidase (PPO) family. Content of both catecholamine precursors in food has a major impact on human health since products rich in L-dopa may replace L-dopa administration in Parkinson's disease patients and high tyramine causes hypertensive crises with patients on monoamine oxidase inhibitors (MAOIs). This work aimed at isolating the genes for enzymes involved in the dopamine biosynthesis in banana. Our work has confirmed that higher levels of dopamine exist in peel than in pulp. Tyrdec and PPO partial genes sequences have been cloned from banana sub variety Grand Nain and the expression level after harvest has been determined and related to ripening parameters. In this cultivar and in six additional cultivars, expression of the transcript levels of both enzymes Tyrdec and PPO was higher in peel in most of the cultivars analyzed than in pulp. Cloning of the gene encoding TH has not been successful even in the additional cultivars and mucuna a plant with high levels of L-dopa. However, through western blot, TH-like protein was detected in the peel of Grand Nain but not in the pulp. Tyrdec expression was highest in green fruit, while PPO expression was highest in the climacteric stage of ripening. Hence, Tyrdec gene expression decreases with ethylene production while PPO was correlated with increase in ethylene production. It is still not clear if the PPO pattern of expression corresponds to PPO activity in the tissue since PPO may exist as a multi gene family in banana. Our results suggest that all these enzymes might participate in dopamine synthesis since all exhibit higher levels in peel than in pulp and their levels decrease at post climacteric stage which was found to be in correlation with

dopamine levels. This work laid the ground for manipulating levels of catecholamines and their precursors in banana fruit.

## **2.0. Introduction**

### **2.1. Catecholamines in animal systems**

Catecholamines are a group of biogenic amines that possess a 3,4-dihydroxy-substituted phenyl ring. These compounds include dopamine, norepinephrine (noradrenaline) and epinephrine (adrenaline) (Smith, 1977; Kuklin and Conger, 1995). Catecholamines are involved in impulse transmission in the central nervous systems in animals. They have been also implicated in hormonal function, in particular regulation of glycogen metabolism by epinephrine and norepinephrine. These catecholamines also function as transmitters in the branch of the peripheral nervous system, known as the autonomic nervous system (Hynes et al., 1995).

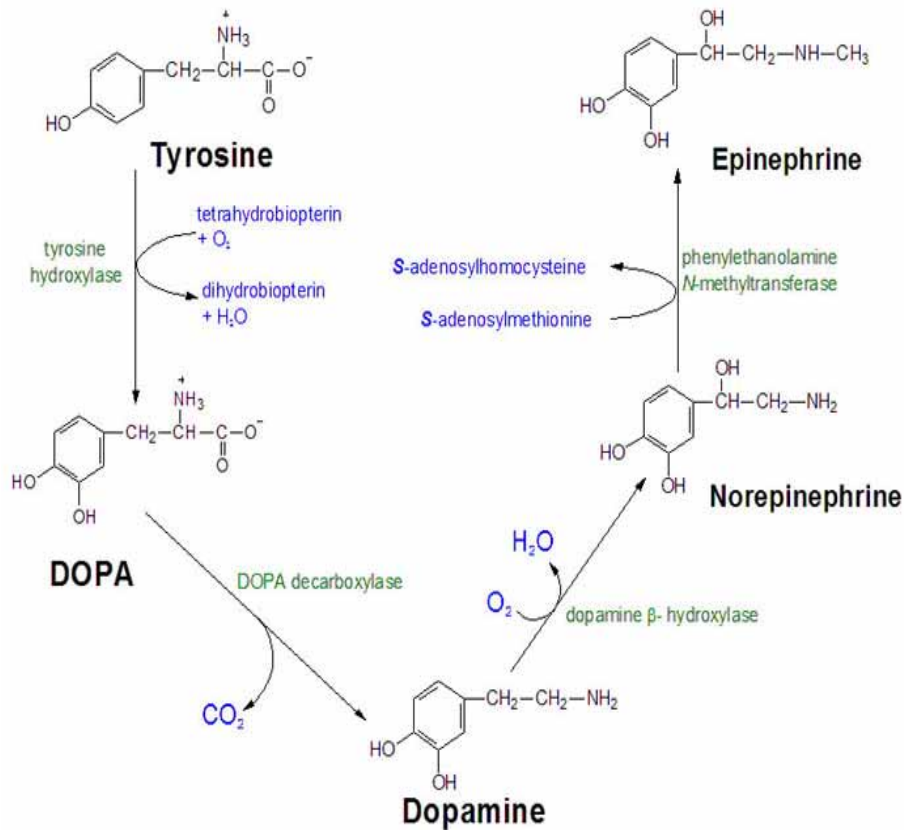
Dopamine has a number of roles acting as a hormone and neurotransmitter in vertebrate and invertebrate animals. It functions as neurotransmitter in the brain where it helps control sleep, attention, mood changes and learning. There are five types of dopamine receptors; D1, D2, D3, D4 and D5 and their variant (Hynes et al., 1995). It is produced in many parts of the brain. Dopamine can be supplied as drug which acts on the sympathetic nervous system producing effects such as increase heart rate and blood pressure. It cannot cross over the blood-brain barrier (BBB) therefore dopamine given as medication has no effect on the central nervous system.

Imbalances of catecholamines, in particular dopamine may lead to a number of disorders including Parkinson's disease (lack of dopamine) and schizophrenia (excess of dopamine) (Harrower et al., 2005) in human. Parkinson disease is one of the most common neurodegenerative movement disorders characterized by resting tremor, rigidity, bradykinesia and postural instability (Calne, 2001). People who suffer from Parkinson disease as a result of lack of dopamine are given its precursor L-dopa which crosses the blood-brain barrier and therefore increase the amount of dopamine in such patients (Hornykiewiez, 2002).

### **2.2. Biosynthesis and metabolism of catecholamines in animals**

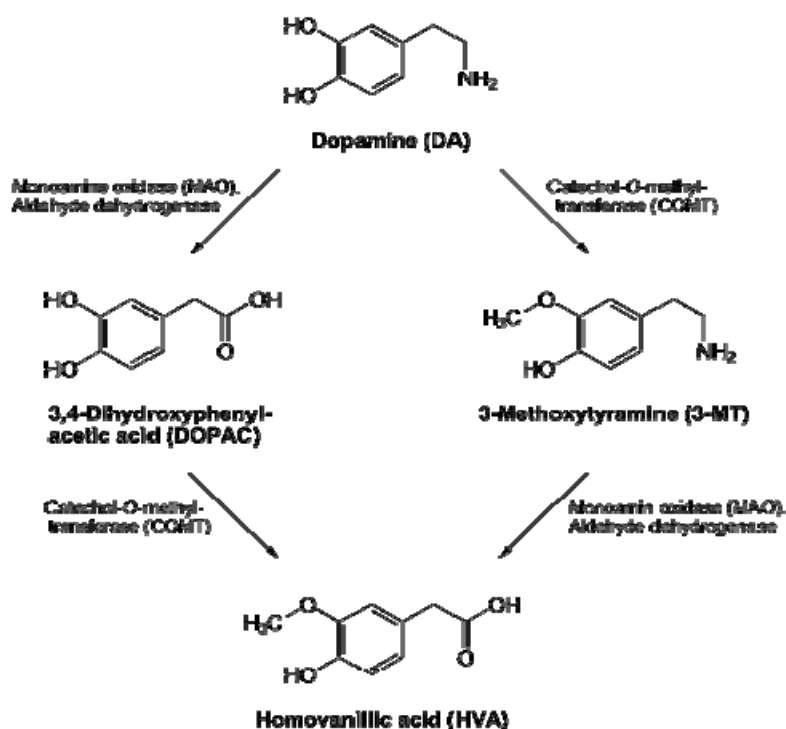
In the synthesis of catecholamine, three major products are formed namely dopamine, epinephrine and norepinephrine (Fig. 1). These compounds are derived from the amino acid tyrosine (Kimura, 1968). In the catecholamines pathway in mammalian cell (Fig. 1), L-tyrosine is converted to L-dopa by the

enzyme tyrosine hydroxylase which requires a tetrahydrobiopterin, then from L-dopa to dopamine by dopa decarboxylase. Dopamine is then converted to norepinephrine by  $\beta$ -hydroxylase and phenylethanolamine N-methyltransferase converts norepinephrine to epinephrine (Roshchina, 2001).



**Fig. 1: Catecholamine biosynthetic pathway in animal system**  
(<http://themedicalbiochemistrypage.org>)

In mammalian cells, dopamine is inactivated by reuptake via the dopamine transporter, then enzymatic breakdown by Catechol-O-methyl transferase (COMT) and Monoamine oxidase (MAO) (Fig. 2). Dopamine that is not broken down by enzymes is repackaged into vesicles for reuse. It may also simply diffuse away from the synapse (Vermeulen, 1994).



**Fig. 2:** Dopamine degradation in animal system (Roshchina, 2001)

### 2.3. Occurrence of catecholamines in plant

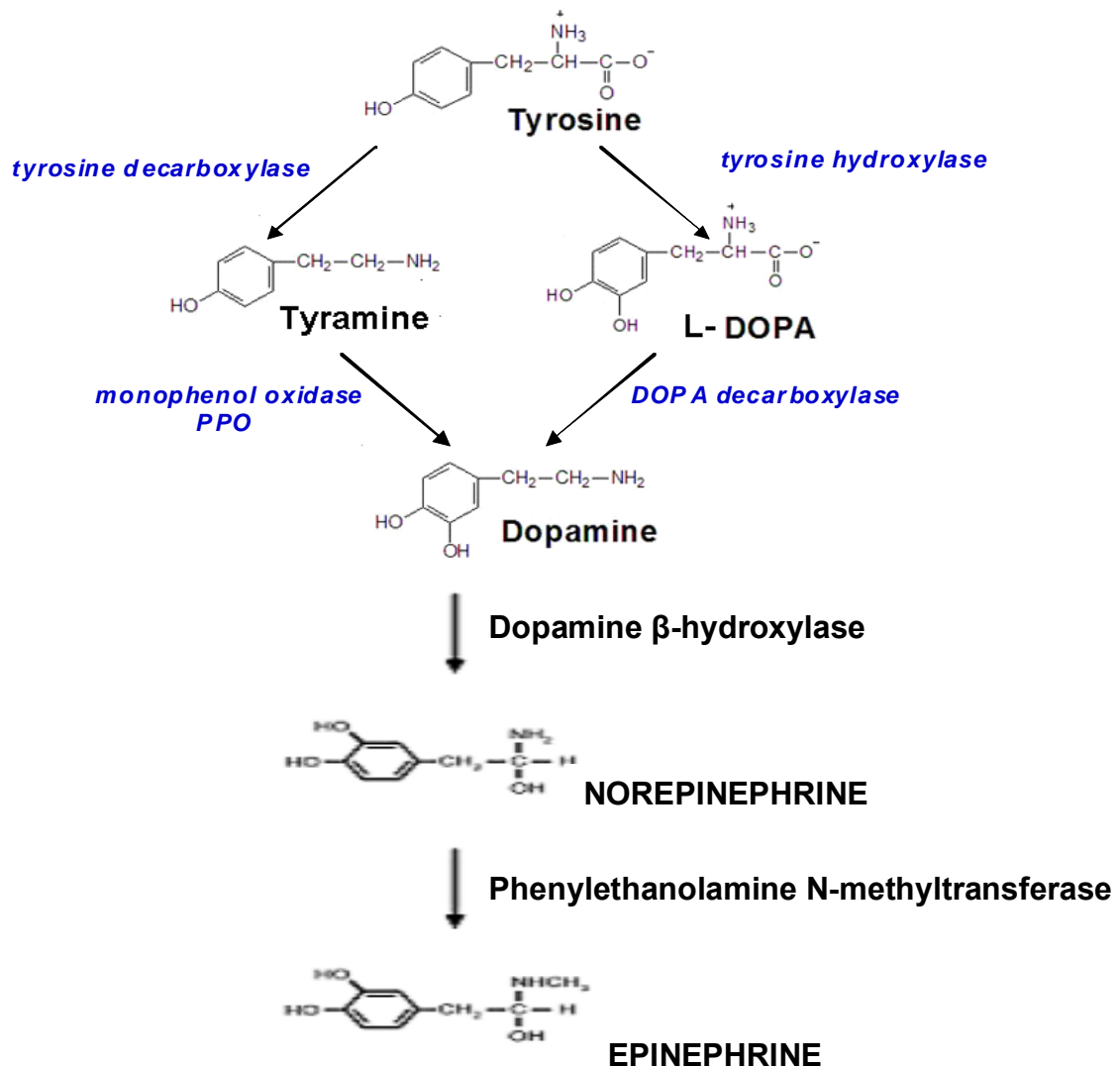
Though typically found in mammals, the catecholamines have been reported to occur in plant. In contrast to the vast amount of knowledge concerning the role and action of catecholamines in mammals, very little is known of the role they play in plants. Catecholamines were first reported to occur in plants during studies of the mechanisms underlying the therapeutic effect of banana fruit (Waakles et al., 1958; Udenfriend et al., 1959). Since then, they have been reported to occur in 44 plant families (Kimura, 1968; Smith, 1977, 1980; Kulma and Szopa, 2007) of which 29 are present in the human diet (Kimura, 1968). These compounds and their precursors as well as other derivatives have been reported to occur in plant families, however no important metabolic role has been assign to them (Kuklin and Conger, 1995). They are however reported to be precursors for benzoic phenanthridine alkaloids which are the active principal ingredients of many medicinal plant extracts (Kuklin and Conger, 1995) such as sanguinarine in *Papaver somniferum*. Dopamine is also a precursor for other types of alkaloids, such as hallucinogen phenethylamine alkaloid mescaline indentified in several species of cacti, and tetrahydroisoquinoline alkaloids (Roshchina, 2001). The catecholamine content in a number of fruit and

vegetables was measured using radio enzymatic assay. The concentration of dopamine in the pulp of yellow banana (*Musa acuminata*), red banana (*Musa sapientum* var. baracoa), plantain (*Musa x paradisiaca*), , and avocado (*Persea americana*) was 42, 54, 5.5 and 4 µg/g respectively (Feldman et al., 1987). Dopamine has been reported to be involved in plant cellular activities leading to suggestions of its physiological role. For instance it was reported to enhance ethylene biosynthesis, promote flowering and somatic embryogenesis and to inhibit indole-3-acetic acid oxidation in various plant (Elstner et al., 1976). The release of dopamine was observed following the seal of wounds in some *Papaver species* by a brown melanin-like substance (Homeyer and Roberts, 1984). An increase in dopamine concentration was observed after the formation of wound tissue in *Carnegiea gigantean* (Kuklin and Conger, 1995). Dopamine has high antioxidant capacity, comparable with potent natural antioxidants, such as ascorbic acid, glutathione and various phenolic compounds (Kanazawa and Sakakibara, 2000). They are shown to also have a possible protective role against insect predators, injuries as well as nitrogen detoxification (Smith, 1980).

#### **2.4. Two pathways of catecholamine biosynthesis and their key enzymes**

Two possible pathways for the biosynthesis of catecholamine in plants have been considered (Fig. 3). The first one, similar to animals, is initiated by the hydroxylation of tyrosine to L-dopa followed by a decarboxylation of L-dopa to dopamine. The second pathway is initiated by the decarboxylation of tyrosine to tyramine and then subsequent hydroxylation of tyramine to dopamine by monophenol hydroxylase (Kulma and Szopa, 2007).





**Fig. 3:** Catecholamine biosynthetic pathway in plant. Adopted from (Kulma and Szopa, 2007)

#### 2.4.1. Tyrosine hydroxylase pathway

Tyrosine hydroxylase, the rate limiting enzyme in catecholamine biosynthesis and the immediate enzyme precursor for dopamine has been studied extensively in mammalian cells and the gene has been identified and characterized (Haavik et al., 1991; Zhou et al., 1995).

The mechanism by which tyrosine hydroxylase functions is poorly understood and there is yet no crystal structure available. Its activity is modulated by several factors involving modification of the enzyme molecule and regulation of its expression. It plays an important role in the etiology of some diseases attributed to the impairment of central catecholaminergic neurons such as

Parkinson's disease and other effective disorders (Kaufman, 1995). It is known to contain 1 iron atom/subunit (Hoeldtke and Kaufman, 1977). No other metal has been found to be catalytically active (Haavik et al., 1991). In human, tyrosine hydroxylase exists as four different isozymes (TH1-TH4) which are generated through alternative splicing of pre-mRNA. The enzyme is distributed in discrete regions of the brain, sympathetic ganglia, adrenal medulla and the central nervous system (CNS) in mammalian cell (Zhou et al., 1995).

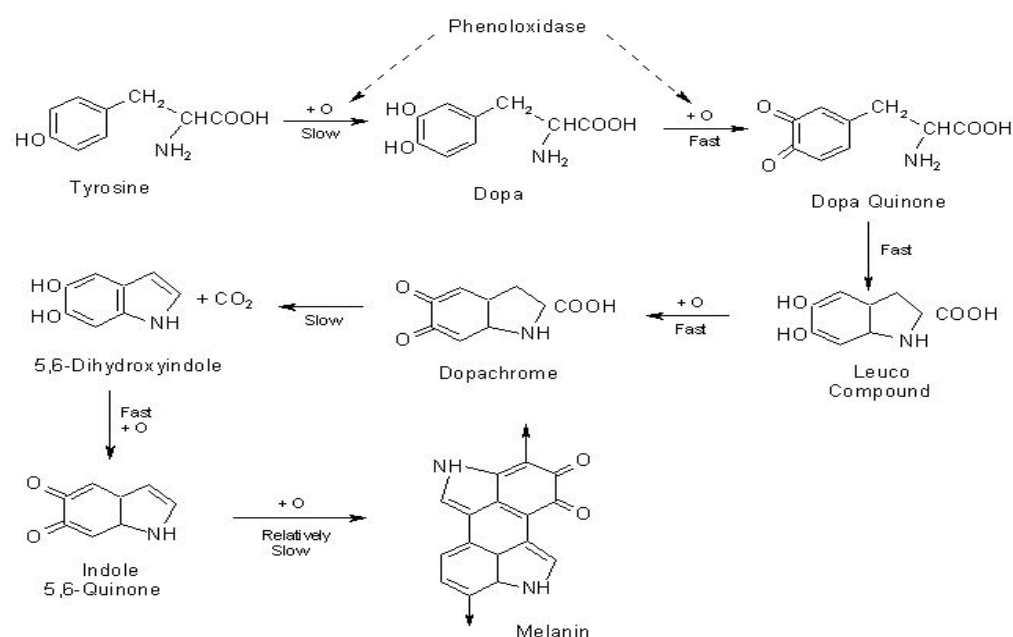
In higher plants, tyrosine hydroxylation has been usually ascribed to polyphenol oxidase. It catalyzes the hydroxylation of monophenols to *o*-diphenols, usually followed by the oxidation of *o*-diphenols to *o*-quinones. Only one research isolated and demonstrated the activity of tyrosine hydroxylase separated from polyphenol oxidase activity from callus cultures of *Portulaca grandiflora* (Yamamoto et al., 2001). The gene or protein of this enzyme has not yet been characterized in plant.

#### **2.4.2. Tyrosine decarboxylase pathway**

Tyrosine decarboxylase (Tyrdec) is an enzyme reported to participate in the biosynthesis of various secondary products in higher plants, in particular diverse alkaloids and auxin (Connil et al., 2002). The enzyme belongs to a group of enzymes known as aromatic amino acid decarboxylases. The gene that encodes this enzyme has been characterized in some higher plants (Marques and Brodelius, 1988). In *Petroselinum crispum* for instance, four different Tyrdec genes were cloned and it was shown that the enzyme activity was significantly influenced by fungal elicitor treatment as a result of rapid gene activation (Kawalleck et al., 1993). This enzyme has been shown to also take part in the biosynthesis of catecholamines as demonstrated (Skiryecz et al., 2005; Swiedrych et al., 2005) using transgenic potato (*Solanum tuberosum*). In this pathway, tyramine is further hydroxylated to dopamine by a monophenol oxidase (Kulma and Szopa, 2007), presumably a non-specific enzyme belonging to polyphenol oxidase (PPO) family.

## 2.5. Dopamine involvement in fruit browning

Dopamine has been reported to be one of the natural substrates responsible for the browning reaction of fruit and vegetables which are catalyzed by the enzyme, PPO (Palmer, 1963; Vamos-Vigyazo, 1981; Mayer, 2006). Browning is mainly attributed to the oxidation of phenolic compounds by PPOs (Fig. 4). These enzymes catalyze the oxidation of different phenols to the corresponding quinones; the highly reactive compounds which polymerize to melanin (Castaner et al., 1996).



**Fig. 4:** Formation of melanin from tyrosine (Lerner, 1953).

Polyphenol oxidases are copper-utilizing enzymes that catalyze the oxidation of aromatic compounds by oxygen. Two types of PPOs have been shown (tyrosinases and laccases). Tyrosinase catalyzes two kinds of reactions; ortho hydroxylation of monophenols such as L-tyrosine yielding L-3,4-dihydroxyphenylalanine (L-Dopa) and the oxidation of other *o*-dihydroxyphenols to *o*-quinones. The quinone product formed can then polymerize and react with amino acid groups of cellular proteins resulting in black or brown pigment deposits known as melanin (Fig. 4).

Laccase (or catechol oxidase), however oxidize mostly *p*-diphenols and methoxy substituted phenols (Thurson, 1994). The PPO found in banana has been shown to be that of tyrosinase (Nematpour et al., 2008). Enzymatic

oxidative browning is probably a part of plant's natural defense system. On the other hand, it causes considerable economic and nutritional loss in the commercial production of fruit and vegetables (Vamos-Vigyazo, 1981). In addition to melanin generation in wounded tissues, the enzymes of polyphenol oxidase family may be involved in biosynthetic reactions not related to wounding, such as formation of betalains and auronones (Strack and Schliemann, 2001; Ono et al., 2006).

## **2.6. Dopamine presence in food and toxicological effect of biogenic amines**

Biogenic amines are basic nitrogenous compounds formed mainly by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones (Maijala et al., 1993). The chemical structure of biogenic amines can either be aliphatic, heterocyclic or aromatic. The aromatic amines include catecholamines and their precursors.

Dopamine and its precursors (L-dopa and tyramine) have been reported to be present in food. The greatest amount of dopamine was reported to occur in the peel of banana while it was much lower in the pulp (Waakles et al., 1958; Fellows and Bell, 1971). Amino acid decarboxylation is the most common mode of synthesis of amines in foods. In food, amines are formed by the action of living organisms through the decarboxylation process of amino acids (Halasz et al., 1994). Conditions that may influence the production of biogenic amines by microorganisms in food include the availability of protein and free amino acids (Brink et al., 1990; Huis et al., 1990; Marklinder and Lonner, 1992).

The precursor for the biogenic amine tyramine is tyrosine and occurs widely in plants and animals. Tyramine is metabolized by the enzyme tyrosine decarboxylase and has been intensively studied due to its toxicological effects derived from their vasoactive and psychoactive properties (Santos-Buelga et al., 1986). The importance of tyramine in food is basically due to its toxicological effect since apart from being slightly toxic by itself, tyramine also reacts with monoamine oxidase inhibitor (MAOI) drugs. The MAOI drugs are used to aid in monoamine metabolism in human (Mower and Bhagavan, 1989), in particular as antidepressants. When food high in tyramine is

ingested by a MAOIs patient, it causes the release of stored monoamines such as dopamine, norepinephrine and epinephrine from the sympathetic nervous system (McCabe, 1986).. Since the presence of MAOIs lead to high accumulation of tyramine, it results in hypertensive crises and severe headaches (Blackwell, 1963; Joosten, 1988).

## **2.7. Presence of catecholamines in banana and changes occurring during ripening of the fruit**

Banana belongs to the family *Musaceae* and the genus *Musa*. There are about 401 species in this genus (Simmonds and Shepherd, 1955). Banana is the fifth largest crop after wheat, maize, rice and soy bean and is the most important fruit crop in the world (Crouch et al., 1998). It comprises an important part in the diet of millions of people. Commercially, it is a very prosperous crop in world trade. In Costa Rica and Honduras, bananas make up more than 25% of their total export (Ortiz et al., 1998).

Grand Nain (AAA) is a high yielding Cavendish sub variety which is highly consume and readily available on the market. Aside the Cavendish groups there are other banana cultivars that have been developed around the world. These cultivars were mostly developed to tackle disease problem in banana plantation ([www.articlesbase.com/advice-articles/the-history-and-evolution-of-banana-hybrids](http://www.articlesbase.com/advice-articles/the-history-and-evolution-of-banana-hybrids)).

The FHIA cultivars were developed in Honduras by the "Fundacion Hondurena de Investigacion Agricolainclude". The cultivars include, FHIA-1, 3 and 21 and many others were developed in a special program to increase banana and plantain production. These cultivars have the characteristics of being resistance to various diseases that attack banana plantation. Hence, they have been introduced into export markets of organic products. The cultivars develop their natural flavor and become softer when allowed to ripe naturally without treatment with ethylene therefore it is recommended not to treat with ethylene. They are able to withstand lower temperatures than that of Cavendish varieties. It tastes like apple when ripened and can be cooked or eaten or fried when green (Alvares and Rosales, 2004).

The cooking banana Matokke (AAB) is a major cultivar that is cultivated in Uganda. It is a major staple for the people.

Blugoe and Silver Blugoe cultivars are triploid cooking banana (ABB group). They are resistant to the bacterial wilt disease called *Marchitez bacteriana* (Moko) and Black sigatoka. It does well in poor soils and in adverse conditions where other varieties cannot thrive. It has good qualities and is consumed either ripe or unripe. It is not adopted for export due to its rapid ripening habit (Escalant et al., 1992).

During ripening of banana, changes in appearance, texture and chemical composition occur. The color of the peel turns from green to yellow. There is migration of water from the peel to the pulp and degradation of starch which then softens the pulp. There is also accumulation and production of low molecular sugars. These changes are a contributing factor to the appearance, desirable sweetness and eating quality of the ripened fruit (Marriot, 1980).

Starch levels in the fruit decreases to very low levels with ripening and eventually the soluble sugars level increases (Hubbard et al., 1990).

Banana has been shown to contain catecholamines including dopamine (Roshchina, 2001) and its precursors, L-dopa and tyramine (Bapat et al., 2000; Romphophak et al., 2005). The concentration of dopamine was found to decrease during ripening of the banana fruit while that of L-dopa and tyramine were seen to increase (Romphophak et al., 2005). Though many studies have indicated the presence of dopamine and its precursors in banana fruit through chemical analysis, no up-to-date investigations have been conducted on their biosynthetic pathways. In the early work (Buckley, 1961) suggested on the basis of indirect evidence that dopamine in banana is produced through the tyramine pathway with tyrosine decarboxylation being the limiting step. The genes that encode the enzymes involved in the biosynthesis pathway have not yet been reported.

This study was aimed at cloning the genes encoding the enzymes involved in the dopamine biosynthetic pathway in banana fruit.

### **3.0. Objective**

The objectives of the study were

- ❖ To identify components of the dopamine biosynthetic pathway from different banana cultivars.
- ❖ Study the dynamic in the expression of biosynthetic components during banana ripening in peel and pulp.
- ❖ To determine ripening parameters existing within different banana cultivars after harvest of the fruit.

## **4.0. Materials and Methods**

### **4.1. Plant material**

Banana (*Musa acuminata*), AAA Cavendish subgroup, Grand Nain (the most common cultivar grown in Israel for consumption) was used in this study. In addition six other cultivars; FHIA-1 (AAAB), FHIA-21 (AAAB), FHIA-3 (AAAB), Matokke (AAB), Bluggoe (ABB) and Silver Bluggoe (ABB) were obtained from Rahan Meristem Ltd. The Cavendish variety was grown in Nahsholim (located south of Haifa) additional cultivars in Rosh Hanikra (located north of Haifa) both along the Mediterranean coast in Israel. Both peel and pulp from the banana of different ripening stages were used.

### **4.2. Determination of ripening parameters**

Ethylene (C<sub>2</sub>H<sub>4</sub>) and carbon dioxide (CO<sub>2</sub>) production were determined by sealing a banana finger in 2-L sealed glass jar at 20°C. Samples were withdrawn from the sealed jars using gas-tight syringes. Ethylene concentration was determined with Varian 3300 gas chromatograph equipped with a flame ionization detector and a C-5000 alumina packed column using helium as the carrier gas. Carbon dioxide concentration was determined by Packard 7500 gas chromatograph (Packard, Downers Grove, Ill.) with thermal conductivity detector and CTR-I packed column using helium as a carrier gas. Peel color from surface area of three individual banana fingers was determined using Minolta CR-300 (Minolta Corporation, New Jersey, USA). Firmness was measured on whole fruit using a Chatillon Force tester/gauge (Ametek Inc., Florida USA).

Determination of total soluble solids (TSS) was done by using protocol developed by (Pesis et al., 2005). TSS was checked in the peel and pulp of the banana by freezing the tissue for several days at -20°C. Thawing the frozen tissue enabled to measure the TSS in the outcome juice using handheld HSR-500 refractometer (Atago Co. Ltd, Japan).

### **4.3. RNA extraction**

Spectrum Plant Total RNA kit (Sigma, UK) was used for the RNA extraction of banana (peel and pulp). Peel and pulp were cut into 1 g and frozen in -80°C to be used later. Approximately 1 g of each frozen tissue was powdered and 100



mg was used for the extraction process. The extraction process as described in ([www.sigmaaldrich.com/sigma/bulletin/STRN250bul.pdf](http://www.sigmaaldrich.com/sigma/bulletin/STRN250bul.pdf)) was used. The tissue was pulverized in a pre chilled porcelain mortar and pestle with continuous presence of liquid nitrogen to prevent thawing of the tissue. To obtain a clean RNA free from DNA contamination, the extracted RNA was digested with TURBO DNase (Applied Biosystems, USA). The cleaning was done using the protocol specified ([www.ambion.com](http://www.ambion.com)). The final concentration of the cleaned RNA was determined using Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., USA). For the concentration determination, 1.5  $\mu$ l of RNA was pipetted onto the spectrophotometer pedestal and measured at an absorbance ratio of 260 and 280 nm. Purity determination of RNA interference by contaminants can be recognized by the calculation of the ratio A<sub>260</sub>/A<sub>230</sub>. A pure and high quality RNA should give a value of 1.8-2.2. Values obtained for the samples used for this study was 1.8-2. RNA concentration ranging between 50-150  $\mu$ g/ $\mu$ l was obtained for the banana fruit with peel and pulp giving different concentration with the advancement of ripening.

#### **4.4. cDNA preparation**

cDNA was prepared from extracted RNA using Verso™ cDNA kit (Thermo Fisher Scientific Inc., USA). For the cDNA preparation, it is recommended that a minimum RNA concentration of 100  $\mu$ g/ $\mu$ l should be used. Since the different tissue gave different concentrations, an amount of 1-5  $\mu$ l of RNA was used for the cDNA synthesis. The reaction was prepared in 0.2 ml eppendorf tube in a total reaction volume of 20  $\mu$ l. The cDNA reverse transcription reaction was performed using the Eppendorf Mastercycler gradient (Biocompare, USA.) programmed to one cycle for each cDNA reaction at 42°C for 30 min followed by inactivation in 95°C for 2 min after which the cDNA was stored in -20°C for future use.

#### **4.5. Primers design**

Degenerate primers were prepared for the different genes of interest (tyrosine hydroxylase, tyrosine decarboxylase and polyphenol oxidase). Gene sequences used for the cloning were obtained from NCBI GeneBank

(www.ncbi.nlm.nih.gov). Gene sequences alignments were prepared based on sequences of various sources. Primers were designed using ortholog information from various sources depending on the gene of interest (see appendix). For tyrosine hydroxylase primer design, the accession numbers for the genes used were; (*Gallus gallus* AJ251387.1, *Rattus norvegicus* NM\_012740.3, *Bos Taurus* NM\_173884.2, *Xenopus laevis* NM\_001097923.1, *Homo sapiens* BC104967.1 and *Pan tryglod* XM\_5082212.2), for tyrosine decarboxylase: (*Papaver somniferum*, AAA97535.1, *Parsley*, AAA33863.1 and *Arabidopsis thaliana*, AAM20115.1), and Polyphenol oxidase (*Populus balsamifer* AF263611.1, *Triticum aestivum* AY515506.1, *Ipomoea batatas* AJ309175.1 and *Vicia faba* Z11702.1).

BioEdit software program (www.mbio.ncsu.edu/bioedit/bioedit.html) was used for the DNA and protein sequence alignment and primer preparation. Table 1 shows the primer sequences used for cloning of the different gene as well as primers prepared for RT-PCR and the reference gene.

**Table 1:** Sequences of primers used for cloning of the various genes and for RT-PCR and Ribosomal gene used as reference.

| Amplified cDNA                     |           | Primers   |
|------------------------------------|-----------|---|
| Tyrosine Hydroxylase<br>(570 bp)   | A         | 5' TTTGAGACRTTYGAAGCYCA 3'<br>5' TGGACAARTGTCAYCAYACM 3'                          |
|                                    | B         | 5' DGAYAYRAARTABACDGRCT 3'<br>5' RYCDGMDGCRTARYTCCTBA 3'                          |
|                                    | C         | 5' WRGABCAYCCBGGCTWY 3'<br>5' TSCGVCCNGTRGCBGGNCT 3'                              |
|                                    | D         | 5' AGNCCVGCYACNGBGCGSA 3'<br>5' RTACTGBGTKSAHTGRAA 3'<br>5' SCCBGCVCCRWAVGCBTT 3' |
| Tyrosine Decarboxylase<br>(470 bp) | A         | 5' GGHRTACDCAYTGGCAAAG 3'<br>5' TCTGAYCARACYCATTSWG C 3'                          |
|                                    | B         | 5' ATACARGCACTTC CAGCATA 5'<br>5' ATTTGCCARTCTT TRTAATC 3'                        |
|                                    | RT<br>PCR | 5' GAGCTCGAGACCATCGTCATG 3'<br>5' GGGCTTAGGAAGGTTGAGCAT                           |
| Polyphenol Oxidase<br>(420 bp)     | A         | 5' GARGAYATGGGHAWYTTYTWYTC3'<br>5' TKGGHRCSARHGHMACC3'                            |
|                                    | B         | 5' TTYCTYTTCTAYRAYGAGRAB3'<br>5' TCHTCYTCYKCYTCYTTCTC3'                           |
|                                    | RT<br>PCR | 5' GACCGCATGTGGTACTTGTGG3'<br>5' GAAGGTGGTGTGAGCCAGTC3'                           |
| Ribosomal                          | RT<br>PCR | 5' GCGAGGCATCATTCAAATTC3'<br>5' TCCGGAATCGAACCTAATTC3'                            |

Single letter code shown below was used to denote multiple nucleotides in the sequence alignment for the degenerate primer preparation. The various letters

indicate; B- C or G or T, D- A or G or T, H- A or C or T, K- G or T, M- A or C, N- A or C or G or T, R- A or G and S- C or G. For each gene more than one set of primers were prepared. Letters in each gene section (Table 1) represents the primer set (forward and reverse).

#### **4.6. Gene cloning**

PCR amplification reaction was performed in a 0.2 ml eppendorf tube in a final volume of 20  $\mu$ l containing 1  $\mu$ l each of forward and reverse primer (100  $\mu$ M) concentration; 1 $\mu$ l cDNA, 10  $\mu$ l PCR Master Mix (Thermo Scientific, UK) and 7  $\mu$ l of double distil water (ddw). The PCR amplifications were carried out in an Eppendorf Mastercycler gradient (Biocompare, USA) using 40 cycles of: denaturation at 95°C for 30 sec, annealing at 52°C for 60 sec and elongation at 72°C for 60 sec. The PCR reaction was loaded on 1% agarose gel and run at a voltage of 100V for 30 min. The PCR product was excised under UV light. The product was further purified from the gel using QIAEX II gel extraction kit (Qiagen Ltd, UK) according to manufacturer's instruction ([www.qiagen.com](http://www.qiagen.com)). The purified PCR product was then ligated to pGMT<sup>®</sup>-T easy vector (Promega Corporation Madison, USA). The reaction was prepared in a 0.2 ml eppendorf tube and incubated over night at 4°C. The ligation reaction was transferred into JM 100 competent E. coli cells ([www.rcbioscience.com](http://www.rcbioscience.com)) (RBC Bioscience, Taiwan). Less than 10% volume of the ligation reaction was added to the competent cells and incubated on ice for 10 min. LB medium (Bacto<sup>®</sup>-trptone, Bacto<sup>®</sup>-yeast extract and NaCl) in addition to agar were prepared with ampicillin (50 mg/ml) and the content pored in Petri dish. Following LB solidification, 80  $\mu$ g/ml X-gal and 0.5 mM Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) were spread on the surface and incubated for 30 min at 37°C. Competent cells containing the cloned product were grown on the LB plates and incubated at 37°C overnight. White colonies which carry the insert were then selected and grown in LB liquid in tubes containing ampicillin (100 mg/ml) to select bacteria carrying the insert. The tubes were incubated at 37°C overnight with shaking. The tubes were centrifuge at 4000 g to concentrate the bacteria. Plasmids were purified from bacteria using Highyield Plasmid mini kit (RBC Bioscience, Taiwan) according to the manufacturer's specification ([www.rcbioscience.com](http://www.rcbioscience.com)). The extracted plasmids were

sequenced using T7 and SP6 as forward and reverse primers respectively. The sequencing was carried out at Hylabs laboratories Ltd, Israel.

#### **4.7. Quantitative Real-Time PCR analysis**

Different concentration of the primer were checked to make sure that the concentration to be used for the reaction is within the linear range of the graph this was to prevent primer dimmer effect that can lead to unspecific reaction. After checking different concentrations, 8  $\mu$ M and 4  $\mu$ M were chosen for the gene of interest and the Ribosomal reference, respectively. The same was done for the cDNA and dilution of 1:20 and 1:5000 was used for the gene of interest and the reference Ribosomal gene respectively. Reaction mixture contained forward and reverse primers (Table 1 denoted as RT PCR) and Power SYBR Green PCR Master mix (Applied Biosystems, USA) in a 20  $\mu$ l total sample volume. Reactions were run in duplicates and analyzed by quantitative real-time PCR on a Rotor-Gene 3000 PCR machine (Corbett Life Research, Sydney, Australia) using 35 cycles of 95°C for 10 sec, 60°C for 15 sec and 72°C for 20 sec. Data obtained was analyzed with Rotor-Gene 6 software (Corbett Research).

#### **4.8. HPLC analysis of dopamine concentration in banana**

High performance liquid chromatography (HPLC) analysis was carried out using protocol described by (Lavizzari et al., 2006) with little modification. Frozen 1 g of tissue (peel and pulp) separated were pulverized in a pre-chilled porcelain mortar and pestle with continuous presence of liquid nitrogen to prevent thawing of the tissue. The pulverized material was quantitatively transferred to 15 ml polypropylene screw- capped test tubes, and 250  $\mu$ l of 17.5% hydrochloric acid (HCl) (concentrated HCl diluted by half) was promptly added to the test tube. The content of each test tube was mixed with a glass rod and the test tubes were incubated for 5 min on a boiling water bath. After taking the test tubes from the bath, 3 ml of 0.6 M perchloric acid (HClO<sub>4</sub>) was added to the test tube. The test tube were shaken for 20 min in room temperature (RT) and centrifuged at 30,000 g for 10 min at 4°C. The supernatant was collected and the pellet extracted twice by re-suspending in 4 ml of 0.6 HClO<sub>4</sub>, shaken for 20 min at RT and centrifugation done as

described above. The supernatant was collected and combined, and total final volume adjusted with 0.6 M HClO<sub>4</sub> to 12 ml. The extract is passed through a 0.45- $\mu$ m filter before HPLC analysis. For the analysis, injection volume of the sample was 20  $\mu$ l and the HPLC (MERCK, HITACHI, USA) specification used; Column; hypersil ODS Ci8, 250 x 10 mm. Isocratic regime; Mobile phase; 50 mM acetate buffer pH 4.3 with addition of 4% methanol and 0.125 mM NaEDTA. Flow rate 1 ml/min. Detector UV/Vis at wavelength 280 nm.

#### **4.9. Total plant protein extraction**

Total protein was extracted from Banana (peel and pulp) using two protocols: developed by (Lavid et al., 2002) and (Xu et al., 2008) with slight changes. For the first protocol, 1 g of tissue was pulverized in the presence of liquid nitrogen in a pre chilled porcelain mortar and pestle. Extra liquid N<sub>2</sub> was added intermittently during the grinding process to prevent the tissue from thawing. Polyvinylpyrrolidone (1 mg PVP-40) was added to the frozen powdered sample. The powdered tissue was resuspended in buffer solution containing 50 mM bis propane, 10% glycerol, 5 mM Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>, 1 mM EDTA, 14 mM  $\beta$ -Mercaptoethanol, 10 mM NaCl, protease inhibitor (1tablet/50 ml). Samples were then centrifuge at 20,000 g in 4°C for 15 min to precipitate cell debris. The supernatant (containing the proteins) was transferred to 2 ml eppendorf tube using a pipette. The protein concentration was determined using BCA protein assay kit (Thermo Scientific, USA) and 30  $\mu$ g of total protein was loaded on the gel.

For the second protocol, 0.25 g liquid nitrogen-frozen ground tissue was weighed into a 2 ml tube which contained 10% Trichloro acetic acid in acetone and then vortexed. Samples were centrifuged at 16,000 g for 3 min at 4°C. Supernatant was discarded and the tube was filled with 80% Methanol (MeOH), 0.1 M ammonium acetate and vortexed. Centrifugation was done at 16,000 g for 3 min at 4°C to precipitate the proteins. Supernatant was discarded and pellet was washed in 80% acetone and vortexed. It was centrifuge at 16,000 g for 3 min at 4°C after which supernatant was discarded again and pellet was air dried. Phenol/SDS solution (1 volume Phenol, pH 8 and 1 volume of SDS buffer containing 30% sucrose, 2% SDS, 0.1 M Tris pH 8.0 and 5%  $\beta$ -Mercaptoethanol) was added to the dried pellet in a total

volume of 2 ml. The dissolved pellet was incubated in room temperature (RT) for 5 min and centrifuged at 16,000 g for 3 min at RT. Supernatant was transferred to a fresh 2 ml tube and filled with 80% MeOH and 0.1 M ammonium acetate to further precipitate the proteins and incubation done overnight at -20°C. Again precipitated proteins were centrifuged at 16,000 g for 5 min at 4°C and supernatant discarded. Pellet was washed with 100% MeOH, vortexed, spinned and supernatant discarded again. Pellet was washed again with 80% acetone, vortexed and supernatant discarded. Pellet was air dried after which it was resuspended in 100 µl of SDS buffer (0.5 M Tris pH 7.0 and 1.4% SDS). Proteins were run on Sodium dodecylsulfate Polyacrylamide gel electrophoresis (SDS-PAGE), using Mini-PROTEAN<sup>®</sup> Cell (BIO-RAD, USA). A 10% and 5% Acrylamide gel buffers were used for resolving and stacking gel, respectively. Prior to loading the sample, 1.5 µl SDS reducing buffer (0.5 M Tris-HCl pH 6.8, glycerol, 10% SDS, 0.5% bromophenol blue, β-Mecaptoethanol and deionized water) was added to the sample. The samples were then heated at 70°C for 5 min after which it was shortly centrifuge to uniformly mix the sample and 30 µg of total protein was loaded on the gel. Gels were run with a 1X running buffer (0.1M Tris base, 1.44% Glycine and 0.1% SDS) at a constant voltage of 120V for 2 hrs. Pre stain protein ladder (Fermentas, USA) was used to monitor the protein separation and determine the size of the protein measured in kilo Dalton (kDa). The protein gels either stained with Commassie solution (40% MeOH, 10% acetic acid and 1% Commassie brilliant blue G250) overnight, followed by distaining with distaining solution (40% MEOH and 10% Acetic acid), or further used for Western blotting.

#### **4.9.1. Western blotting and protein detection by monoclonal antibody**

Blotting was done by Mini Trans-Blot Cell (BIO-RAD, USA). Gel used for blotting was equilibrated in transfer buffer (250 ml 1X SDS Running buffer, 200 ml MeOH and 550 ml ddw) for 10-15 minutes. Nitrocellulose membrane (Schleicher and Schuell, USA) with 0.2 µm pore size was cut to the size of the gel and soaked for few minutes in transfer buffer. A precut filter paper (3 MM) 2 per gel and fiber pads were also soaked in transfer buffer. The gel holder cassette was placed in a tray containing transfer buffer with the black side of

the holder down. For the arrangement, first fiber pad was placed on the gel holder followed by filter paper, gel, membrane (care was taken to take out any air bubble trapped between the gel and the membrane). The second filter paper and additional fiber pad were placed on it, after which the gel holder was closed. The gel cassette was then placed inside the electrode kit, with the black side of the gel cassette facing the black side of the electrode panel. A cooling unit was added to the tank and transfer buffer was poured until gel cassette was submerged and it was run at a voltage of 100V for 90 minutes. The blotted membrane was stained with Ponceau S solution (0.1 % Ponceau S (w/v) in 5% acetic acid (v/v)) (Sigma, UK) to determine protein transfer from the gel to the membrane. The membrane was then incubated in a blocking solution containing 1% low fat milk and Tris buffer saline containing Tween-20 (TBS-T) (50 mM Tris base, 150 mM NaCl adjusted to a pH of 7.4 with HCl and 1% Tween-20) over night at 4°C. For the primary antibody, Monoclonal Anti-Tyrosine hydroxylase (mouse IgG1 isotope) (Sigma, USA) in a ratio of 1:100 was added to TBS-T and 1% low fat milk. The solution was poured over the membrane and kept in room temperature on a shaker for 1.5 hrs. The membrane was then washed thrice for 10 min each with TBS-T. The secondary antibody anti-mouse Alkaline Phosphatase (KPL, USA) in a ratio of 1:500 was mixed with TBS-T and 1% low fat milk poured over the membrane and kept for 1.5 hrs in room temperature with shaking. The membrane was washed thrice with TBS-T, 1 ml each of Nitro-Blue Tetrazolium Chloride (NBT) and 5-Bromo-4-Chloro-3'-Indolyphosphate BCIP (KPL, USA) were mixed with 10 ml Tris buffer in a dark tube after which it was poured over the membrane with shaking at RT for the signal to develop. The membrane was photographed using Image Master (Pharmacia Biotech, Sweden).

#### **4.10. Results analysis**

DNA and protein sequences were analyzed using BioEdit software program version 7.0.9. Sigma plot 2000 software was used for graphic description of the results. qBase version 1.3.5 software was also used for analyzing the RT-PCR results.

## **5.0. Results**

### **5.1. Isolation of Tyrosine decarboxylase and Polyphenol oxidase genes**

Tyrosine hydroxylase (TH), tyrosine decarboxylase (Tyrdec), and polyphenol oxidase (PPO) are enzymes taking part in phenol modification and possibly in that of tyrosine. The genes of these enzymes were cloned to help understand their role in dopamine production in banana fruit.

Degenerate primers based on mammalian cells used for cloning TH are described in Table 1. However the attempts using various combinations of degenerate primers have failed. A mixture of cDNA from other six cultivars was also used to clone the TH, with no success. Using the same primers we were also unsuccessful in cloning the gene from *Mucuna* plants, known to have high levels of L-DOPA (Buckles, 1995). cDNA for these trials was prepared from *Mucuna* seeds (old and new), leaves (early and late stage) stem and root .

Nevertheless, we were able to clone the gene of Tyrdec, another enzyme that participate in the biosynthesis pathway of catecholamine in plants (Fig. 3). Tyrdec is responsible for converting tyrosine to tyramine. The gene encoding Tyrdec was cloned from Cavendish banana cultivar Grand Nain using degenerate primers (described in Table 1) prepared from conserved regions around the putative pyridoxal phosphate (PLP) binding region of several plants. The partial cloned transcript (208 putative aa) shares about 70% homology with other Tyrdec genes from other plant families (Fig. 5).



```

Try Dec. Cavendish Banana -----GVTHWQSPNYFAYF 14
Try Dec. Papaver somniferum SQANPGSQ-QTLPETAPNHSESIETILQDVQNDIIPGITHWQSPNYFAYF 97
Try Dec. Parsley SQVSPGYLREILPESAPYNPESELETILQDVQTKIIPGITHWQSPNFFAYF 100
Try Dec. Arabidopsis thaliana SQVQPGYLKLLPDSAPDHPETLDQVLDVRAKILPGVTHWQSPFFAYF 91
Tyr Dec. Oryza sativa SQVQPGYLKEVLPDSAPRQPDLDLDFDDIQQKIIPGVTHWQSPNYFAYF 96
Clustal Consensus *:*****.:***:

Try Dec. Cavendish Banana PSSGSVAGFLGEMLSVGFNVVGFNWMSSPAATELETIVMDWLKMLNLPK 64
Try Dec. Papaver somniferum PSSGSVAGFLGEMLSVGFNVVGFNWMSSPAATELESIVMNLWGQMLNLPK 147
Try Dec. Parsley PSSGSTAGFLGEMLSVGFNVVGFNWMVSPAATELENVVDWFGKMLQLPK 150
Try Dec. Arabidopsis thaliana PSNSSVAGFLGEMLSAGLGVGFVSWVSPAATELEMIVLDWVAKLLNLPK 141
Tyr Dec. Oryza sativa PSNSSTAGFLGEMLSAAFNIVGFVSWITSPAATELEVIVLDWFAKMLQLPS 146
Clustal Consensus **.*.*****. :.:***.*: ***** :* :*.:*:** 50

Try Dec. Cavendish Banana PFLFSG-----GGGGVLQGTTCGILCTVTAARDKVLNKGDRDRIGDLV 108
Try Dec. Papaver somniferum SFLFSSDDNAGSSGGGVLQGTTCGAILCTLTASRDKMLNKGRENINKLV 197
Try Dec. Parsley SFLFSG-----GGGGVLQGTTCGAILCTLVAARDKNLRQHGMNIGKLV 194
Try Dec. Arabidopsis thaliana QFMSKG-----NGGGVIQGSASEAVLVVLIARDKVLRSVGNNALEKLV 185
Tyr Dec. Oryza sativa QFLSTA-----LGGGVIQGTASEAVLVALLAARDRALKKHGKHSLEKLV 190
Clustal Consensus *: .. ****:*:.*:* : :*:*: *.. * : : **

Try Dec. Cavendish Banana VYCSdqTHCALKKAQIAGIHPDNIRALPthQRDAFGLCPETLGAALAAD 158
Try Dec. Papaver somniferum VYASDqTHCALQKAAQIAGINPKNFRAIATSKATDFGLSPQALLSTILAD 247
Try Dec. Parsley VYCSdqTHSALQKAAQIAGIDPKNFRAIETTKSSNFKLCBKRLESAILYD 244
Try Dec. Arabidopsis thaliana VYSSDqTHSALQKACQIAGIHPENCRVLTDSSTNYALRPESLQEAVSRD 235
Tyr Dec. Oryza sativa VYASDqTHSALQKACQIAGIFSENVVRVVIADCNKNYAVAPEAVSEALSID 240
Clustal Consensus **.******.*:***:**** ..* *.: : : :*: : : : * 104

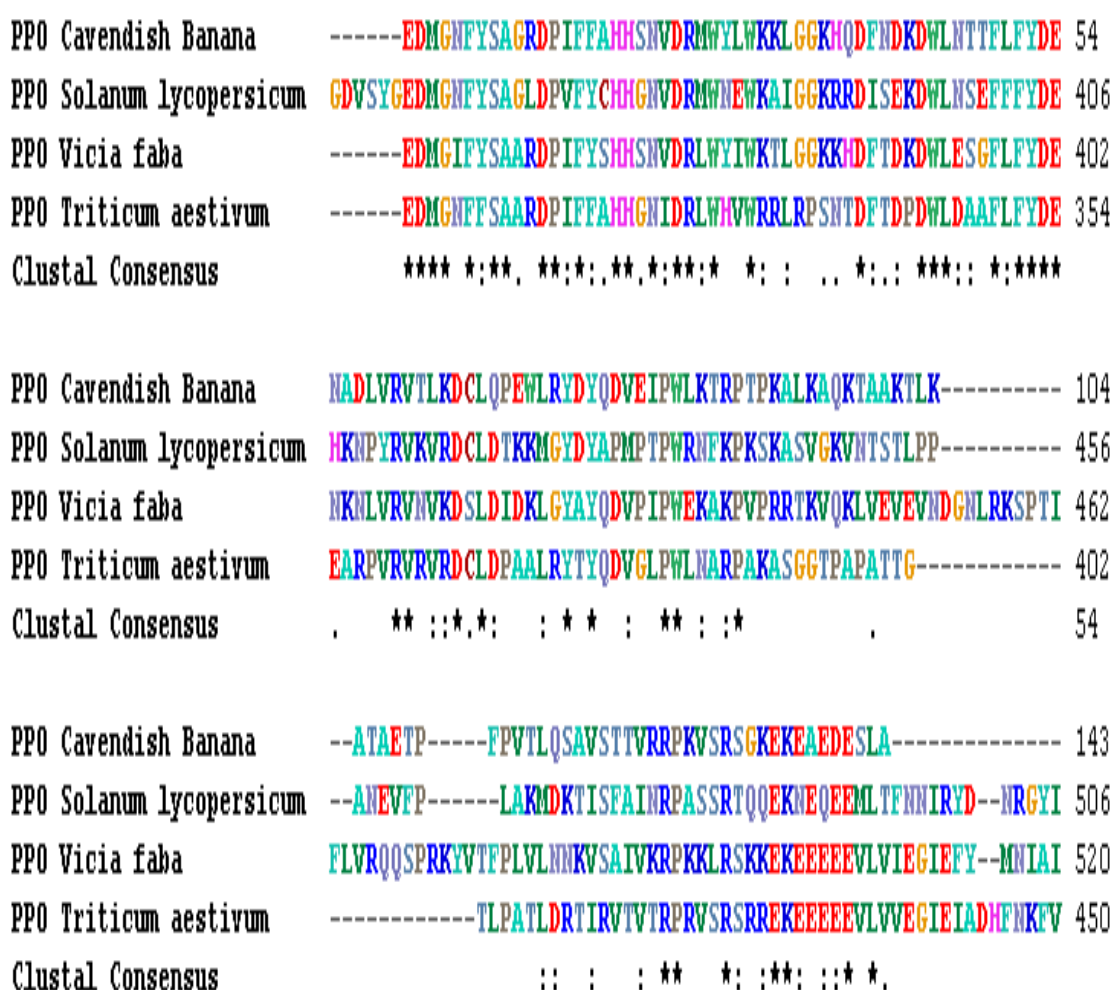
Try Dec. Cavendish Banana VSEGLVPLYLCSTVGTSSSTAVDPLRALCEVAEYDIWVHVDAAYAGSAC 208
Try Dec. Papaver somniferum IESGLVPLFLCATVGTSSSTAVDPIGPICEVAKQFGIWHVHVDAAYAGSAC 297
Try Dec. Parsley LQNGLIPLYLCATVGTSSSTVDPALTEVAKKYDLWVHVDAAYAGSAC 294
Try Dec. Arabidopsis thaliana LEAGLIPFFLCANVGTSSSTAVDPLAALGKIANSNGIWFHVDAAYAGSAC 285
Tyr Dec. Oryza sativa LSSGLIPFFICATVGTSSSAVDPLPELGQIAKSNMWFHIDAAYAGSAC 290
Clustal Consensus :. **.*:.*:.******:***: * :.* . :.*.****** 143

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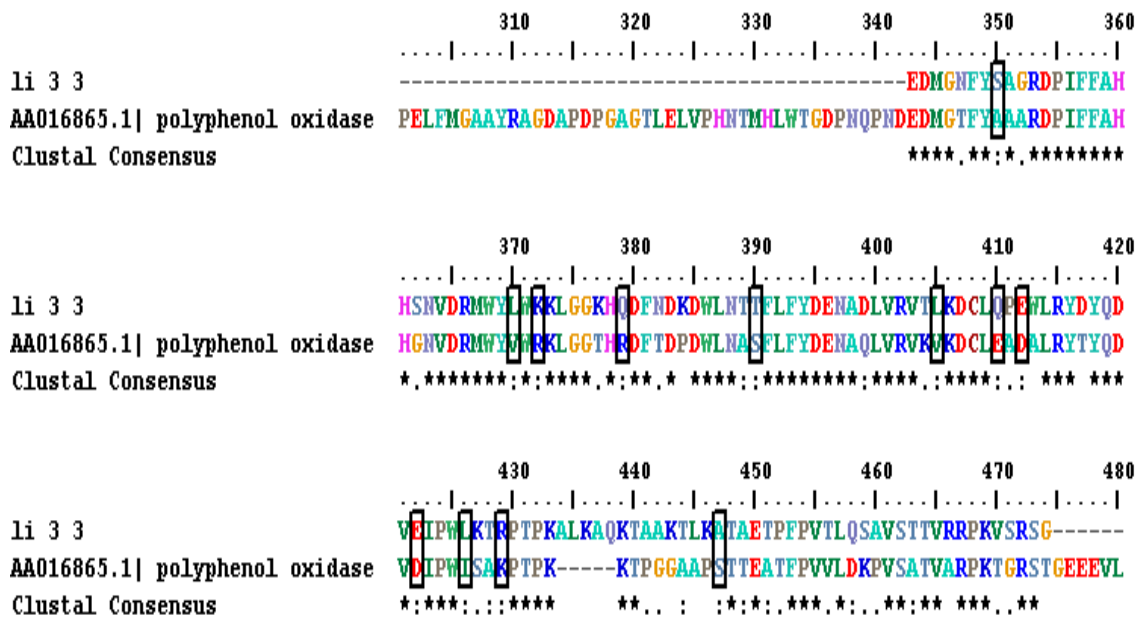
**Fig. 5:** Isolation of tyrosine decarboxylase (Tyrdec) gene from Cavendish banana sub variety Grand Nain. A putative proteins alignment of Trydec sequences isolated from banana fruit with sequences from other species used for the primer design; *Papaver somniferum* (AAA97535.1), *Petroselinum crispum* (AAA33863.1), and *Arabidopsis thaliana* (AAM20115.1) in addition with sequence of *Oryza sativa* (BAD30830.1), a gene that was not used for the primer design. The fragment sequence was deposited in [www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST) (EU880276).

Polyphenol oxidase an enzyme responsible for blackening in banana and which uses dopamine as one of its substrates has been extensively studied and the gene cloned in other plants. Degenerate primers complimentary to conserved regions around the putative copper-binding residues of PPO genes were designed based on tyrosinase enzymes that mainly show tyrosine

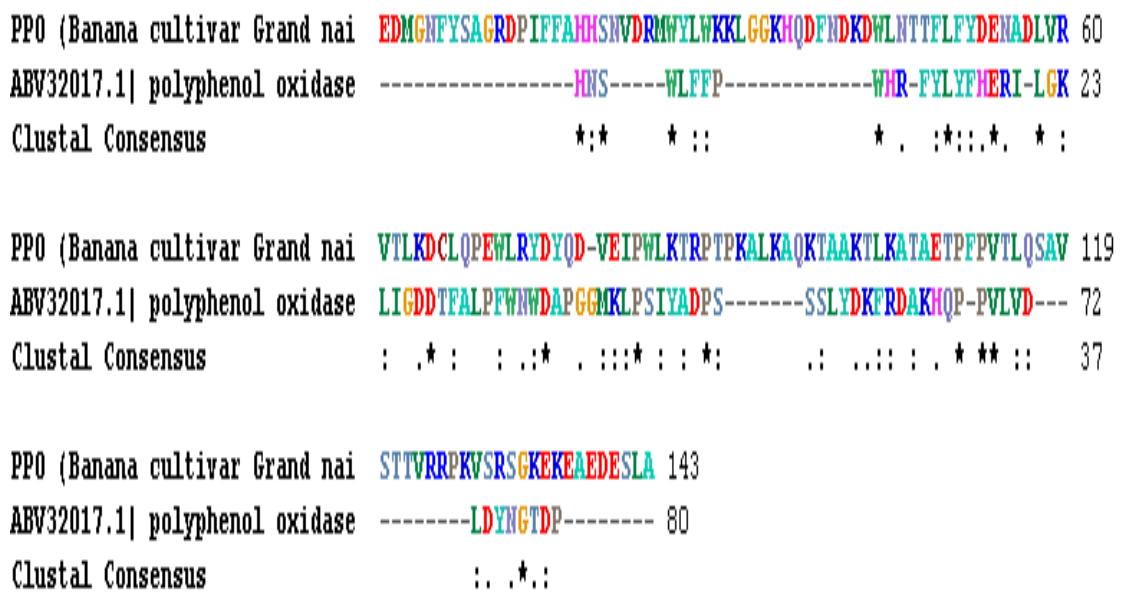
hydroxylase activity ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)). Primers are described in Table 1. The cloned fragment was of 141 amino acids (aa) and shared a very low homology (10%) with PPO from plants used for primer preparation (Fig. 6) but shared about 64% similarity with PPO from *Ananas comosus* (Fig. 7). Interestingly, an alignment of the putative amino acid sequence of the cloned PPO gene shows with another PPO gene cloned from banana deposited in the Genebank during the course of this study (Promyou and Ketsa, 2007), shows very low similarity (Fig. 8).



**Fig. 6:** Isolation of polyphenol oxidase (PPO) gene from Cavendish banana sub variety Grand Nain. A putative protein alignment of PPO isolated from banana fruit and sequences used for primer design; *Solanum lycopersicum* (CAA78296.1), *Vicia faba* (CAA77764.1) and *Triticum aestivum* (AAS00454.1). The gene sequence was deposited in [www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST) (EU880277).



**Fig. 7:** Alignment of putative cloned PPO gene from Cavendish banana Grand Nain with PPO from *Ananas comosus* (AA016865.1).

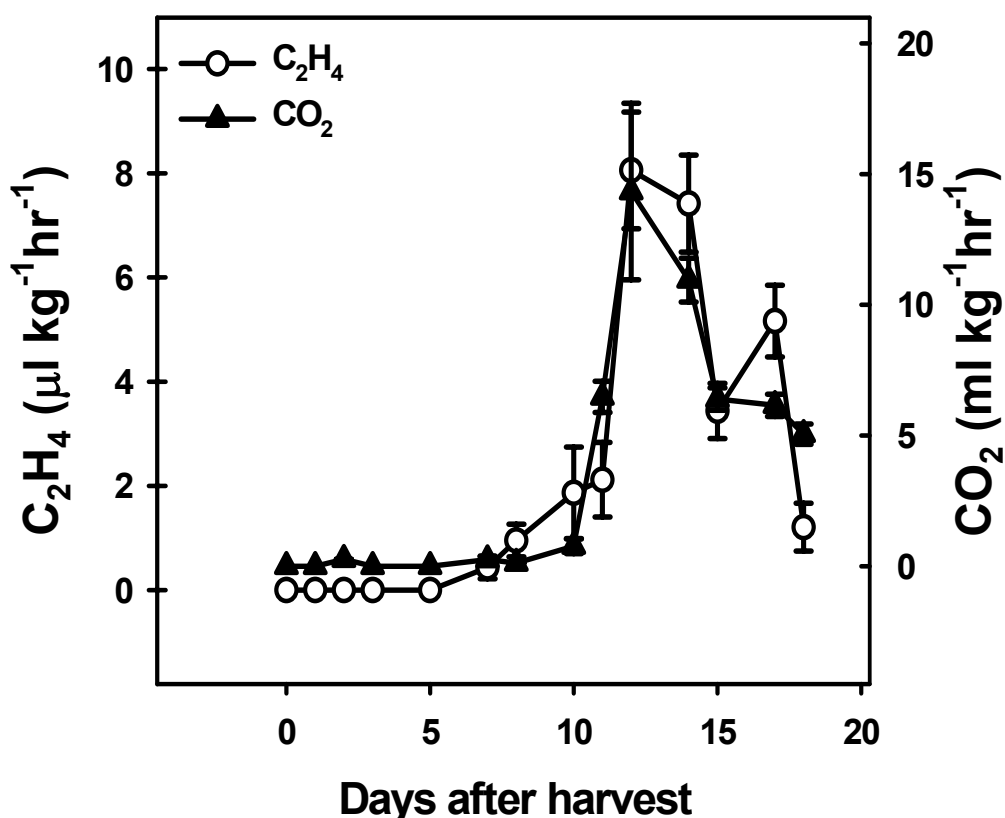


**Fig. 8:** Alignment of a putative amino acid of cloned PPO gene from Cavendish banana Grand Nain cloned in this study with PPO cloned from banana (*Musa acuminata*) in previous study (ABV32017.1) (Promyou and Ketsa, 2007).

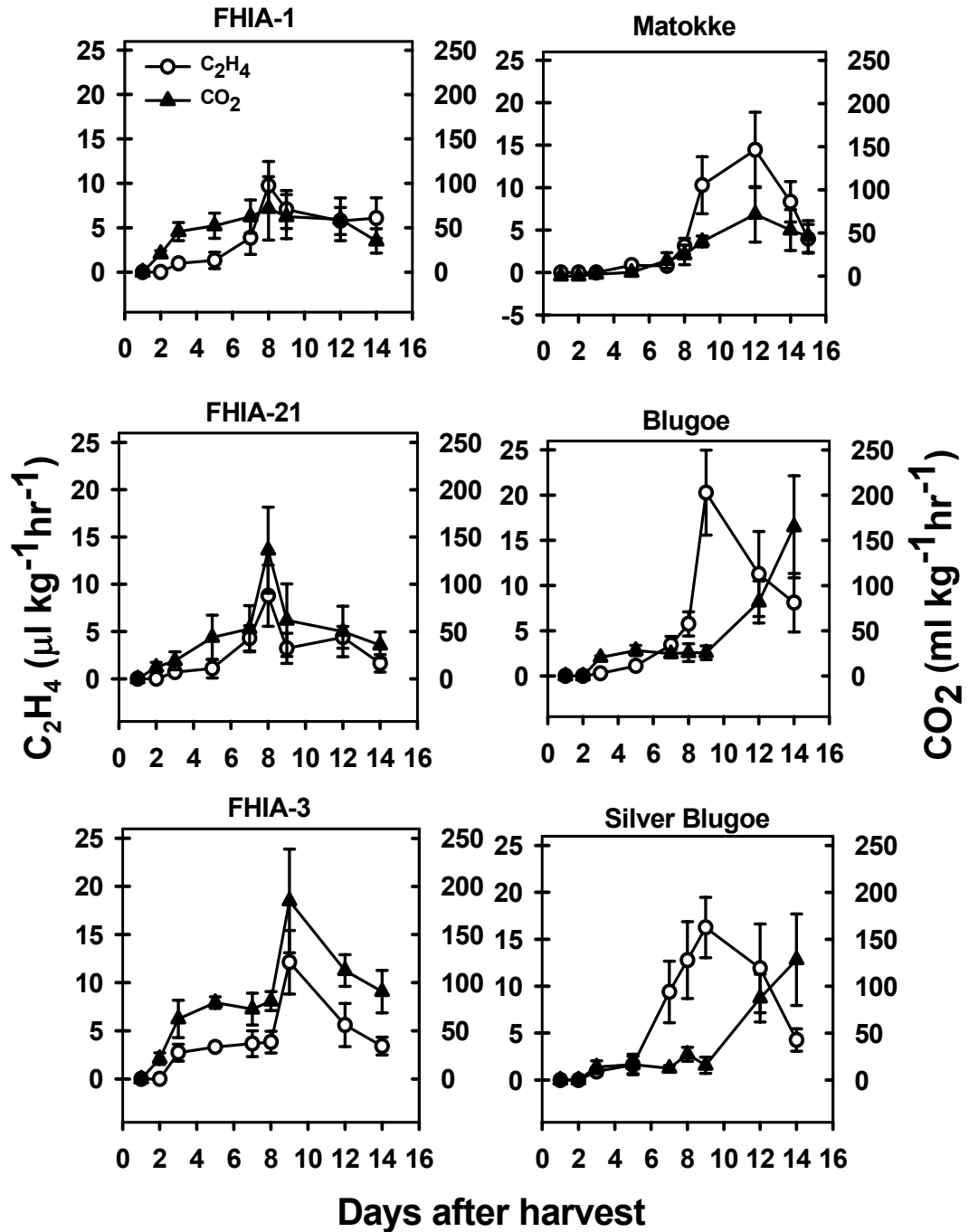
## 5.2. Determination of ethylene, carbon dioxide production and ripening parameters of banana after harvest

Grand Nain and six other cultivars (FHIA-1, FHIA-3, FHIA-21, Matokke, Blugoe and Silver Blugoe) were used for expression analysis of gene cloned that might be involved in the dopamine biosynthesis pathway. For this purpose the ripening of these cultivars was described by following ethylene ( $C_2H_4$ ), carbon dioxide ( $CO_2$ ) production, firmness, color and total soluble solids (TSS).

Increase in ethylene production was observed 12 days after harvest (DAH) for the cultivar Grand Nain (Fig. 9). The  $CO_2$  and  $C_2H_4$  productions in the fruit increased at the same time and the rate of increase was very similar. The increase was followed by a decrease which correlated with over ripening and appearance of black spots.



**Fig. 9:** Ethylene and carbon dioxide production in whole fruit of Cavendish banana sub variety Grand Nain during ripening. The parameters were determined immediately following harvest and at consecutive days during storage at 20°C and 75% relative humidity (RH).



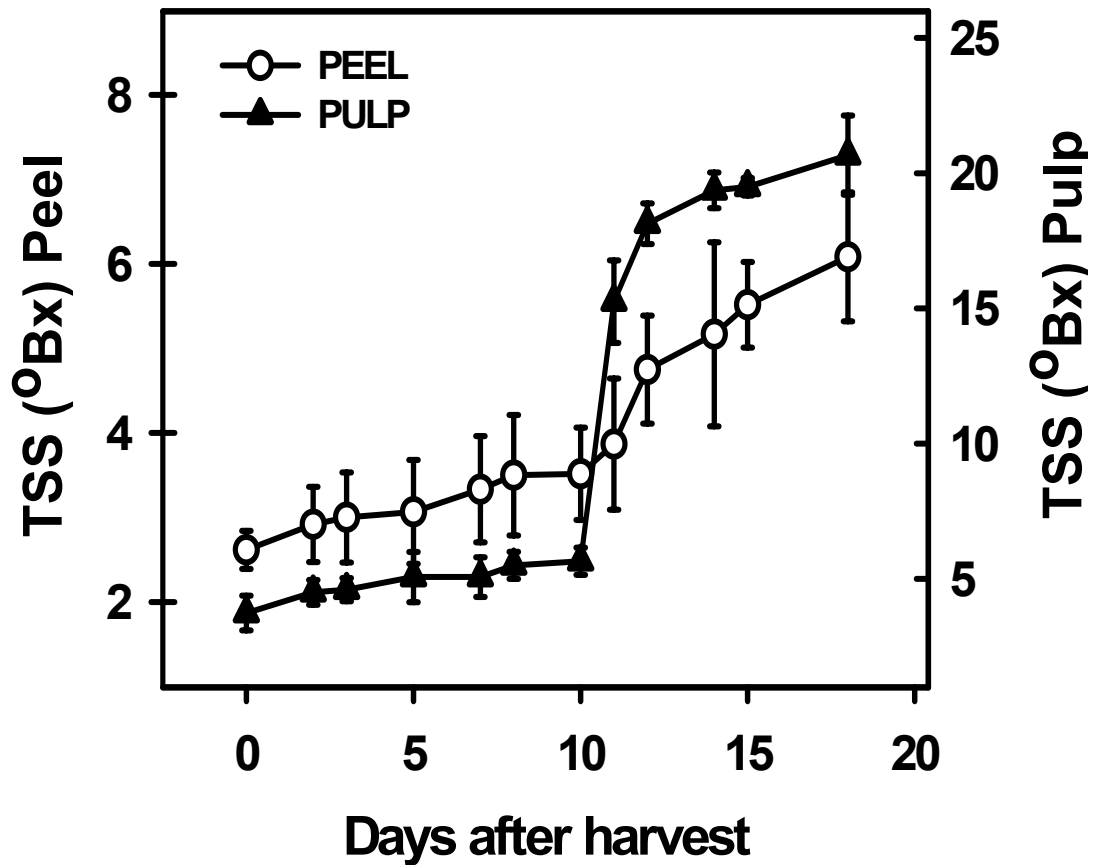
**Fig. 10:** Ethylene and carbon dioxide production in whole fruit of six banana cultivars after harvest.

Ethylene and carbon dioxide levels were also determined for the other six banana cultivars. In most cultivars the ethylene peak appeared 10 days after harvest however, in the cultivars FHIA-1 and FHIA-21 it appeared earlier on the eighth day and in Matokke at the twelve day. In three cultivars (FHIA-1, FHIA-21 and FHIA-3),  $C_2H_4$  production coincided with  $CO_2$  production. However, a lag occurred in cultivars (Matokke, Blugoe and Silver Blugoe) but

increase in  $C_2H_4$  and  $CO_2$  production occurred the same time in Matokke while  $C_2H_4$  increase preceded  $CO_2$  increase in Blugoe and Silver Blugoe (Fig. 10). Ethylene and carbon dioxide concentration in the fruit decreased after the climacteric peak in all samples except Blugoe and Silver Blugoe in which the high levels of  $CO_2$  at the climacteric peak was sustained until full ripening: yellow fruit with appearance of brown spot on the skin.

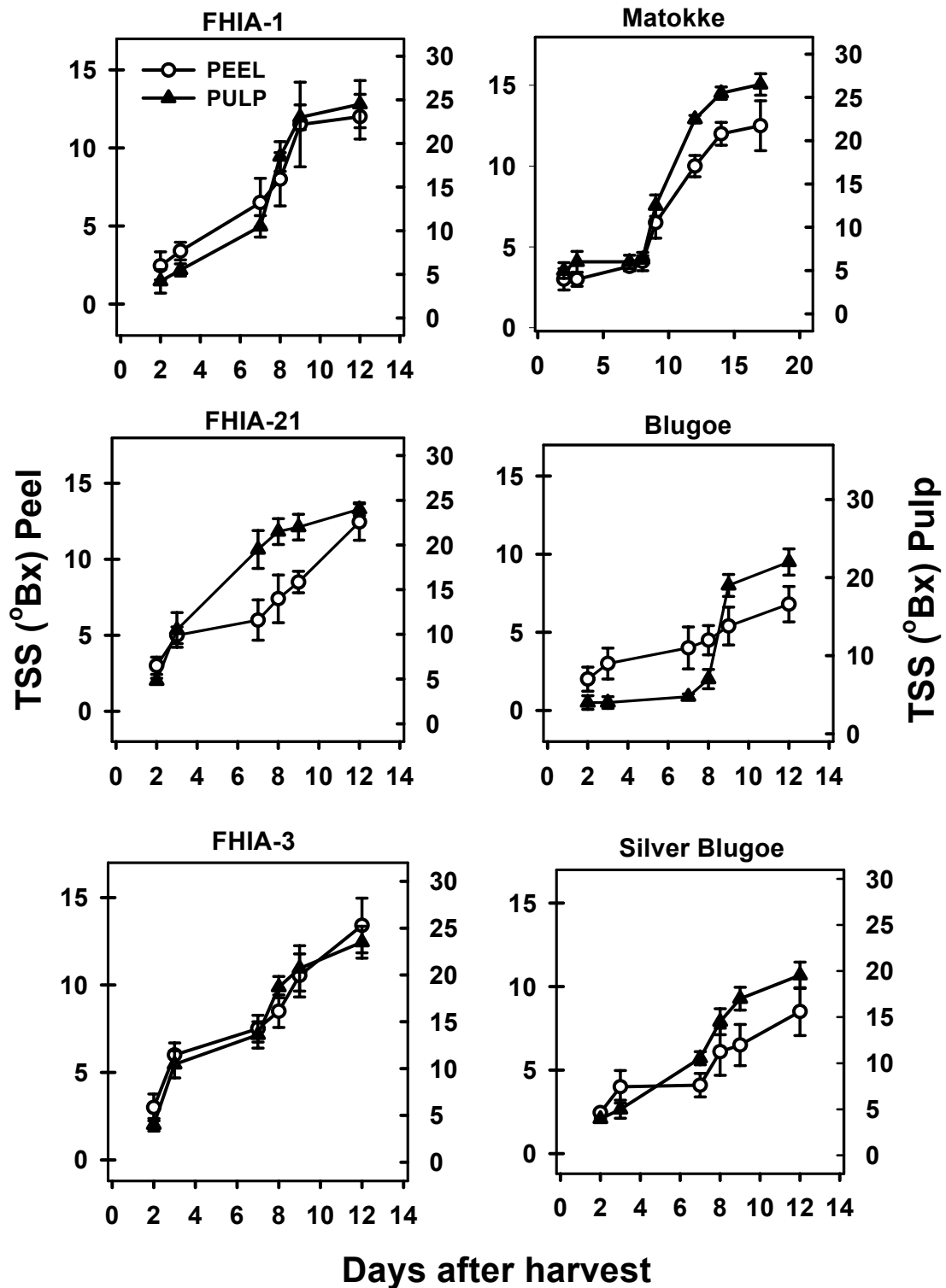
Total soluble solids (TSS) were determined for Grand Nain and in the additional six cultivars at various stages after harvest (Figs. 11 and 12). It was observed in Grand Nain that TSS concentration increased during ripening in the pulp and peel but the levels in pulp increased at a higher rate and reached 3.3 times higher levels than that in peel. The highest TSS level in the pulp in Grand Nain occurred at day 12 after harvest (Fig. 11) concomitantly with ethylene increase (Fig. 9).

A gradual increase in TSS levels was observed in both the peel and pulp of cultivars (FHIA-1, FHIA-21 and FHIA-3) analyzed. Lag in TSS levels was observed for Matokke and Blugoe in both the peel and pulp. A lag phase in the accumulation of TSS was observed in the peel of Silver Blugoe while the levels in the pulp increased gradually (Fig. 12). Only in two cultivars (Matokke and Blugoe) did the TSS increase sharply after a lag period parallel to  $C_2H_4$  production (Figs. 10 and 12). In Silver Blugoe there was no correlation between the gradual increase in TSS in the pulp and the lag in ethylene production, however, the TSS increase in the other three cultivars was gradual in parallel to the gradual increase in ethylene before the peak of  $C_2H_4$ .



**Fig. 11:** Total soluble solids (TSS) concentration during ripening of Cavendish banana sub variety Grand Nain. Brix ( $^{\circ}\text{Bx}$ ) was determined in peel and pulp at different days after harvest.

Ripening was followed by monitoring the color development and firmness of the fruit (Figs. 13 and 14). In the cultivar Gran Nain a lag in color was observed however there was a gradual decrease in firmness (Fig. 13). A sharp decrease in firmness at 12 DAH harvest correlated with increase in  $\text{C}_2\text{H}_4$  production (Fig. 13).

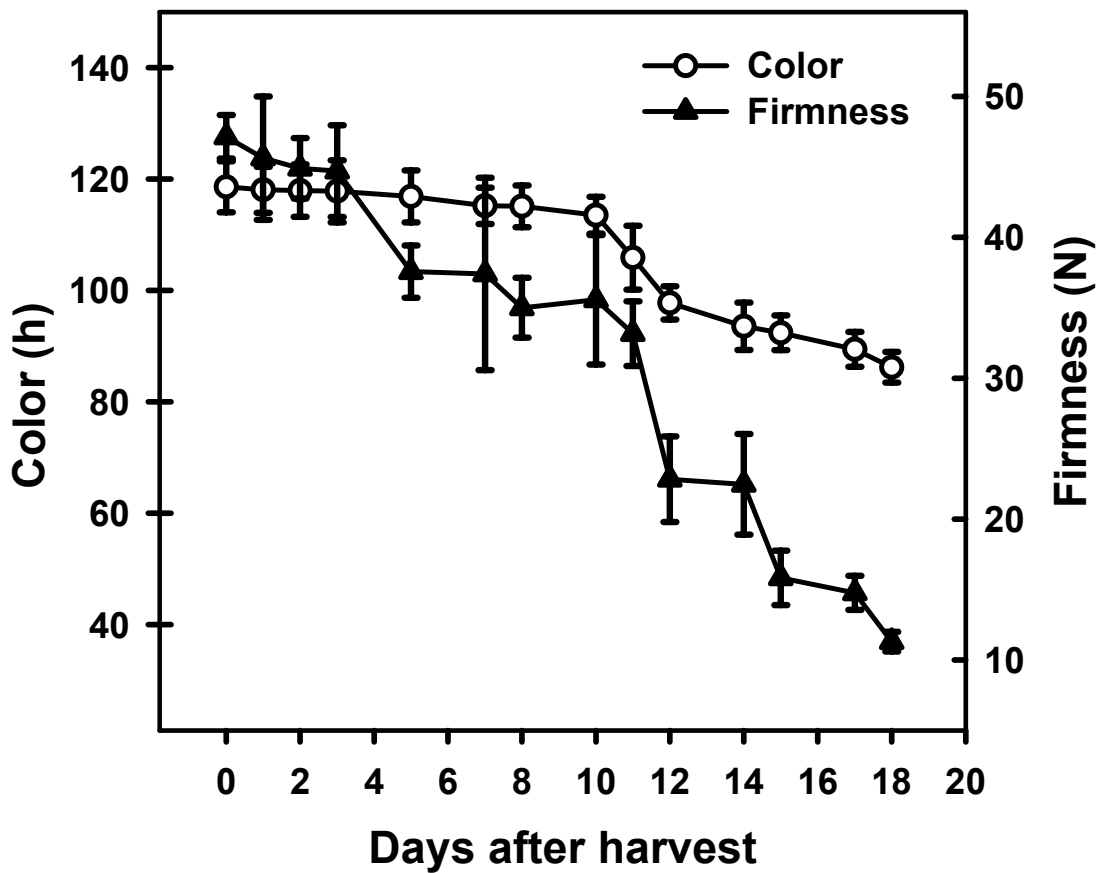


**Fig. 12:** Total soluble solids (TSS) concentration during ripening of six banana cultivars. Samples used for TSS are same from ethylene production determination

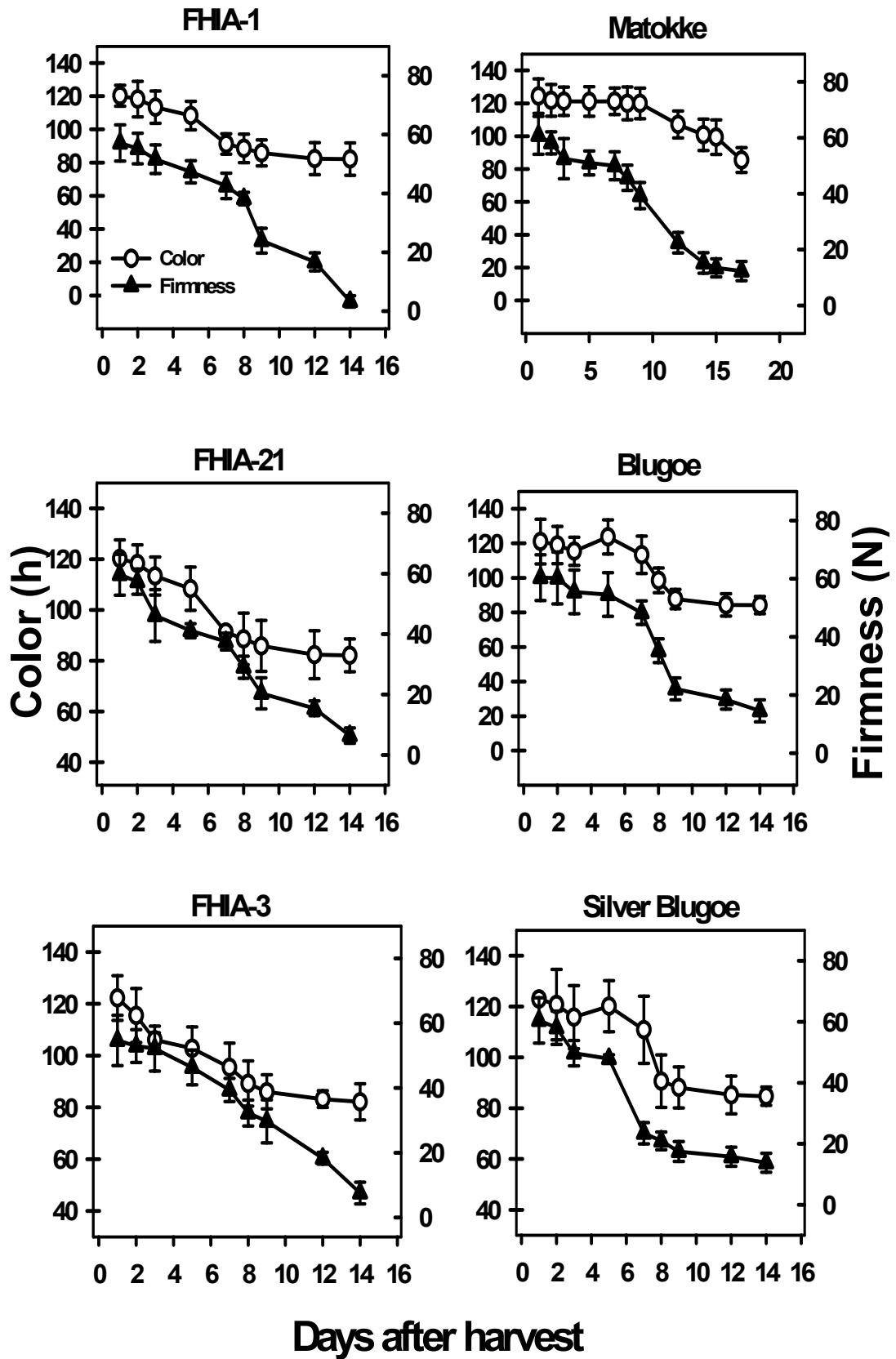
In three cultivars (FHIA-1, FHIA-21, FHIA-3) there was gradual decrease in color (Fig. 14). In Matokke, Blugoe and Silver Blugoe there was a lag in color development and it correlated with the lag in ethylene production (Figs. 10



and 14). The gradual decrease in color of the other cultivars (FHIA-1, FHIA-21 and FHIA-3) preceded the ethylene peak but coincided with a slow increase in ethylene and CO<sub>2</sub> production. There was a gradual decrease in firmness for all six cultivars analyzed (Fig. 14). Indicating that in some cultivars it coincided with the gradual increase in ethylene production (FHIA-1, FHIA-21, FHIA-3) however, in others, (Matokke, Blugoe and Silver Blugoe) it preceded the sharp increase in ethylene production.



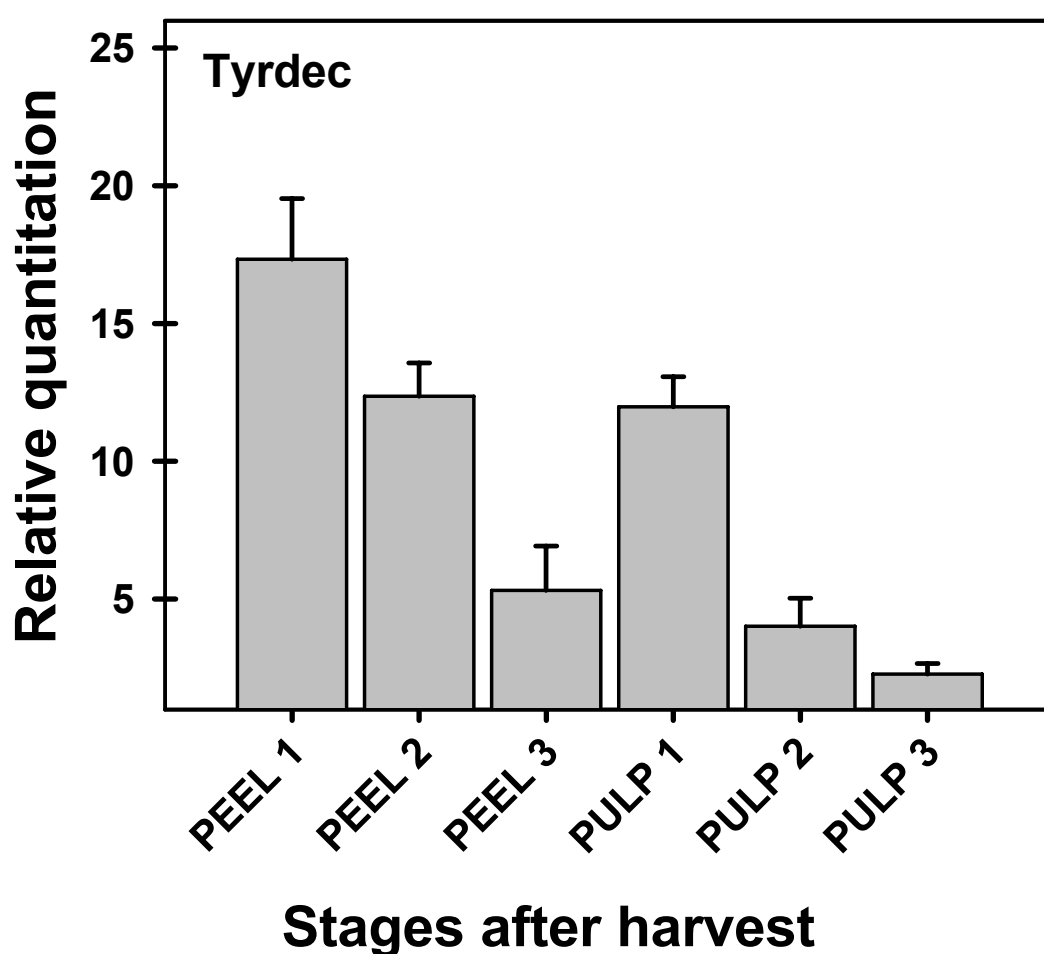
**Fig. 13:** Ripening parameters determined for Cavendish banana sub variety Grand Nain. Parameters determined were peel color (hue o) and firmness (N) of the whole fruit. Results are of a representative experiment, and represent an average of three replicates  $\pm$  SE.



**Fig. 14:** Ripening parameters determined for six banana cultivars. Parameters determined were peel color (hue o) and firmness (N) of the whole fruit.

### 5.3. Expression of tyrosine decarboxylase gene in banana fruit

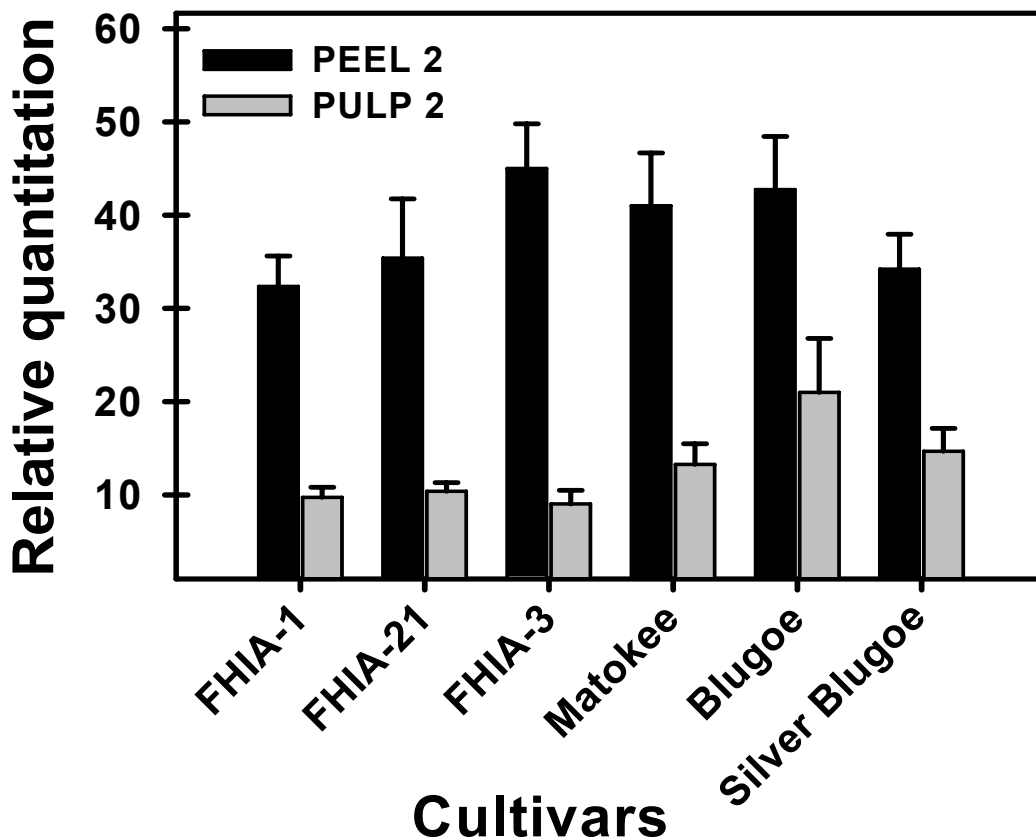
The expression level of tyrosine decarboxylase (Tyrdec) gene was determined in peel and pulp of the banana cultivar Grand Nain. Three ripening stages 1, 2 and 3 representing green (pre-climacteric), yellow with green edges (climacteric) and yellow with brown spot (post-climacteric) respectively were used for the expression level determination. The gene was expressed at all stages of ripening, but the level in peel was higher than in pulp in all stages (Fig. 15). The gene expression was highest in green (pre-climacteric) stage of the fruit in both tissues (peel and pulp) and the expression decreased with progression of ripening (Fig. 15).



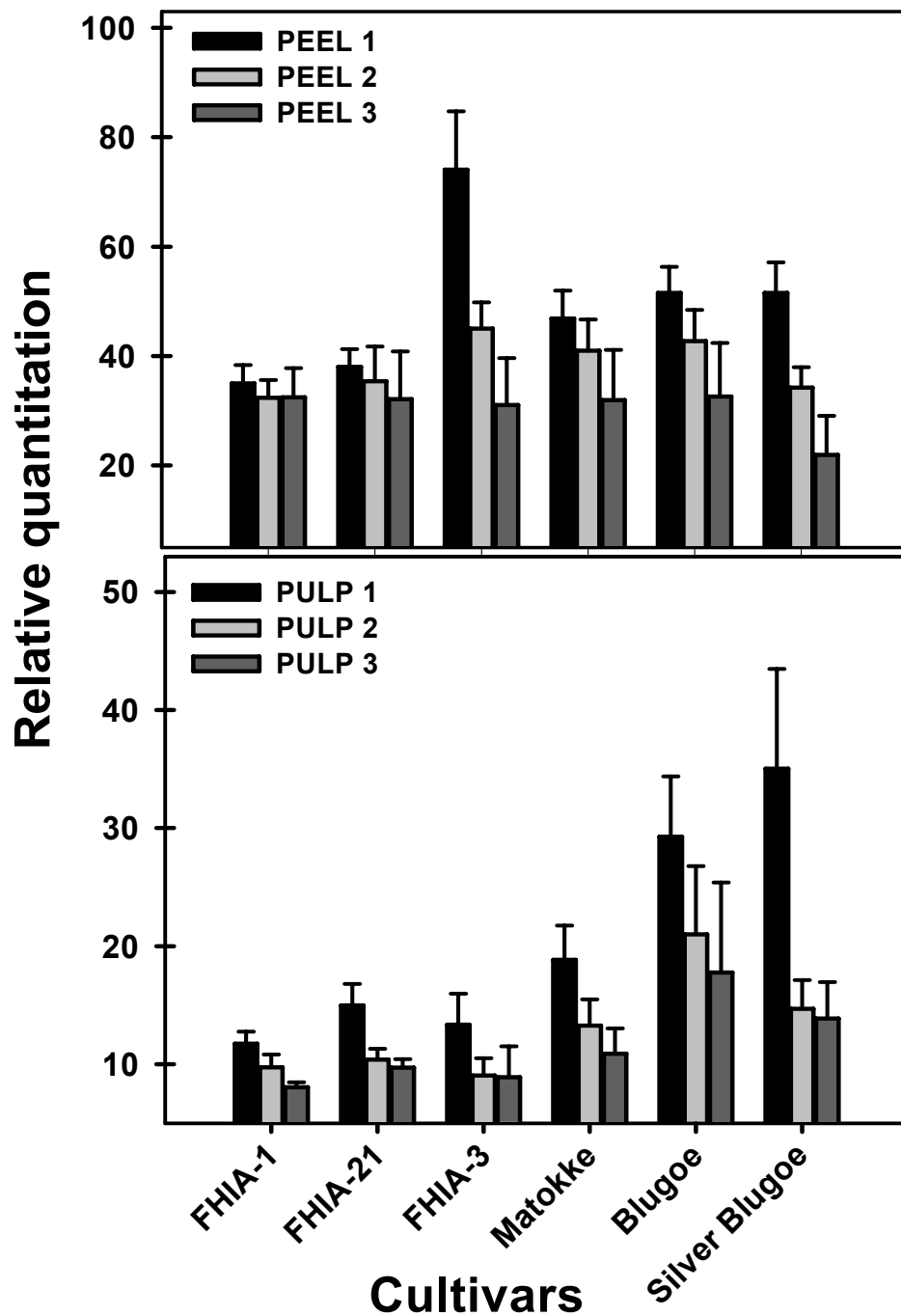
**Fig. 15:** Expression patterns of tyrosine decarboxylase (Tyrdec) gene in Cavendish banana sub variety Grand Nain after harvest. Samples were prepared from Peel (PE) and Pulp (PU) and numbers 1, 2, 3 representing stages after harvest. 1- Green stage, 2- yellow with green edges and 3- yellow with brown spot. Transcript expression was determined by RT-PCR as relative quantization. Results are of a representative experiment, and represent an average of three replicates  $\pm$  SE.

The gene expression was determined also for the six banana cultivars separately for the peel and pulp at all stages as above (Fig. 17). For clarity the expression at climacteric stage is presented (Fig. 16). The gene expression was highest in the peel for all cultivars at the climacteric stage (Fig. 16). However, no significant difference was observed for the expression in the pulp in all the cultivars.

Taking the three stages of ripening into consideration in peel of all cultivars, it was observed that the gene transcript was expressed in all stages in the peel. The expression was observed to decrease gradually with the advancement of ripening for all cultivars except FHIA-1 and FHIA-21 which had the expression at all stages very similar. The peel of FHIA-3 had a higher expression of the gene compared with the other cultivars at pre-climacteric. The gene expression in the pulp was also observed to exhibit a gradual decrease trend in all cultivars (Fig. 17), and the highest levels observed for Blugoe and Silver Blugoe at pre-climacteric stage



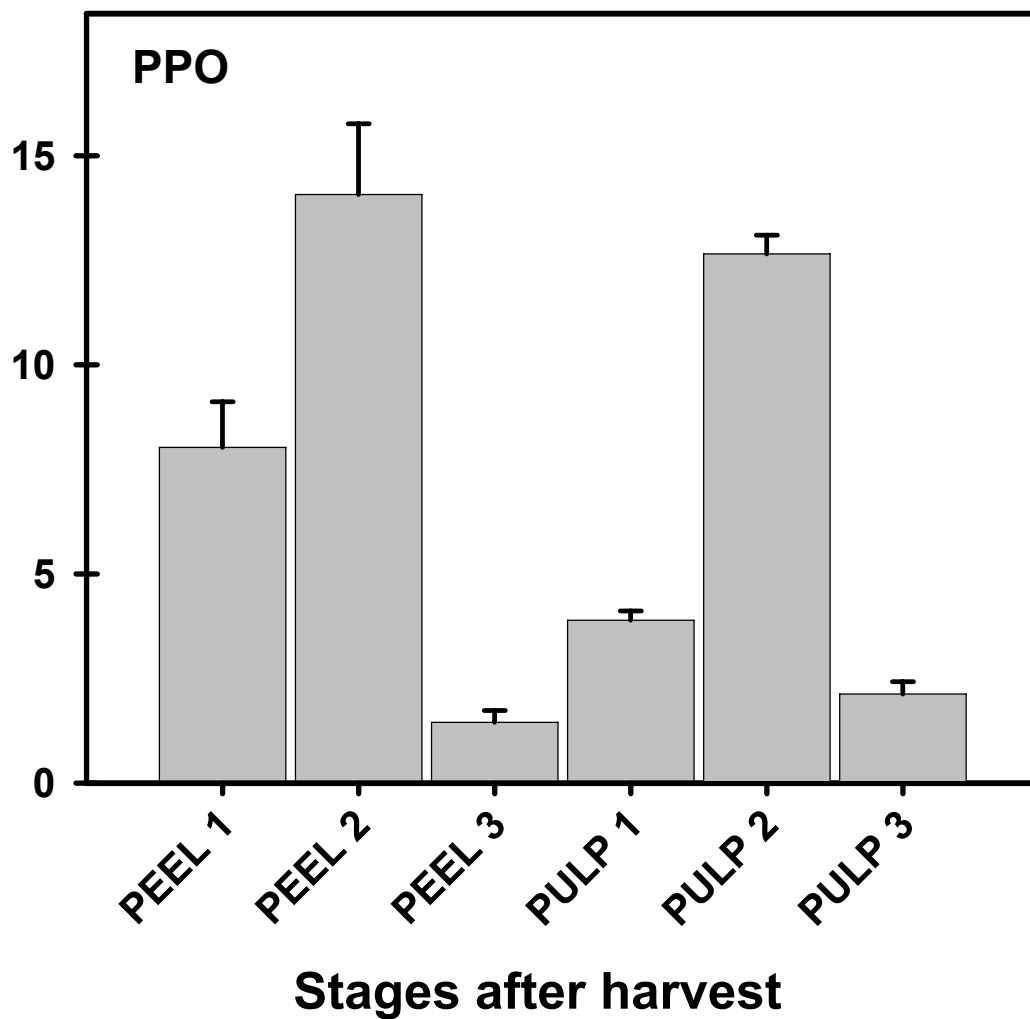
**Fig. 16:** Comparison of Tyrdec gene in peel and pulp of six banana cultivars during the ethylene climacteric peak (stage 2).



**Fig. 17:** Expression patterns of tyrosine decarboxylase gene in tissue of six banana cultivars after harvest.

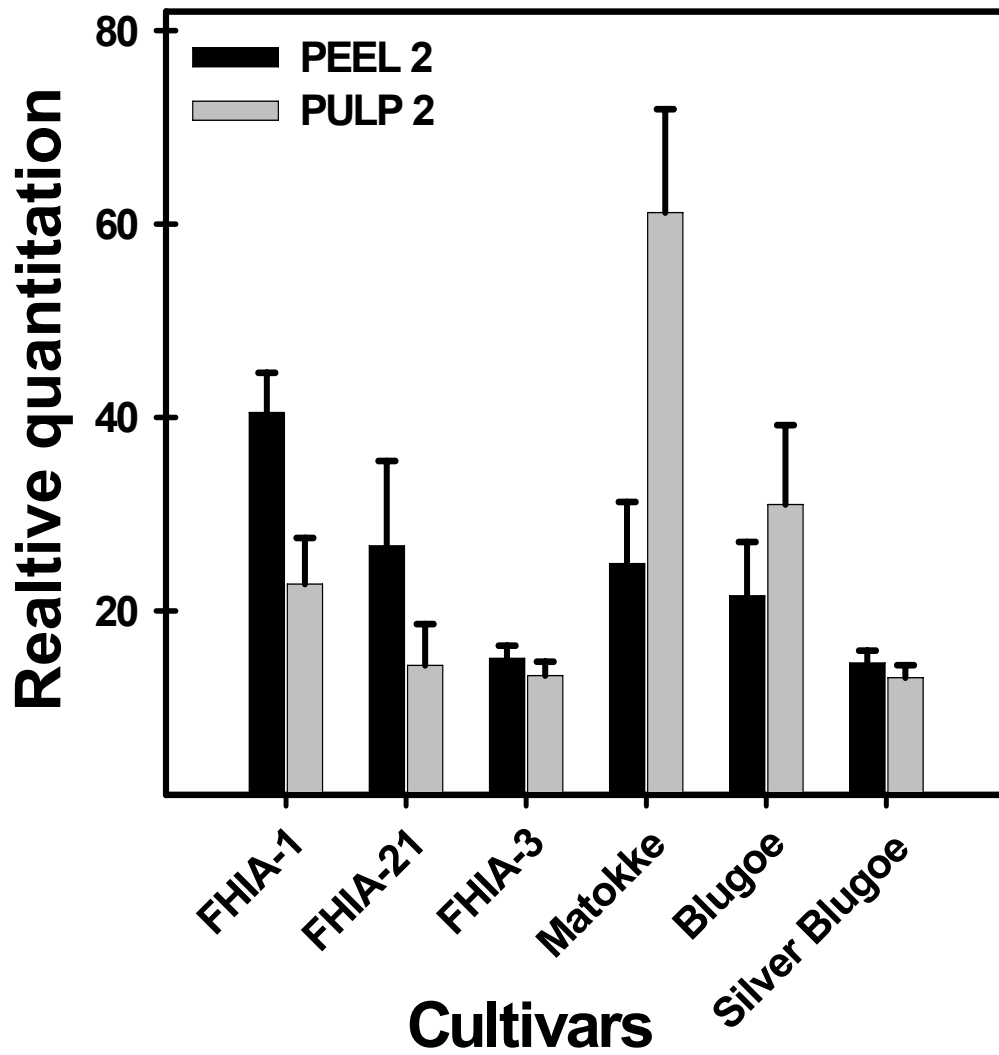
#### 5.4. Expression of polyphenol oxidase gene in banana fruit

The expression of the PPO gene increased at climacteric- yellow with green edges corresponding in both the peel and the pulp of Grand Nain (Fig. 18). The level of the PPO transcript was only slightly higher in the peel than in the pulp of the green stage (PEEL 1 versus PULP 1).

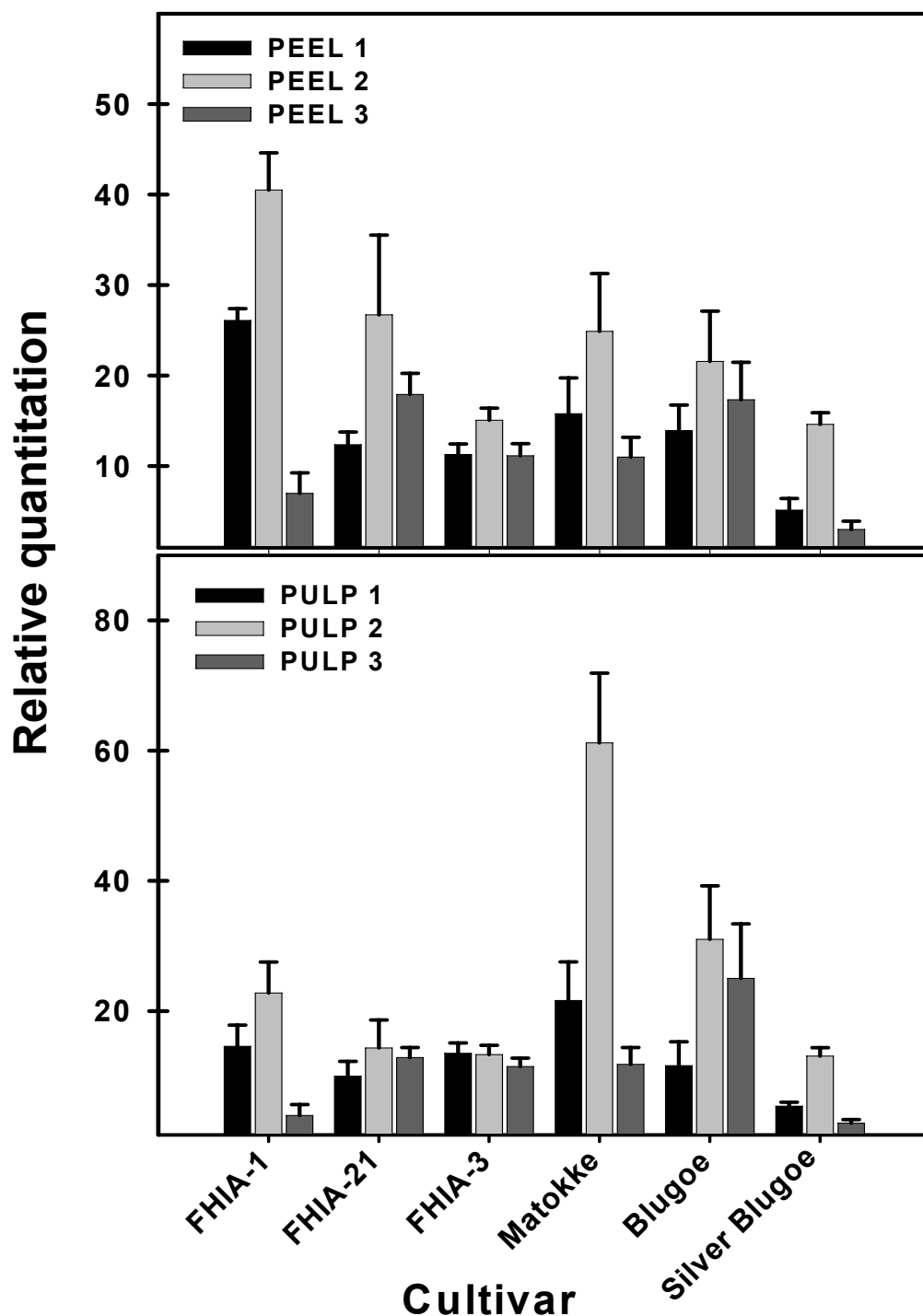


**Fig. 18:** Expression patterns of polyphenol oxidase (PPO) gene in peel and pulp of Cavendish banana sub variety Grand Nain after harvest.

Comparing the expression level of PPO at climacteric stage for all six banana cultivars is presented in (Fig. 19). This data was obtained from Fig. 20. It was observed that the gene was expressed higher in the peel for FHIA-1 and FHIA-21. However in cultivars FHIA-3 and Sliver Blugoe, the gene expression in the peel was similar to that in pulp. However, in Matokke and Silver Blugoe the gene was expressed higher in the pulp than in the peel. The pulp of Matokke had the highest expression of the gene in comparison to peel and pulp of the other five cultivars at the climacteric stage (Fig. 19).



**Fig. 19:** Comparison of PPO gene in peel and pulp of six banana cultivars during the ethylene climacteric peak (stage 2).



**Fig. 20:** Expression patterns of PPO gene in six banana cultivars after harvest.

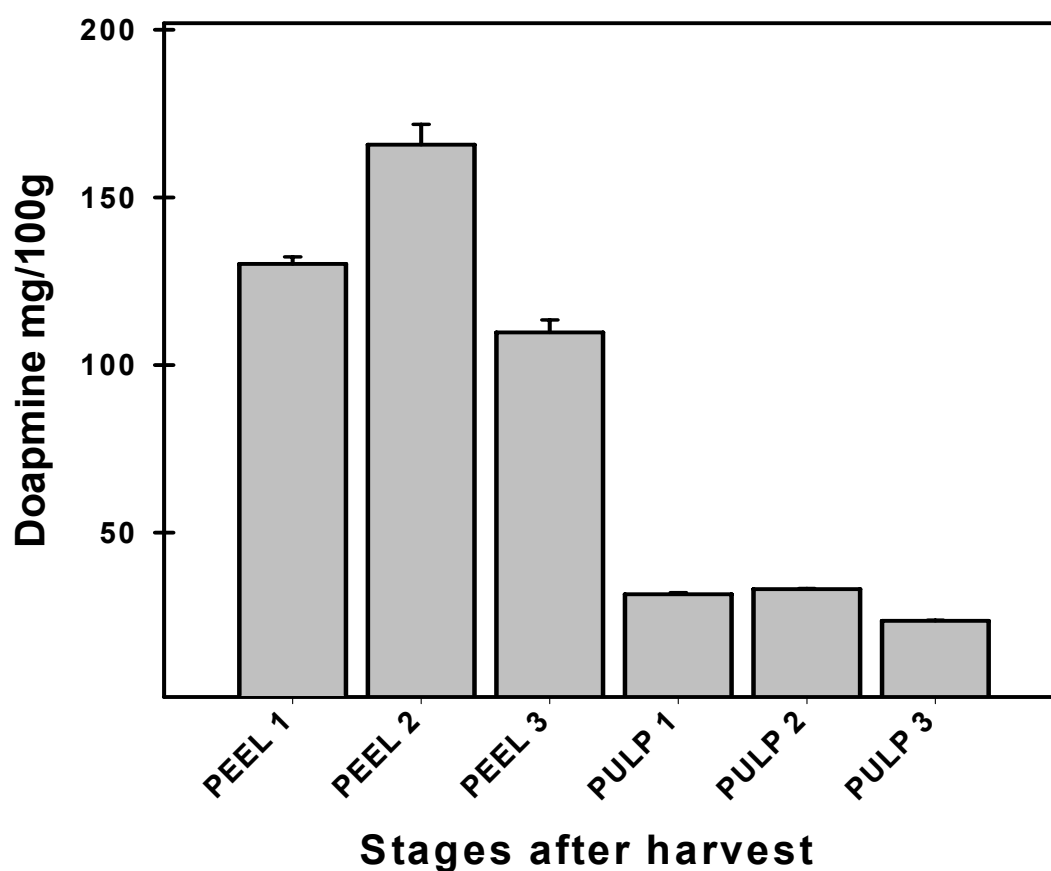
The gene was expressed in all 3 stages in the peel, and the highest expression appeared in the climacteric stage in all the cultivars. The highest expression in the peel appeared at green fruit of FHIA-1 (Fig. 20). In the pulp the gene was expressed in all three stages with higher expression of the gene



in climacteric stage for all cultivars except FHIA-3 and FHIA-21 which had similar expression in all stages and for Blugoe which had similar expression in 2 and 3 being the same (Fig. 20).

### 5.5. Accumulation of dopamine in banana fruit

The concentration of dopamine in three different stages during ripening of banana cultivar Grand Nain was determined with HPLC (Fig. 21). Dopamine was present in all the stages of ripening in peel and pulp. However the amount in the pulp was lower in comparison to the amount in the peel.



**Fig. 21:** HPLC determination of dopamine concentration in peel and pulp of Cavendish banana sub variety Grand Nain during three ripening stages of the fruit. PEEL and PULP 1, 2 and 3 represents green (pre-climacteric), yellow with green edges (climacteric) and yellow with brown spot (post-climacteric).

The amount of dopamine was higher in climacteric stage - yellow with green edges in the peel and the lowest in post-climacteric stage- yellow with brown spot in both peel and pulp.

It was detected that there was higher accumulation of dopamine in the climacteric stage corresponding to the climacteric peak of ethylene (Fig. 9), higher than pre-climacteric-green banana and post-climacteric-ripe banana with brown spots.

### 5.6. Banana protein analysis for immuno-detection of tyrosine hydroxylase

Proteins were extracted from peel and pulp from Cavendish banana sub variety Grand Nain by two methods described in 'Materials and Methods' section. Proteins extracted with method developed by (Lavid et al., 2002) and (Xu et al., 2008) at consecutive days after harvest are described in Figs. 22 and 23 respectively. Protein separation on the gel was observed to be different for the two methods though the same amount of protein concentration was loaded.

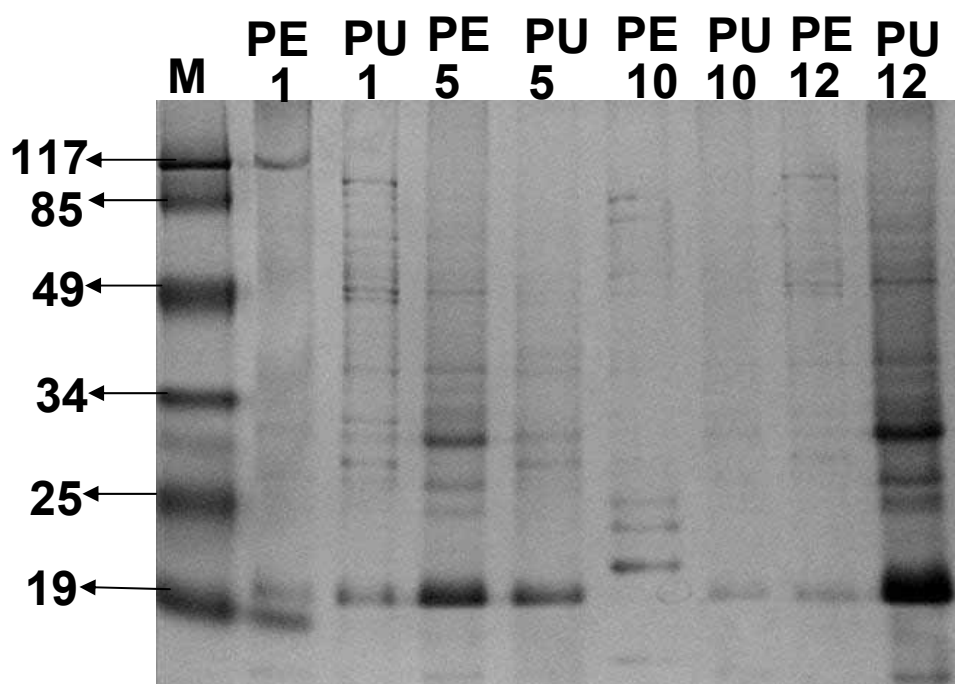
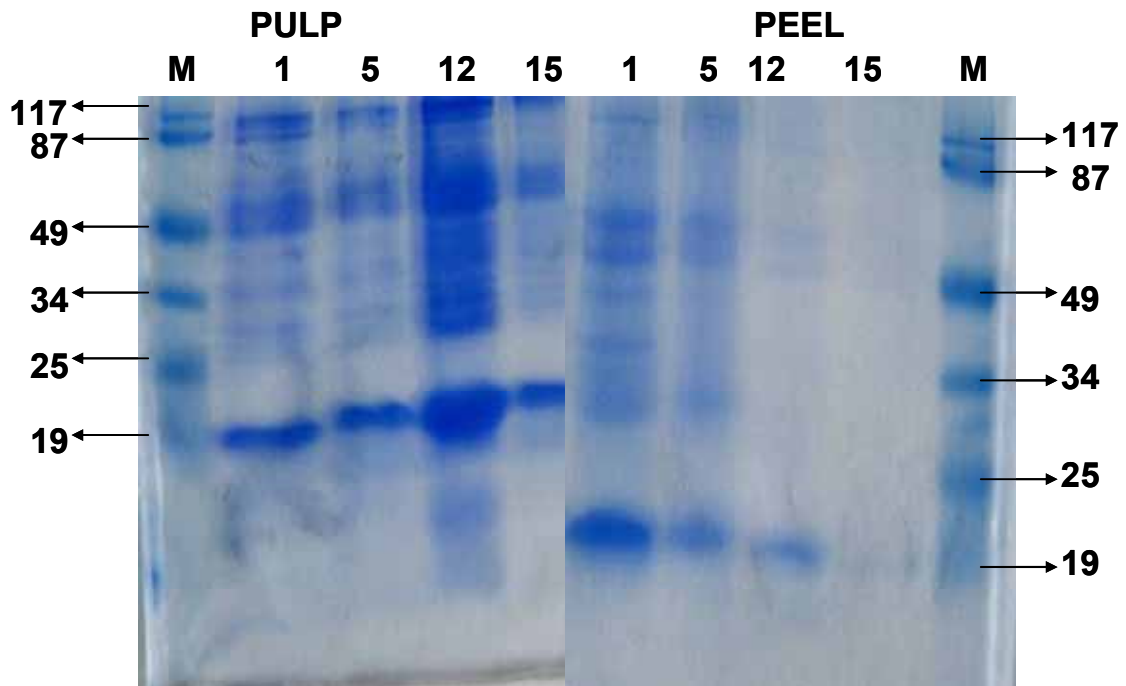
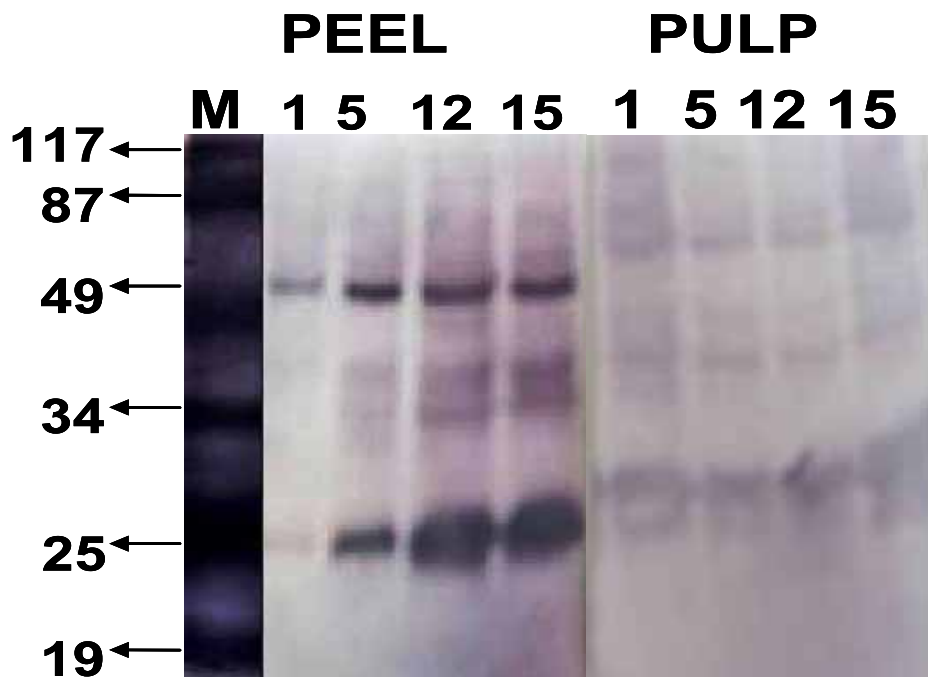


Fig. 22: SDS-PAGE of banana protein extract stained with coomassie blue. Marker (M)- used as standard sizes measured in kilo Dalton (kDa). A total protein (40  $\mu\text{g/ml}$ ) was loaded. PE-Peel, PU- Pulp and numbers denote different day after harvest of the fruit.



**Fig. 23:** SDS-PAGE of banana proteins stained with coomassie blue. A total protein (40  $\mu\text{g/ml}$ ) was loaded. Marker (M) - used as standard sizes measured in kilo Dalton (kDa). Each lane follows in the order peel and pulp with numbers indicating day after harvest of the fruit



**Fig. 24:** Western blot analysis of banana (peel and pulp) at different stages of ripening using tyrosine hydroxylase antibody. Marker (M) was used for monitoring the protein size. Peel and Pulp, numbers indicates day after harvest.

The gel of the method described in (Fig. 23) was transfer to a membrane for a western analysis with antibody against tyrosine hydroxylase. A product size of 49 kDa was detected in the peel of the banana fruit at all ripening stages (Fig. 24). This band was not visible in the pulp at all the stages. The level of the protein was similar at all stages. Similar results were observed in other western analysis.

## **6.0. Discussion**

### **6.1. Dopamine concentration in banana cultivar Grand Nain during ripening of the fruit**

Though the presence of dopamine and its alleged precursors L-dopa and tyramine in banana has been reported in several studies (Deacon and Marsh, 1970; Kanazawa and Sakakibara, 2000), the biosynthesis of this catecholamine in the fruit has not been conclusively elucidated yet. The discovery of the tyrosine decarboxylase gene in banana witnesses in favor of the tyramine pathway in agreement with previous suggestions (Buckley, 1961; Szopa et al., 2001). On the other hand, finding a TH-like protein in banana peel indicates that this pathway cannot be completely ignored at this stage. Dopamine level in the fruit (both peel and pulp) underwent an increase during the transition from unripe to ripening (climacteric) fruit, and further declined with over-ripening and senescence. This dynamics somewhat differs of the data presented in the literature (Kanazawa and Sakakibara, 2000; Romphophak et al., 2005) which reported steady decline of the dopamine level from unripe towards over-ripe fruit. However, the referred studies dealt at ripening stages with bananas subjected to artificial ethylene treatment causing sharp changes in the fruit physiological behavior. On the contrary, the fruit in our study underwent more gradual natural ripening. Changes in dopamine accumulation in Grand Nain observed in our study may be interpreted as a result of balance between the processes of tyramine biosynthesis (Tyrdec), and its conversion into dopamine on one hand, and PPO-tyrosinase activity and further oxidation on the other hand (see below). Also further degradation of dopamine by additional PPO can lead to the reduction in the levels of dopamine.

It has been noted that senescent spotting in banana is a result of phenolic oxidation (Romphophak et al., 2005). According to this study, dopamine is used as substrate of PPO for the formation of brown pigments of senescent spotting which is mostly visible on the peel of the fruit. The material contributing to brown/black color is most likely quinones (Yoruk and Mashall, 2003). The decline of dopamine after the climacteric peak may suggest that indeed this material is oxidized at the stage of over-ripening to the corresponding quinones which eventually polymerize to melanin the

compound causing the brown/black pigment on the peel. The fact that the dopamine levels in peel are higher than in pulp (Fig. 21) may contribute to protection of the fruit from external conditions that may include fungus and other external injuries. It has been established that dopamine has antioxidant capacity (Kanazawa and Sakakibara, 2000), and they may act by this action in the peel.

## **6.2. Polyphenol oxidase from banana fruit**

Polyphenol oxidases (PPOs) are enzymes involved in oxidation of phenol compounds like dopamine and its precursors; tyrosine, L-dopa and tyramine. When the substrate is oxidized, it can lead to browning of the tissue (Vamos-Vigyazo, 1981; Nematpour et al., 2008) and most evidences come from wounded tissues. On the other hand, some enzymes of the PPO family may perform biosynthetic functions in non-wounded tissues (Strack and Schliemann, 2001; Ono et al., 2006). The peel contains double the total polyphenol oxidase transcript than in the pulp at harvest (Fig. 19) and our results with dopamine correlate with this observation. However, the expression of PPO does not correlate with dopamine at later stages.

In this study, degenerate primers complimentary to conserved regions around the putative copper-binding residues of PPO gene were prepared from three plant species. The cloned putative PPO gene shared a very low (10%) homology with PPO from plants used for the primer preparation though the primers were from the highly conserved copper binding residue. However, it shared about 64% with PPO from *Ananas comosus* (Fig. 7). Interestingly, alignment of the putative amino acid (144 aa) did not share any similarity with PPO gene cloned from *Musa acuminata* during this study (Promyou and Ketsa, 2007) (Fig. 8). This suggests that in banana there are multiple PPO genes which may share similarity or differences with PPO from other plant families. Recently another study reported the cloning of additional four PPO from banana, which their sequence is not revealed yet in the GenBank (Gooding et al., 2001). Indeed in other plants including potato (*Solanum tuberosum*) it was reported that, the PPO genes constitute a family of genes and each of the genes had a different expression levels in the different developing tissues (Thygesen et al., 1995). Seven distinct PPO genes have

also been isolated from tomato previously (Newman et al., 1993). It is possible that different PPO exist in different banana species as well as their cultivars. It is however important to clone the full gene sequence and possibly the other forms of PPO that may exist in banana fruit and study the expression of the genes during ripening in the indicated cultivars in order to remark on the contribution of each PPO to the process of dopamine accumulation or development of dark spots. Full length cloning will enable to express the genes in yeast or bacteria to help determine the specificity of their substrates. This may enable us to determine if one of the PPO has a tyrosine hydroxylase activity and can be used for manipulation of the production of L-dopa as suggested (Chu et al., 1993).

### **6.3. Tyrosine hydroxylase and tyrosine decarboxylase in banana fruit**

Tyrosine hydroxylase (TH) is the rate limiting enzyme involved in the first step in the biosynthesis of catecholamine in mammals (Fig. 1), and there were several attempts to clone it by using degenerate primers prepared from a highly conserve regions in both the N and C terminals, of these genes from mammalian cells. These trials were not successful. It is possible that TH from mammal and plant do not share any sequence homology. It is also possible that TH from plant is an enzyme belonging to a different family since it was demonstrated that several plants harbour a tyrosinase with an abnormally high tyrosine hydroxylase/dopa ratio (Hernandez-Romero et al., 2005).

Though not successful with cloning the gene encoding TH, through western blot analysis, we were able to determine the existence of a protein specifically binding with mammal TH antibodies. Monoclonal anti-tyrosine hydroxylase prepared from mouse using purified rat tyrosine hydroxylase as the immunogen was used for the western blot analysis of the detection of the protein. The protein was detected in all the days after harvest in the peel without any product detected in the pulp (Fig. 24). This may suggest that some sections of the TH are similar between the mammalian and the plant TH. Further analysis is needed to verify this result.

However, we were able to clone the gene that encodes for Trydec, another enzyme that has been found to participate in the first step in catecholamine biosynthesis in plant (Fig. 3 for schematic representation) (Kulma and Szopa,

2007). The enzyme catalyzes tyrosine to produce tyramine which is then hydroxylated to dopamine (Kulma and Szopa, 2007)

Tyrdec partial gene sequence (Fig. 5) was cloned from Cavendish banana sub variety Grand Nain using degenerate primers prepared from three plant species. The gene was expressed in three stages after harvest of the banana fruit. To the best of our knowledge, this is the first cloned Trydec gene from banana fruit. Alignment of the cloned putative amino acid (208 aa) shared about 70% homology with ortholog genes of different plant families (Fig. 5). It was observed that there was a high conserved region around the putative pyridoxal phosphate (PLP) binding region and this was conserved among the genes from different plants families including the cloned gene from banana. The clone gene sharing a high similarity with Tyrdec from other plant families does agree with the report of (Facchini et al., 2000) emphasizing the high conserve regions of the gene among plant families.

#### **6.4. Determination of ripening parameters of different banana cultivars**

In this study we have followed the ripening of several cultivars in order to determine if all cultivars are using similar enzymes of the dopamine biosynthetic pathway. The different banana cultivars showed differences in the climacteric stage and ripening parameters after harvest. For instance, ethylene and carbon dioxide increased production occurred at the same pattern in the cultivar Grand Nain (Fig. 9) and three other cultivars (Matokke, Blugoe and Silver Blugoe ) (Fig. 10). In all of them a lag occurred in the pre-climacteric peak, however  $C_2H_4$  and  $CO_2$  appeared at the same time for Grand Nain and Matokke but in Blugoe and Silver Blugoe,  $C_2H_4$  increase preceded  $CO_2$  increase (Fig. 10). However in the cultivars (FHIA-1, FHIA-21 and FHIA-3) there was a gradual increase in  $CO_2$  and  $C_2H_4$  from the time of harvest (Fig. 10). This suggests that different control of ripening exist in these two groups. Aside  $C_2H_4$  and  $CO_2$ , other noticeable changes did occur simultaneously during the ripening process. The pulp accumulation of TSS was much higher than in the peel and it was true for all cultivars. In most of the cultivars the TSS increase gradually in the peel, however in the pulp only in FHIA-1, FHIA-21 and FHIA-3 there was a gradual increase, but in the cultivars Grand Nain Matokke Blugoe and Silver Blugoe there was a lag which followed by a sharp



increase. The patterns of TSS accumulation in the pulp of all the cultivars paralleled ethylene accumulation; a gradual increase in TSS coincided with increase in ethylene and a lag in TSS was correlated with a lag in ethylene production. This suggests that the control of TSS accumulation in the pulp in all the cultivars is under ethylene control. Hence, the results where 1-MCP (inhibitor of ethylene) inhibited various ripening parameters but not accumulation of sugars (Golding et al., 1998) are surprising in light of our results. There is higher accumulation of TSS levels in most of the cultivars than observed for Grand Nain, suggesting that some of the cultivars might be sweeter at the ripening stage.

During ripening the fruit changes color and gradually lost its firmness. In Grand Nain, a lag in color was observed which was followed by an abrupt color change during the climacteric peak. Decrease in firmness was observed to be gradual in Grand Nain (Fig. 13). Color change occurring, correlated with  $C_2H_4$  production, since both had a lag period of similar duration, but firmness did not coincide with  $C_2H_4$  production in Grand Nain, since it started before the increase in ethylene (Figs. 9 and 13).

In the cultivars (FHIA-1, FHIA-21 and FHIA-3), a gradual change in color occurred while in Matokke, Blugoe and Silver Blugoe, there was a lag which was followed by an abrupt change in color. Color change in all cultivars correlated with  $C_2H_4$  production since in the cultivars FHIA-1, FHIA-21 and FHIA-3 color decrease gradually at the same duration as ethylene increased, and in Matokke, Blugoe and Silver Blugoe there was a lag period of similar duration to that of lag of ethylene production. Decrease in firmness was gradual in all six cultivars (Fig. 14). Hence, in the cultivars FHIA-1, FHIA-21 and FHIA-3 it correlated with  $C_2H_4$  production, but no correlation with  $C_2H_4$  was observed for cultivars Matokke, Blugoe and Silver Blugoe. Lack of correlation between increase in ethylene and reduction in firmness suggest that cell wall degradation is partially independent on ethylene (Pech et al., 2008).

### **6.5. Expression of Tyrosine decarboxylase in banana fruit of different cultivars after harvest**

Cloned sequences of Trydec gene from Cavendish cultivar Grand Nain was used for the real time quantitative PCR (RT-PCR). The expression of Trydec gene in Grand Nain and the six banana cultivars was observed to decrease gradually with the onset of ripening in both the peel and the pulp (Figs. 15 and 16). The highest expression did occur in the green fruit in all cultivars analyzed. In general, the expression of the gene was higher in the peel than observed for the pulp. Our results clearly show that the expression of the gene coding for Tyrdec is either independent of ethylene production or down regulated as the highest expression occurred in the green fruit. From this it will be interesting to determine if ethylene increase reduces the gene expression.

### **6.6. Expression of polyphenol oxidase in banana fruit of different cultivars after harvest**

Polyphenol oxidase gene was observed to be expressed higher at the climacteric peak of ethylene in Grand Nain (Fig. 18) in both the peel and the pulp and decreased again at stage 3 (post climacteric). The expression at the climacteric peak was almost at the same level for both peel and pulp with no significant difference between them. The gene expression was compared among the six cultivars at the climacteric peak (Fig. 19). It was observed that, in two cultivars the expression was higher in the peel than in the pulp (FHIA-1, FHIA-21), in FHIA-3 and Silver Blugoe the expression was similar, however, in Matokke and Blugoe, the expression was higher in the pulp than in the peel.

It may seem PPO gene is dependent on ethylene since higher PPO levels correlated with increase ethylene production (Fig. 20). It is possible that high levels at the climacteric stage lead to higher accumulation of dopamine and or melanin leading to brown/black color. The expression of the PPO gene resembled the overall pattern of dopamine accumulation (Figs. 18 and 21). However, considering versatility of the PPO enzymes and multiple functions of these enzymes, such comparison should be done with great caution.

Unfortunately, we neither determined the dopamine nor the black color development in the various cultivars and we could not correlate the PPO levels to development of either dopamine or spots after ripening.

Moreover, since the PPO gene cloned is most likely one of several genes within the family, it is difficult at this stage to determine that one cultivar contain higher PPO activity than the other since other types may be expressed differently in the various cultivars. In other case the PPO activity was not determined in the cultivars. In addition PPO activity is dependent on various conditions like pH and the post transcriptional conditions might modulate their activity (Ayaz et al., 2007).

## **7.0. Conclusion**

Dopamine did occur in higher amount in the peel than in the pulp with certain increase towards the climacteric stage. All enzymes described in this work; TH, Tyrdec and PPO were found to be elevated in peel compare to pulp and this correlated with higher levels of dopamine in the peel. It is possible that more than one pathway exists in banana for the biosynthesis of dopamine. Our evidence so far supports the involvement of tyrosine decarboxylase (Tyrdec) as the first enzyme participating in dopamine biosynthesis in banana fruit in combination with PPO activity as tyrosinase.

Due to the growing interest in natural product especially plant in the treatment of Parkinson disease, further research on the dopamine biosynthetic pathway in banana will help to determine whether the fruit biosynthetic capacities can be manipulated in the direction of enhanced L-dopa production.

Due to cloning partial gene encoding Trydec, it will be of interest to determine tyramine level in the banana fruit since the enzyme that catalyzes the formation of tyramine is Tyrdec and tyramine has been reported to react with monoamine oxidase inhibitor (MAOIs) drugs (McCabe, 1986). This will help in breeding fruit either without tyramine or with lower levels that will not interact with MAOIs.

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## Appendix

### Sequence used fro Tyrosine hydroxylase primer preparation

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AJ251387.1| Gallus gallus mRNA -----GCACGAGTCGGCACGAGCGGG
NM_012740.3| Rattus norvegicus -----CAG
NM_173884.2| Bos taurus tyrosi -----CCGGACCTCG
NM_001097923.1| Xenopus laevis
BCI04967.1| Homo sapiens tyros A-TAAGAGGCTGCTGGGCCAGGGCTGTGGAGA--CGGAGCCCCGACCTCC
XM_508221.2| PREDICTED: Pan tr GCTGGGAGACAGCAGGGGGCCGAGCGGTAGGCTTCCCTACCCCCAGGCTCC
Clustal Consensus

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          360          370          380          390          400
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
AJ251387.1| Gallus gallus mRNA ACGAGTGAAGATGCCAACCCCCAACATCTCCACCTCTGCAGCC--AAAGG
NM_012740.3| Rattus norvegicus CTTGC--ACTATGCCCCACCCCCAGCGCCCCCTCGCC-ACAGCC-CAAGGG
NM_173884.2| Bos taurus tyrosi CCGGC--ACCATGCCCTACCCCCAACGCGCCTCGCC-GCAGGC-CAAGGG
NM_001097923.1| Xenopus laevis -----AAGTCATGCCGACCCCCAACATCTCTGGATCTGCAGGG--AAGAG
BCI04967.1| Homo sapiens tyros ACACTGAGCCATGCCACCCCCGACGCCACCAAGCC-ACAGGC-CAAGGG
XM_508221.2| PREDICTED: Pan tr CCATC-AGGCATCCCCCTCCCCAGGGGGCCCCGGGG-CCAGCCTCACAGG
Clustal Consensus
          * * * * *

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          410          420          430          440          450
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
AJ251387.1| Gallus gallus mRNA CTTCCGCAGGGCGGTGTCTAG-AGCTGGA--CTCCAAGCAAGCTGAGGGC
NM_012740.3| Rattus norvegicus CTTCAGAAGGGCCGTCTCAG-AGCAGGA--TGCCAAGCAGGCCGAGGGC
NM_173884.2| Bos taurus tyrosi CTTCCGCAGGGCCGTCTCCG-AGCTGGA--CGCCAAGCAGGGCTGAGGGC
NM_001097923.1| Xenopus laevis CTTCAGGAGGGCTGTGTCTG-AACTGGA--CCCTAAACAAGCTGAAGC
BCI04967.1| Homo sapiens tyros CTTCCGCAGGGCCGTGTCTG-AGCTGGA--CGCCAAGCAGGCCAGAGGGC
XM_508221.2| PREDICTED: Pan tr CTCTCCGTGGCCTGGAAGTGCAGCCCCAGCTGCATCCTACACCCCCAGCC
Clustal Consensus
          * * * * *

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**F1**

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          460          470          480          490          500
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
AJ251387.1| Gallus gallus mRNA CATCATGTCTCCACGGTTCATTGGTAGACGCCAAAGCCCTATTGAAGATG
NM_012740.3| Rattus norvegicus TGTCCGTCGCCAAGGTTCATCGGACGCGCAGAGTCTCATCGAGGATG
NM_173884.2| Bos taurus tyrosi CATCATGTCCCCACGGTTCGTGGGAGCGGCAGAGCCCTATCCAGGACG
NM_001097923.1| Xenopus laevis CATTCTGTCCGCTCGGTTCCTGGGCCGAGGCAAAAGTCTGATAGAAGACG
BCI04967.1| Homo sapiens tyros CATCATGTCCCCCGGGTTCATTGGGGCAGGCAGAGCCCTCATCGAGGACG
XM_508221.2| PREDICTED: Pan tr CA--AGGTCCCGCGGTTCATTGGGGCAGGCAGAGCCCTCATCGAGGACG
Clustal Consensus
          * * * * *

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          510          520          530          540          550
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
AJ251387.1| Gallus gallus mRNA CCAGGAAGGAACGAGAGGGCTGCAGCTGC-----AGCCACCGAT
NM_012740.3| Rattus norvegicus CCCCAGGAGAGCGGGAGGCGGCGGCAGCTGCAGCAGCAGCAGCGGTAGCC
NM_173884.2| Bos taurus tyrosi CACGCAAGGAGCGGGAGAAGGCGGAGGC-----CGCGGCTCA
NM_001097923.1| Xenopus laevis CTGCAAGGACAGGGAAAGTGGCAGCTGC-----AG-----GG
BCI04967.1| Homo sapiens tyros CCCCAGGAGAGCGGGAGGCGGCGGTGGC--AGCAGCGCCGCTGCAGTC
XM_508221.2| PREDICTED: Pan tr CCCCAGGAGAGCGGGAGGCGGCGGTGGC--AGCAGCGCCGCTGCAGTC
Clustal Consensus
          * * * * *

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          560          570          580          590          600
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
AJ251387.1| Gallus gallus mRNA GCTGCAGAGTCCA-----CAGAGCAATTGTTTTGAGGAGAAGGA
NM_012740.3| Rattus norvegicus TCCTCGGAACCTGGGAACCCACTGGAGGCTGTGGTATTTGAGGAGAGGGA
NM_173884.2| Bos taurus tyrosi TCCTCGGAGTCCG-----CGGAGGCGGGCAGCCTGGTGGAGAGGGA
NM_001097923.1| Xenopus laevis GCTGTGTAGTCCG-----CCGATTCGCTGGTTTTCGAGGAGAAGGA
BCI04967.1| Homo sapiens tyros CCCTCGGAGCCCCGGGACCCCCCTGGAGGCTGTGGCCTTTGAGGAGAAGGA
XM_508221.2| PREDICTED: Pan tr CCCTCGGAGCCCGGGGACCCCCCTGGAGGCGCTGGCCTTTGAGGAGAAGGA
Clustal Consensus
          * * * * *

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          610          620          630          640          650
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
AJ251387.1| Gallus gallus mRNA GGGGAGAGCCATGCTGAACCTGTCTTTCATGCTCAAGGGTGCCAAAGCCT
NM_173884.2| Bos taurus tyrosi CGGGAAGGCGGTGCTGACCTGCTGTTCCGCCCTGCGGCCACCAAGCCCC
NM_001097923.1| Xenopus laevis CGGCAAGCCATCGTCAGTCTCCTCTTCTCCCTCAAGGGCAGCAAACTT
BCI04967.1| Homo sapiens tyros GGGGAAGGCCATGCTAAACCTGCTCTTCTCCCCGAGGGCCACCAAGCCTT
XM_508221.2| PREDICTED: Pan tr GGGGAAGGCCGTGCTAAACCTGCTCTTCTCCCCGAGGGCCACCAAGCCTT
Clustal Consensus
          * * * * *

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## F2

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      660      670      680      690      700
AJ251387.1| Gallus gallus mRNA  CTCCGCTCTCACGAGCGCTTAAGGTGTTGAGACATTTGAAGCTAAAATC
NM_012740.3| Rattus norvegicus CCTCCTTGTCTCGGGCTGTAAAAGTATTTGAGACATTTGAAGCCAAAAT
NM_173884.2| Bos taurus tyrosi CTGCGCTGACCCGGGCTATCAAGGTGTTGAGACATTCGAAGCCCACCTC
NM_001097923.1| Xenopus laevis CCTCCTTATCCGCGCACACTTAAGGTGTTGAGACATTTGAAGCCAAAATC
BC104967.1| Homo sapiens tyros CGGCGCTGTCCGAGCTGTGAAGGTGTTGAGACGTTTGAAGCCAAAATC
XM_508221.2| PREDICTED: Pan tr CCGCGCTGTCCGAGCTGTGAAGGTGTTGAGACGTTTGAAGCCAAAATC
Clustal Consensus          * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

      710      720      730      740      750
AJ251387.1| Gallus gallus mRNA  CACCACCTTGGAGACAAGACTCAGCCGAAAGCCCGTGAAGGGACTGCTGA
NM_012740.3| Rattus norvegicus CACCACCTTAGAGACCCGGCCTGCCAGAGGCCACTGGCAGGAAGCCCCCA
NM_173884.2| Bos taurus tyrosi CACCACCTTGGAGACCCGACCCGCCAGCCACTCGGGGGGGGAGCCCCGC
NM_001097923.1| Xenopus laevis CAACACCTTGGAGACACGACCCGGGAGAAAATCAGTGGGGACGGCTGAGGA
BC104967.1| Homo sapiens tyros CACCATCTAGAGACCCGGCCGCCAGAGCCGCGAGCTGGGGGCCCCCA
XM_508221.2| PREDICTED: Pan tr CACCATCTAGAGACCCGGCCGCCAGAGCCGCGAGCTGGGGGCCCCCA
Clustal Consensus          ** * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

      760      770      780      790      800
AJ251387.1| Gallus gallus mRNA  ACTGGAGTATTTTGTGCGCTGTGAGGTGCACAGCTCTGACCTCAATACTT
NM_012740.3| Rattus norvegicus CCTGGAGTATTTTGTGCGCTTTCGAGGTGCCAGTGGAGACCTGGCTGCC
NM_173884.2| Bos taurus tyrosi CCTGGAGTGTCTCGTGCCTGTGAGGTGCCCGCCAGTGGTGCCTGCC
NM_001097923.1| Xenopus laevis GCTGGAATACCTTGTGCGCTGTGAGGTGCATCGTTTACAGCTGAGCACCC
BC104967.1| Homo sapiens tyros CCTGGAGTACTTCTGTGCGCTCGAGGTGCCCGAGGGGACCTGGCCGCC
XM_508221.2| PREDICTED: Pan tr CCTGGAGTACTTCTGTGCGCTCGAGGTGCCCGTAGGGGACCTGGCCGCC
Clustal Consensus          ***** * * * * * * * * * * * * * * * * * * * * * * * * * * * *

      810      820      830      840      850
AJ251387.1| Gallus gallus mRNA  TCATTAGCTCCATCAAGAGGGTAGCAGAAGATGTGAGGACCCTAAGGAA
NM_012740.3| Rattus norvegicus TCCTCAGCTCTGTGCTCGGGTGTCTGACGACGTGCCAGTGCAGAGAG
NM_173884.2| Bos taurus tyrosi TGCTGAGCGCCCTGCGCCCGTGGCAGAAGATGTGCTGCCCGCGGGGAG
NM_001097923.1| Xenopus laevis TAATGAGTCCGTGAGGAGAGTTTGTGAAGATGTGAGGAGCACTCGGGAG
BC104967.1| Homo sapiens tyros TGCTCAGTGGTGTGCGCCAGGTGTGAGAGGACGTGCCAGCCCCGCGGGG
XM_508221.2| PREDICTED: Pan tr TGCTCAGTGGTGTGCGCCAGGTGTGAGAGGACGTGCCAGCCCCGCGGGG
Clustal Consensus          * * * * * * * * * * * * * * * * * * * * * * * * * * * *

      860      870      880      890      900
AJ251387.1| Gallus gallus mRNA  GACAAATTTCACTGGTTTCCCAAGAAAATATGTGAACCTGGACAAGTGTCA
NM_012740.3| Rattus norvegicus GACAAGGTCCCTGGTTCCCAAGAAAAGTGTCCGAATTGGACAAGTGTCA
NM_173884.2| Bos taurus tyrosi AGCAAAGTCCCTCTGGTTCCCAAGGAAAGTGTCCGAGCTGGACAAGTGTCA
NM_001097923.1| Xenopus laevis GATAAAGTTCACTGGTTTCCAACCAAAATTTGCTGAATTGGACAAGTGTCA
BC104967.1| Homo sapiens tyros CCCAAGGTCCCTGGTTCCCAAGAAAAGTGTGAGAGCTGGACAAGTGTCA
XM_508221.2| PREDICTED: Pan tr CCCAAGGTCCCTGGTTCCCAAGAAAAGTGTGAGAGCTGGACAAGTGTCA
Clustal Consensus          * * * * * * * * * * * * * * * * * * * * * * * * * * * *

      910      920      930      940      950
AJ251387.1| Gallus gallus mRNA  CCATTTGGTCAACAAGTTTGACCCCTGACCTTAGACTTGGATCATCCGGGCT
NM_012740.3| Rattus norvegicus CCACCTGGTCAACAAGTTTGACCCCTGACCTGGACTGGATCATCCGGGCT
NM_173884.2| Bos taurus tyrosi CCACCTCGTCAACAAGTTTGACCCCTGACCTGGACTTGGATCATCCGGGCT
NM_001097923.1| Xenopus laevis CCACCTAGTAACAAGTTTGACCCGGACCTGGATTTGGAGCACCCGGGCT

BC104967.1| Homo sapiens tyros  TCACCTGGTCAACAAGTTTCGACCCCTGACCTGGACTTGGACCACCCGGGCT
XM_508221.2| PREDICTED: Pan tr  TCACCTGGTCAACAAGTTTCGACCCCTGACCTGGACTTGGACCACCCGGGCT
Clustal Consensus          * * * * * * * * * * * * * * * * * * * * * * * * * * * *

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AJ251387.1| Gallus gallus mRNA  
 NM\_012740.3| Rattus norvegicus  
 NM\_173884.2| Bos taurus tyrosi  
 NM\_001097923.1| Xenopus laevis  
 BC104967.1| Homo sapiens tyros  
 XM\_508221.2| PREDICTED: Pan tr  
 Clustal Consensus

```

          960          970          980          990          1000
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
ACTCTGACCAGGTATATCGCCAGAGGAGGAAATCAATAGCAGAAATCGCT
TCTCTGACCAGGTGTATCGCCAGCGTCGGAAGCTGATTGCAGAGATTGCC
TCTCCGACCAGGCGTACCGCCAGCGCAGGAAGCTGATCGCCGAGATCGCC
TTTCAGACCAAGAATATCGCAAGAGGAGGAAGATGATAGCAGACATAGCA
TCTCGGACCAGGTGTACCGCCAGCGCAGGAAGCTGATTGCTGAGATCGCC
TCTCGGACCAGGTGTACCGCCAGCGCAGGAAGCTGATTGCTGAGATCGCC
  ** * * * * * * * * * * * * * * * * * * * * * * * * * * * *

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AJ251387.1| Gallus gallus mRNA  
 NM\_012740.3| Rattus norvegicus  
 NM\_173884.2| Bos taurus tyrosi  
 NM\_001097923.1| Xenopus laevis  
 BC104967.1| Homo sapiens tyros  
 XM\_508221.2| PREDICTED: Pan tr  
 Clustal Consensus

```

          1010          1020          1030          1040          1050
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
TTTCACTATAAGCATGGGGACCCAATCCCTCGTGTGGAGTACACAGCAGA
TTCCAGTACAAGCACGGTGAACCAATCCCCATGTGGAATACACAGCGGA
TTCCAGTACAAGCAAGGCGACCCCATCCCCACGTGGAGTACACAGCCGA
TTCAACTACACCCATGGTGACCCCATCCAGCGAGTGGAAATATCTGCTGA
TTCCAGTACAGGCACGGCGACCCGATTCCCCGTGTGGAGTACACCGCCGA
TTCCAGTACAGGCATGGCGACCCGATTCCCCGTGTGGAGTACACCGCCGA
  ** * * * * * * * * * * * * * * * * * * * * * * * * * * * *

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AJ251387.1| Gallus gallus mRNA  
 NM\_012740.3| Rattus norvegicus  
 NM\_173884.2| Bos taurus tyrosi  
 NM\_001097923.1| Xenopus laevis  
 BC104967.1| Homo sapiens tyros  
 XM\_508221.2| PREDICTED: Pan tr  
 Clustal Consensus

```

          1060          1070          1080          1090          1100
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
GGAGACTGCTACCTGGAAGGAGGTTTACAGCACTGAAGAGCCTGTATC
AGAGATTGCTACCTGGAAGGAGGTATATGTCACCGCTGAAGGGCCTCTATG
GGAGACAGCCACCTGGAAGGAGGTCTACTCCACGCTGCGGGGCCCTGTACC
AGAAATTGCAACATGGAAGGAGGTATATCCACCCTCAAGAGCCTGTACC
GGAGATTGCCACCTGGAAGGAGGTCTACACCACGCTGAAGGGCCTCTACG
GGAGATTGCCACCTGGAAGGAGGTCTACACCACGCTGAAGGGCCTCTACG
  ** * * * * * * * * * * * * * * * * * * * * * * * * * * * *

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AJ251387.1| Gallus gallus mRNA  
 NM\_012740.3| Rattus norvegicus  
 NM\_173884.2| Bos taurus tyrosi  
 NM\_001097923.1| Xenopus laevis  
 BC104967.1| Homo sapiens tyros  
 XM\_508221.2| PREDICTED: Pan tr  
 Clustal Consensus

```

          1110          1120          1130          1140          1150
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
CAACCCATGCTGCAAGGAGTACCTAGAGCTTTAATCTACTGGAGAAA
CTACCCATGCGTGCCGGGAGCACCTGGAGGGTTCCAGCTTCTGGAACGG
CCACCCACGCTGCCCGAGCACCTGGAGGCCCTCCAGCTGCTGGAGCGC
CAACTCATGCTGTAAAGGAATACCTGGAGGCCCTCCAGCTGCTGGAGGCA
CCACGCACGCTGCCGGGAGCACCTGGAGGCCCTTGTCTTGTGGAGCGC
CCACGCACGCTGCCGGGAGCACCTGGAGGCCCTTGTCTTGTGGAGCGC
  ** * * * * * * * * * * * * * * * * * * * * * * * * * * * *

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AJ251387.1| Gallus gallus mRNA  
 NM\_012740.3| Rattus norvegicus  
 NM\_173884.2| Bos taurus tyrosi  
 NM\_001097923.1| Xenopus laevis  
 BC104967.1| Homo sapiens tyros  
 XM\_508221.2| PREDICTED: Pan tr  
 Clustal Consensus

```

          1160          1170          1180          1190          1200
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
TTCTGCGGCTACAATGAGAACAACATCCCCAGCTGGAGGAGTCTCCCG
TACTGTGGCTACCGAGAGGACAGCATCCACAGCTGGAGGACGTCTCCCG
TTCTGCGGGTACCGCGAGGACCGAATCCCGCAGCTGGAGGACGTCTCACG
CATTGTGGTTATAGTGAAGACAATATCCACAGCTTCCAGGATGTATCCAA
TTACGCGGCTACCGGGAAGACAATATCCCCAGCTGGAGGACGTCTCCCG
TTACGCGGCTACCGGGAAGACAATATCCCCAGCTGGAGGACGTCTCCCG
  ** * * * * * * * * * * * * * * * * * * * * * * * * * * * *

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AJ251387.1| Gallus gallus mRNA  
 NM\_012740.3| Rattus norvegicus  
 NM\_173884.2| Bos taurus tyrosi  
 NM\_001097923.1| Xenopus laevis  
 BC104967.1| Homo sapiens tyros  
 XM\_508221.2| PREDICTED: Pan tr  
 Clustal Consensus

```

          1210          1220          1230          1240          1250
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
CTTCCTGAAAGAGAGGACTGGCTTCCAGCTCCGGCCTGTGGCTGGCCTGT
CTTCCTGAAAGAGAGGACTGGCTTCCAGCTCCGACCCGTGGCCGGTCTAC
CTTCCTGAAAGAGAGGACTGGCTTCCAGCTCCGACCCGTGGCCGGTCTGC
CTTCCTAAAAGAGAAGAGTGGGTTACGGTTCGACCTGTAGCCGGACTTT
CTTCCTGAAAGAGCGCACGGGCTTCCAGCTCCGGCCTGTGGCCGGCCTGC
CTTCCTGAAAGAGCGCACGGGCTTCCAGCTCCGGCCTGTGGCCGGCCTGC
  ** * * * * * * * * * * * * * * * * * * * * * * * * * * * *

```

AJ251387.1| Gallus gallus mRNA  
 NM\_012740.3| Rattus norvegicus  
 NM\_173884.2| Bos taurus tyrosi  
 NM\_001097923.1| Xenopus laevis  
 BC104967.1| Homo sapiens tyros  
 XM\_508221.2| PREDICTED: Pan tr  
 Clustal Consensus

```

      1560      1570      1580      1590      1600
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
CTTCCATGCGGAGGCTCATACACTCCTTGTCTGATGAAACCAGAGGTACGA
CTTCCATGCGGAGGCTCCTGCACTCCCTGTCTGAGGAGCCTGAGGTCCGA
CCTCCTACGGGGAGGCTCCTGCACTCCCTTTCCGAGGAGCCCGAGATCCGG
CCTCATTGGGGAACGTGCTGCACGCATTGTCTGATGAACCAGAGGTTCCG
CCTCCTACGGGGAGGCTCCTGCACTGCCTGTCTGAGGAGCCTGAGATTCGG
CCTCCTACGGGGAGGCTCCTGCACTGCCTGTCTGAGGAGCCCGAGATTCGG
* * * * *

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**R1**

AJ251387.1| Gallus gallus mRNA  
 NM\_012740.3| Rattus norvegicus  
 NM\_173884.2| Bos taurus tyrosi  
 NM\_001097923.1| Xenopus laevis  
 BC104967.1| Homo sapiens tyros  
 XM\_508221.2| PREDICTED: Pan tr  
 Clustal Consensus

```

      1610      1620      1630      1640      1650
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
GACTTTGATCCCTGATGCTGCTGCAGTTCAGCCCTACCGAGGACCAGAACTA
GCCTTTGACCCAGACACAGCAGCTGTGACGCCCTACCAAGATCAAACTA
GCCTTCGACCCCTGATGCAGCGGCCGTGCAGCCCTATCAGGACCAGACTA
CCCTTCAACCCAAATGAGGCGGCAGTGCAGCCATACCAAGACCAGAACTA
GCCTTCGACCCCTGAGGCTGCGGCCGTGCAGCCCTACCAAGACCAGACTA
GCCTTCGACCCCTGAAGCTGCAGCCGTGCAGCCCTACCAAGACCAGACTA
*** * * * *

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**R2**

AJ251387.1| Gallus gallus mRNA  
 NM\_012740.3| Rattus norvegicus  
 NM\_173884.2| Bos taurus tyrosi  
 NM\_001097923.1| Xenopus laevis  
 BC104967.1| Homo sapiens tyros  
 XM\_508221.2| PREDICTED: Pan tr  
 Clustal Consensus

```

      1660      1670      1680      1690      1700
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
CCAGCCTGTGTATTTTGTGTCTGAGAGCTTCAGTGTGCCCCAAAACAAGC
CCAGCCTGTGTACTTTGTGTCCGAGAGCTTCAATGACGCCAAGGACAAGC
CCAGCCCGTCTACTTTCGTCTGAGAGCTTACCGGATGCCAAGGACAAGC
CCAGCCTGTATATTTTATATCAGAGAGTTCCATGATGCCAAAAGAAAAGC
CCAGTCAGTCTACTTTCGTCTGAGAGCTTCAGTGTGCCCCAAGGACAAGC
CCAGTCAGTCTACTTTCGTCTGAGAGCTTCAGTGTGCCCCAAGGACAAGC
***** * * * *

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**R2**

AJ251387.1| Gallus gallus mRNA  
 NM\_012740.3| Rattus norvegicus  
 NM\_173884.2| Bos taurus tyrosi  
 NM\_001097923.1| Xenopus laevis  
 BC104967.1| Homo sapiens tyros  
 XM\_508221.2| PREDICTED: Pan tr  
 Clustal Consensus

```

      1710      1720      1730      1740      1750
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
TGAGGAATACGCAGCACATATCAAGAGGCCCTTCTCAGTGAATACGAG
TCAGGAATATGCCTCTCGTATCCAGCGCCCATCTCTGTGAAGTTTGAC
TCAGGAGCTACGCCTCCCGCATCCAGCGCCCTTCTCTGTGAAGTTTGAC
TAAGGAGTTACGCTTCCATATAAAGCGCCCATTTGCAGTCCGTTTGTAT
TCAGGAGCTATGCCTCAGCATCCAGCGCCCTTCTCCGTGAAGTTTGAC
TCAGGAGCTATGCCTCAGCATCCAGCGCCCTTCTCCGTGAAGTTTGAC
* * * * *

```

AJ251387.1| Gallus gallus mRNA  
 NM\_012740.3| Rattus norvegicus  
 NM\_173884.2| Bos taurus tyrosi  
 NM\_001097923.1| Xenopus laevis  
 BC104967.1| Homo sapiens tyros  
 XM\_508221.2| PREDICTED: Pan tr  
 Clustal Consensus

```

      1760      1770      1780      1790      1800
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
CCCTACACCCACAGCATCGAGCTCCTGGACAGCCCCAGACCATCTGCCA
CCGTACACACTGGCCATGACGCTACTGGACAGCCCTCACACCATCCAGCG
CCGTACACACTGGCCATCGACGTGCTGGACAGTCCCCACGCCATCCGGCG
CCATACACTGTGAGCATGACGTTCTGAAC--TCACCGGGCCGGATTCAA
CCGTACACGCTGGCCATCGACGTGCTGGACAGCCCCAGGCCGTGCGGCG
CCGTACACGCTGGCCATCGACGTGCTGGACAGCCCCAGGCCGTGCGGCG
* * * * *

```

AJ251387.1| Gallus gallus mRNA  
 NM\_012740.3| Rattus norvegicus  
 NM\_173884.2| Bos taurus tyrosi  
 NM\_001097923.1| Xenopus laevis  
 BC104967.1| Homo sapiens tyros  
 XM\_508221.2| PREDICTED: Pan tr  
 Clustal Consensus

```

      1810      1820      1830      1840      1850
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
CT--CCCTGGAGAGTGTAAAGGACGAGCTTCATTCGCTGATTAACGCGCT
CT--CCTTGGAGGGGGTCCAGGATGAGCTGCACACCCCTGGCCCACGCACT
CG--CCCTGGACGGCGTCCAGGACGAGATGACGGCCCTGGCCCATGCTCT
ACGACTCTGGAGGAGCTAAGAGATGAGCTACAGACCCCTTTCAAGTGCTCT
CT--CCCTGGAGGGTGTCCAGGATGAGCTGGACACCCCTTGCCCATGCGCT
CT--CCCTGGAGGGTGTCCAGGATGAGCTGGACACCCCTTGCCCATGCGCT
* * * * *

```

AJ251387.1| Gallus gallus mRNA  
 NM\_012740.3| Rattus norvegicus  
 NM\_173884.2| Bos taurus tyrosi  
 NM\_001097923.1| Xenopus laevis  
 BC104967.1| Homo sapiens tyros  
 XM\_508221.2| PREDICTED: Pan tr  
 Clustal Consensus

```

CAATGTGATCAGTTAATAAGAGAAGCTGGGCTCTCCATCCCCCTGCC
GAGTGCCATTAGCTAAATGCATAGGGTACCACCCACAGGTGCCAGGGCCT
GAACGCCATCAGTAGACGCA--GGGCACCCCCGAGCCCCAGTGGGCC
CAGTACATTGCTATAACACACA-----
GAGTGCCATTGGCTAGGTGCA--CGGCGTCCCTGAGGGCCCTTCCCAACC
GAGTGCCATTGGCTAGGTGCA--CGGCGTCCCTGAGGGCCCTTCCCAACC
* * * * *

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## Sequence used for Tyrosine decarboxylase primer preparation

```

10      20      30      40      50      60      70
U16804.1|PSU16804 Papaver somn AAGCTTAGTACCTCTCTCTGAAACCAACTCTACTCATATATCTCTCTTCTCTTATAGTTCAATTAATTT
M95685.1|PUMTRYDC4X Parsley ty                                     CAAAGCA
M96070.1|PUMTRYDC2X Parsley ty
M96069.1|PUMTRYDC1X Parsley ty
AY096475.1| Arabidopsis thalia
Clustal Consensus

80      90      100     110     120     130     140
U16804.1|PSU16804 Papaver somn TGTAGCAATAAAGGTAGGAATATGGGCAGTCTTCCAACTGATTAACCTTCAGAGCATGTCAATATGTTCT
M95685.1|PUMTRYDC4X Parsley ty AAGCAAGACAAAAATGGGCTCCATCGATAATCTTATGGCTCAAAAAATTAACTCCCAATTTCCGAT
M96070.1|PUMTRYDC2X Parsley ty AAAAAATGGGCTCCATCGATAATCTTAACTGAAAAATTAGCATCCCAATTTCCCAAT
M96069.1|PUMTRYDC1X Parsley ty
AY096475.1| Arabidopsis thalia ATGGAAAAATGGAGCCGGGAGGTTGT
Clustal Consensus

150     160     170     180     190     200     210
U16804.1|PSU16804 Papaver somn CAAAAACCACATGATCTCTGATGAGTTTCAGAAAGCAAGGTCACATGATCATGATTTTCCAGCCGATTAAT
M95685.1|PUMTRYDC4X Parsley ty GAATACACTTCGAGCCTGAAAGATTCAGAAAGCAAGGCCACCTGATGATAGATTTTCTTCTGCTGATTAAT
M96070.1|PUMTRYDC2X Parsley ty GAATACACTTCGAGCCTGAAAGATTTCCGAAAGCAAGGCCACATGATGATAGATTTTCTTCTGCTGATTAAT
M96069.1|PUMTRYDC1X Parsley ty GAATTCAGAAAGCAAGGCCACCTGATGATAGATTTTCTTCTGCTGATTAAT
AY096475.1| Arabidopsis thalia GAAGCCGATGGACTCAGAGCAATTCAGAGAGTACGGACATCTGATGGTTGATTTTCAATTCGATTAAT
Clustal Consensus
* * * * *

220     230     240     250     260     270     280
U16804.1|PSU16804 Papaver somn ACAAAAATGTAAGGATTCAGT AGAAGCCAGG CGAACCCGGG ATCTCAGC AAACGTTTACCAGA
M95685.1|PUMTRYDC4X Parsley ty ATCGTAAAGTTGAGAAATTCAGTTAGAAATCAGGTCACCCCTGGTTATCTTCCGCAAAATTTTACCAGA
M96070.1|PUMTRYDC2X Parsley ty ATCGTAAAGTTGAGAAATTCAGTTAGAAATCAGGTCACCCCTGGTTATCTTCCGCAAAATTTTACCAGA
M96069.1|PUMTRYDC1X Parsley ty ATCGTAAAGTTGAGAAATTCAGTTAGAAATCAGGTCACCCCTGGTTATCTTCCGCAAAATTTTACCAGA
AY096475.1| Arabidopsis thalia ACAAAACCATCGAAGATTTCCCTGTTCTTAGCCAAAGTTCCAGCTGGTTATCTTCAATGAGCTTTGCTGA
Clustal Consensus
* * * * *

290     300     310     320     330     340     350
U16804.1|PSU16804 Papaver somn AACTGCTCTCAATCATCTCAGTCCATTGAGACCAATTCCTCAAGATGTCAAAAATGATCATCCCTGGT
M95685.1|PUMTRYDC4X Parsley ty ATCTGCCCAATCAAACTCCGAATCTCTTGAACAATTCCTCAAGATGTCAAAAATGATCATCCCTGGC
M96070.1|PUMTRYDC2X Parsley ty ATCTGCCCAATCAAACTCCGAATCTCTTGAACAATTCCTCAAGATGTCAAAAATGATCATCCCTGGC
M96069.1|PUMTRYDC1X Parsley ty ATCTGCCCAATCAAACTCCGAATCTCTTGAACAATTCCTCAAGATGTCAAAAATGATCATCCCTGGC
AY096475.1| Arabidopsis thalia TTCTGCCCTTGACCCTTGAACCTCTGACCAAGTTCCTGATGATGTTTCGAGCAAAATGATTTGCTGGA
Clustal Consensus
* * * * *

360     370     380     390     400     410     420
U16804.1|PSU16804 Papaver somn ATTACTCACTGGCAAAATCCAAATTCCTTGTCTTATTTTCCCTTCTAGTGGTCCGTTGCCGGATTCCTTG
M95685.1|PUMTRYDC4X Parsley ty ATCACACATGGCAAAATCCCAACTTCCTTGTCTTATTTTCCCTTCCAGTGGTAGTCTGCTGGTTTCTTG
M96070.1|PUMTRYDC2X Parsley ty ATCACACATGGCAAAATCCCAACTTCCTTGTCTTATTTTCCCTTCCAGTGGTAGTCTGCTGGTTTCTTG
M96069.1|PUMTRYDC1X Parsley ty ATCACACATGGCAAAATCCCAACTTCCTTGTCTTATTTTCCCTTCCAGTGGTAGTCTGCTGGTTTCTTG
AY096475.1| Arabidopsis thalia GTAACGCAATGGCAAAATCCGAGTTCCTTGTCTTATTTTCCCTTACAGCAGTGTCTGGTCTGG
Clustal Consensus
* * * * *

430     440     450     460     470     480     490
U16804.1|PSU16804 Papaver somn GGGAAATGCTTAGTAGTGGATTTAATGTCGTTGGGTTCAATGGATGTCATCACCTGCTGCCACTGAGTT
M95685.1|PUMTRYDC4X Parsley ty GTGAAATGCTTAGTAGTGGATTTCAATGTTGTTGGCTTTAACGGATGGTTTCCACCTGCTGCTACGGAGCT
M96070.1|PUMTRYDC2X Parsley ty GTGAAATGCTTAGTAGTGGATTTCAATGTTGTTGGCTTTAACGGATGGTTTCCACCTGCTGCTACGGAGCT
M96069.1|PUMTRYDC1X Parsley ty GTGAAATGCTTAGTAGTGGATTTCAATGTTGTTGGCTTTAACGGATGGTTTCCACCTGCTGCTACGGAGCT
AY096475.1| Arabidopsis thalia GTGAGATGTTGAGTCTGGTCTGGCAATGTTGGTTTGTGGTTTACTTCTCCAGCTGCCACAGAGCT
Clustal Consensus
* * * * *

500     510     520     530     540     550     560
U16804.1|PSU16804 Papaver somn AGAAAATGCTGATGAACTGGCTCGGACAGATGCTTAACTTCCAAAGTCAATCCCTTTTCTCATCAGAT
M95685.1|PUMTRYDC4X Parsley ty CGAAAACGTTGTCACCGATTTGGTTCCGAAAATGCTTCAACTTCCCAAAATCCCTTTTCTCTC-----T
M96070.1|PUMTRYDC2X Parsley ty CGAAAACGTTGTCACCGATTTGGTTCCGAAAATGCTTCAACTTCCCAAAATCCCTTTTCTCTC-----T
M96069.1|PUMTRYDC1X Parsley ty CGAAAACGTTGTCACCGATTTGGTTCCGAAAATGCTTCAACTTCCCAAAATCCCTTTTCTCTC-----T
AY096475.1| Arabidopsis thalia AGAAAATGCTGATGAACTGGCTCGGACAGATGCTTCAACTTCCCAACTTCCCAAGCAGTTTATGTTCAA-----A
Clustal Consensus
* * * * *

570     580     590     600     610     620     630
U16804.1|PSU16804 Papaver somn GATTAATGCAAGGATTCAGCTGGAGGATTTTCAAGGGACTACTTGTGAGCCATTCATATGTTACTCTGA

```

M95685.1|PUMTRYDC4X Parsley ty GGTGGT-----GTGGCGGTCTTCAAGGGACACTTGGAGGCCATTGTTGTTACACTTG  
M96070.1|PUMTRYDC2X Parsley ty GGTGGT-----CGGGGGTCTTCAAGGGACACTTGGAGGCCATACATGTTGTTACACTTG  
M96069.1|PUMTRYDC1X Parsley ty GGTGGT-----GTGGCGGTCTTCAAGGGACACTTGGAGGCCATATGTTGTTACACTTG  
XY096475.1| Arabidopsis thalia GGAATG-----GAGGTGGAGTTTCAAGGGTCAGCTAGTGAAGCTGTTCTTGTTCCTGA  
Clustal Consensus \*

F2

U16804.1|PSU16804 Papaver somm  
M95685.1|PUMTRYDC4X Parsley ty CCGCATCAAGAGATBAAAAGCTCAACAAAAATGGAGAGAAAAATCAATTAAGTTAGTAGTTTACCGTTC  
M96070.1|PUMTRYDC2X Parsley ty TGCCAGCTAGAGACAAAAACCTGAGGCCAACATGGCATGGATAATTTGGCAAGTTGGTCGTTATTTGTC  
M96069.1|PUMTRYDC1X Parsley ty TGCCAGCAAGAGACAAAAACCTGAGGCCAACATGGCATGGATAATTTGGCAAGTTGGTCGTTATTTGTC  
XY096475.1| Arabidopsis thalia TTTGTCACCGGACAAGGTTTATAGAACCTTTGGCAAAAACGGCTTGAAGAGCTCTGTTCTCTCTCC  
Clustal Consensus \*

F2

U16804.1|PSU16804 Papaver somm  
M95685.1|PUMTRYDC4X Parsley ty TGATCAAAACCATTGCTCACTTCAAAAAGCTGCTCAAAATGGCCGGTATAAACCCGAAGAAATTCCCGCGT  
M96070.1|PUMTRYDC2X Parsley ty TGACCAAACCTCATTCTGCCCTGCAAAAAGCTGCAAAAATGCTGGGAATTGATCCCAAGAAATTCCGTC  
M96069.1|PUMTRYDC1X Parsley ty TGACCAAACCTCATTCTGCCCTGCAAAAAGCTGCAAAAATGCTGGGAATTGATCCCAAGAAATTCCGTC  
XY096475.1| Arabidopsis thalia TGACCAGACTCATTCAAGCTTTTTCAGAAAGCCCTGACAGATTGCTGGAAATCCATCAGAGGCTG  
Clustal Consensus \*

U16804.1|PSU16804 Papaver somm  
M95685.1|PUMTRYDC4X Parsley ty ATTGCAACGCTAAGGCCAATGACTTTGGGCTCTCTCCACAACTTCTTCAACAATTTGGCCGATA  
M96070.1|PUMTRYDC2X Parsley ty ATCGAAACATCAAAATCCTCAAAATTCGAGCTCTGTCCTAAGCGACTGGAAATCGCTATTTTGTATGATT  
M96069.1|PUMTRYDC1X Parsley ty ATCGAAACATCAAAATCCTCAAAATTCGAGCTCTGTCCTAAGCGACTGGAAATCGCTATTTTGTATGATT  
XY096475.1| Arabidopsis thalia CTGCAACAGACTCTTCTCAAAATTCGCTCTGGCTCCAGAAATCGCTTCAAGAGCTGTTCTTGGGATC  
Clustal Consensus \*

U16804.1|PSU16804 Papaver somm  
M95685.1|PUMTRYDC4X Parsley ty TGAATTCGGGTAGTGCCTTTGTTCTATTTGCTACTGTGGGTACAACTTCATCAACCGCAGTTGATCC  
M96070.1|PUMTRYDC2X Parsley ty TGCAAAAATGGGCTTATTCATTGTAAGTGTCTTTGTGCCACTGTTGGGACAACTCCATCAACAGATGATCC  
M96069.1|PUMTRYDC1X Parsley ty TGCAAAAATGGGCTTATTCATTGTAAGTGTCTTTGTGCCACTGTTGGGACAACTCCATCAACAGATGATCC  
XY096475.1| Arabidopsis thalia TTTGAAGCTGGATTGATTCATTCTCTTATTTGCTAAAGTTGGGACCACTTCCTCAACCGCAGTTGATCC  
Clustal Consensus \*

R1

U16804.1|PSU16804 Papaver somm  
M95685.1|PUMTRYDC4X Parsley ty AATCGGGCCACTTTGAGGTTCGAAAAAGTTCGGATTTTGGGTTGATGGATGCGCGTTATGCTGGA  
M96070.1|PUMTRYDC2X Parsley ty ATTGCCCGCTTTACAGAGGTGGCAAAAAGTTCGATTTTATGGGTCATGATGGATGCTGCAATATGCTGGA  
M96069.1|PUMTRYDC1X Parsley ty ATTGCCCGCTTTACAGAGGTGGCAAAAAGTTCGATTTTATGGGTCATGATGGATGCTGCAATATGCTGGA  
XY096475.1| Arabidopsis thalia ATTAGCTGCCTTGGGAGATTTGCATAAGCAATGGAAATGGTTTCACGTGGAATGATGCTGGA  
Clustal Consensus \*

R1

U16804.1|PSU16804 Papaver somm  
M95685.1|PUMTRYDC4X Parsley ty AGTGTCTGTAATTTGCCCGAGTTCCAGGCACATCATTGATGGTGTCCGAGGAGGACAGACTCTGTAGCCTTA  
M96070.1|PUMTRYDC2X Parsley ty AGTGTCTGTAATTTGCCCGAGTTCCAGGCATTCATTGACGGTGTGGAAAAATGACAGATTCTTTAAGTTGA  
M96069.1|PUMTRYDC1X Parsley ty AGTGTCTGTAATTTGCCCGAGTTCCAGGCATTCATTGACGGTGTGGAAAAATGACAGATTCTTTAAGTTGA  
XY096475.1| Arabidopsis thalia AGTGTCTGTAATTTGCCCGAGTTCCAGGCATTCATTGACGGTGTGGAAAAATGACAGATTCTTTAAGTTGA  
Clustal Consensus \*

U16804.1|PSU16804 Papaver somm  
M95685.1|PUMTRYDC4X Parsley ty ATGCACACAAATGGTTCCTCACACTTGGATTGTTGTTTATGGGTTAAGACTCTAATGCTCTTGT  
M96070.1|PUMTRYDC2X Parsley ty ATGCACACAAGTGGTTTTTGACAACTTAGACTGTTTGTGCTTTTGGGTGAGGGAATCCGAGTCTCTTAT  
M96069.1|PUMTRYDC1X Parsley ty ATGCACACAAGTGGTTTTTGACAACTTAGACTGTTTGTGCTTTTGGGTGAGGGAATCCGAGTCTCTTAT  
XY096475.1| Arabidopsis thalia ATGCTCACAAATGGTTCCTCACACTTGGATTGTTCCCTTTTGGGTAAAGGATCAAGATTCTCTTAC  
Clustal Consensus \*

R2

U16804.1|PSU16804 Papaver somm  
M95685.1|PUMTRYDC4X Parsley ty CAAAGCCTTATCCCAAAGCCAGAGTATTTGAAGAACAAAGCAACTGATTCAAAGCAGGTTATTTGATTAC  
M96070.1|PUMTRYDC2X Parsley ty AAGTCTCTTTCCACATATCCGAGTCTTTGAAGAAATATGCTAGTGAAAACAAACAGGTTGGTGAATAT  
M96069.1|PUMTRYDC1X Parsley ty AAGTCTCTTTCCACATATCCGAGTCTTTGAAGAAATATGCTAGTGAAAACAAACAGGTTGGTGAATAT  
XY096475.1| Arabidopsis thalia AAGTCTCTTTCCACATATCCGAGTCTTTGAAGAAATATGCTAGTGAAAACAAACAGGTTGGTGAATAT  
Clustal Consensus \*

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AY096475.1| Arabidopsis thalia ATTAGCTCTTCAACAAATCCAGAGTTTCTCAAAAACAAGGCCCTCAGGCAAAC TTGGTTTGATTAC
Clustal Consensus
* * * * *
R2
1200 1210 1220 1230 1240 1250 1260
AAAGATTGGCAAATAGCACTCAGCAGAAGATTTAGGTCAATGAACTTTGGTTAGTACTTCGAAGCTATG
M95685.1|PUMTRYDC4X Parsley ty AAAGCTGGCAAATATATTTGAGCAGGCGATTTCGAGCATTGAATTATGGTTTGATTTGAGAAGCATATG
M96070.1|PUMTRYDC2X Parsley ty AAAGCTGGCAAATATATTTGAGCAGGCGATTTCGAGCATTGAATTATGGTTTGATTTGAGAAGCATATG
M96069.1|PUMTRYDC1X Parsley ty AAAGCTGGCAAATATATTTGAGCAGGCGATTTCGAGCATTGAATTATGGTTTGATTTGAGAAGCATATG
AY096475.1| Arabidopsis thalia AAAGTTGGCAAATCCCTCTCGGACGAAGATTCAGATCATTGAACTATGGATGGTTTAAAGGCTCTATG
Clustal Consensus
*****
1270 1280 1290 1300 1310 1320 1330
GAGTTGCTAATCTBAGAAGTTCTCGAGGAGCCATGTGAAGATGGCTBAGCATTCGACGGGCTCATAGC
M95685.1|PUMTRYDC4X Parsley ty GAGTTGGTCAGCTTGAGGGATTCATAAGAGGGGCATGTAGGCATGGCCAAGTATTTTTGAGGGTCTAGTGGG
M96070.1|PUMTRYDC2X Parsley ty GAGTTGGTCAGCTTGAGGGATTTATAAGAGGGGCATGTAGGCATGGCCAAGTATTTCGAAGGGCTAGTGGA
M96069.1|PUMTRYDC1X Parsley ty GAGTTGGTCAGCTTGAGGGATTTATAAGAGGGGCATGTAGGCATGGCCAAGTATTTCGAAGGGCTAGTGGG
AY096475.1| Arabidopsis thalia GATCTGAGACCTTGAAGAGCTATATAGAAAACCATATCAAACTGGCTAAAGAATTCGAACAACTTTCTCC
Clustal Consensus
** * * * * *
1340 1350 1360 1370 1380 1390 1400
AATGGACAAAAGATTTGAAATAGTGGTTCTTACACACTTCGCCATGGTGTCTTTCGATTAAAACCCGCT
M95685.1|PUMTRYDC4X Parsley ty CTTGGACAAGAGGTTCGAAGTTGTGGCCGAGACTTTTTCCAATGGTCTGTTTCGGATTAAGCCATCT
M96070.1|PUMTRYDC2X Parsley ty CATGGACAAGAGGTTCGAAGTTGTAGCTCCTAGACTATTTTCTATGGTCTGTTTTAGGATAAGCCATCT
M96069.1|PUMTRYDC1X Parsley ty CATGGACAAGAGGTTCGAAGTTGTGGCCTAGACTATTTTCAATGGTCTGTTTTAGGATAAGCCATCT
AY096475.1| Arabidopsis thalia TCAAGATCCTAACTTCGAGATGTTCACACCTCGGATCTTTGTCTTTGTCTGTTTCCGCCTGTGC---T
Clustal Consensus
* * * * *
1410 1420 1430 1440 1450 1460 1470
GCCATTTTCCAACGGAAAATTAGGTGAAAATGGCGTGGACTACAATTGCATTGAGGGAGAAAACAAATGAGA
M95685.1|PUMTRYDC4X Parsley ty GCCATGTATC-----GGAAAAATGA-----TGAGATGAAGTAATGAAA
M96070.1|PUMTRYDC2X Parsley ty GCCATGTATC-----GGAAAAATGA-----TGAGATGAAGTAACGAGA
M96069.1|PUMTRYDC1X Parsley ty GCCATGTATC-----GGAAAAATGA-----TGAGATGAAGTAACGAGA
AY096475.1| Arabidopsis thalia GTGA-----AAGACGA-----AGAAAGAGAGCTTAATTAAC
Clustal Consensus
* * * * *

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## Sequence used for Polyphenol oxidase primer preparation

AF263611.1| Populus balsamifer  
 AY515506.1| Triticum aestivum  
 AJ309175.1| Ipomoea batatas pa  
 Z11702.1| V.faba mRNA for poly  
 Clustal Consensus

ATTGAGACCCACCCCTCACAAATAATTCACAGA--TGGACCGGTGATCCAACCTCAAGAA  
 CTGGAGAACGTGCCGCCAGGCA--CGGTCCACGTC--TGGACTGGCGACCCGGCCAGCCG  
 TCGTACACATATTTAGATAATGGCTGCTCATACAGGTGGTGGGTGACCCGAGAAACACC  
 ATAGAGAAATGTTCCACATGCAC--CCGTTCACT--TGGACAGGTGACAAATCTCAAAC  
 \* \* \* \* \*

F1

AF263611.1| Populus balsamifer  
 AY515506.1| Triticum aestivum  
 AJ309175.1| Ipomoea batatas pa  
 Z11702.1| V.faba mRNA for poly  
 Clustal Consensus

1150 1160 1170 1180 1190 1200  
 AATAACGAAGACATGGGCAATTTTACTCAGCTGCGAGAGATCCAATATTTTTTGTCTAT  
 AACCTTGAGGACATGGGCAACTTCTTCTCGCGCGCGCGACCCCACTTCTTCTCGGCAC  
 AACCAATGAAGACATGGGCAACTTCTACTCGCGCGGAAAGGACATAGCCTTCTACTGCCAC  
 AACATTGAAGACATGGGAAATTTTCTATTCACTGCAAGAGATCCTATTTTTACTCTCAC  
 \*\* \* \* \* \* \*

AF263611.1| Populus balsamifer  
 AY515506.1| Triticum aestivum  
 AJ309175.1| Ipomoea batatas pa  
 Z11702.1| V.faba mRNA for poly  
 Clustal Consensus

1210 1220 1230 1240 1250 1260  
 CACTCAAATGTTGACCGAATGTTGGACGATATGGAAGACTATACCTTGA---GGAACTAGG  
 CACGGCAACATCGACCCGCTGTGGCAGCTCTGGCCCG---CCTCCG---CCGAGCAAC  
 CACTCAAATGTTGACCGTATGTTGGCCATATGGCAGCAGCTCGCCGGAAACCAAGAAAG  
 CATTCCAACTGATAGGTTATGTTATATATGGAAGAAC---ACTTGGT---GGGAAAAA  
 \*\* \* \* \* \* \*

F2

AF263611.1| Populus balsamifer  
 AY515506.1| Triticum aestivum  
 AJ309175.1| Ipomoea batatas pa  
 Z11702.1| V.faba mRNA for poly  
 Clustal Consensus

1270 1280 1290 1300 1310 1320  
 AGGGATATCAGTGATCCTGATTTGGCTTAATTCAGAGTTTCTTTTCTATAATGAGAAAGCA  
 ACCGACTTACCAGCCCGACTGGCTCGACGCGCGCTTCTCTCTACGACGAGGAGGCC  
 CGAGACTACCCGATTCGACTGGTTAAACGCCCTTCTCTTCTACGACGAGAAAGGC  
 CATGATTTCACTGACAAAGATTTGGTTAGAATCAGGGTTTCTTTTCTATGATGAGAAAG  
 \*\* \* \* \* \* \*

AF263611.1| Populus balsamifer  
 AY515506.1| Triticum aestivum  
 AJ309175.1| Ipomoea batatas pa  
 Z11702.1| V.faba mRNA for poly  
 Clustal Consensus

1330 1340 1350 1360 1370 1380  
 GAGCTTGTTCGTTGTAAGGTTAGCGATTGCTTGAACAATACAGGGCTAAGGTACTTTAT  
 CGCCCCGTGCGCGTGCCTGCGGACTGCTCGACCCGCGCGCTGCGGTACACGTAC  
 CAGGCAGTGAAGGTCGGATTGGAGACTCCCTAGACAAACGAAAAATGGGTACAAATAC  
 AATCTTGTAGGTTAATGTGAAGGACAGTCTTGACATAGATAAACTAGGTTATGCTTAT  
 \*\* \* \* \* \* \*

AF263611.1| Populus balsamifer  
 AY515506.1| Triticum aestivum  
 AJ309175.1| Ipomoea batatas pa  
 Z11702.1| V.faba mRNA for poly  
 Clustal Consensus

GAGCTTGTTCGTTGTAAGGTTAGCGATTGCTTGAACAATACAGGGCTAAGGTACTTTAT  
 CGCCCCGTGCGCGTGCCTGCGGACTGCTCGACCCGCGCGCTGCGGTACACGTAC  
 CAGGCAGTGAAGGTCGGATTGGAGACTCCCTAGACAAACGAAAAATGGGTACAAATAC  
 AATCTTGTAGGTTAATGTGAAGGACAGTCTTGACATAGATAAACTAGGTTATGCTTAT  
 \*\* \* \* \* \* \*

AF263611.1| Populus balsamifer  
 AY515506.1| Triticum aestivum  
 AJ309175.1| Ipomoea batatas pa  
 Z11702.1| V.faba mRNA for poly  
 Clustal Consensus

1390 1400 1410 1420 1430 1440  
 CAAAAATGTTGAAATTCCTTGGCTAGAAATCAAAACCAATTCGAGGAGG  
 CAGGACGTCGCGCTGCGTGGCTCAACGCCAGGCGCG---CAAAGCGG  
 GCCAAAAACCCCTCCCATGGCTCGACTCAAAGCCAGTCCCCA---C  
 CAAGATGTTCCATTCATGGGAAAAAGCTAAACCTGTGCCAGGAAACAAAAGTACAA  
 \* \* \* \* \*

AF263611.1| Populus balsamifer  
 AY515506.1| Triticum aestivum  
 AJ309175.1| Ipomoea batatas pa  
 Z11702.1| V.faba mRNA for poly  
 Clustal Consensus

1450 1460 1470 1480 1490 1500  
 GTTGGG---AAAGAAAGCAGCTGAAACAAA---AACTGCATT  
 TCCGGCGGACGCCGCGCCCG---C  
 CAAGAAAGAGGGCGGCTACGCTCCAAAGTCCAAGGCCCCCTT  
 AAATTTGGTGGAGTTGAGGTTAATGATGGCAACTTAAGAAAATCACCAGCTATCTTTCTA  
 \* \* \* \* \*

AF263611.1| Populus balsamifer  
 AY515506.1| Triticum aestivum  
 AJ309175.1| Ipomoea batatas pa  
 Z11702.1| V.faba mRNA for poly  
 Clustal Consensus

1510 1520 1530 1540 1550 1560  
 AACCCCGATCA---CTGCATTCCCTTTAGTCTTAGACAAAACCCATA  
 CACAACCGG---TACCTTCCCTGCCACCTTGGACAGGACCCATA  
 CGTCGCCAG---CGTCTTCCCGTACGCTCGACAAAGGTCGTC  
 GTTCGACAAAGAGTCCAAGAAAATATGTTTACGTTTCCATTGGTTTGAATAATAAAGTG  
 \* \* \* \* \*

R2

AF263611.1| Populus balsamifer  
 AY515506.1| Triticum aestivum  
 AJ309175.1| Ipomoea batatas pa  
 Z11702.1| V.faba mRNA for poly  
 Clustal Consensus

1570 1580 1590 1600 1610 1620  
 GTCACTGTAGTTTCAAGACCAAGAAAATCAAGAAAGCAGGAAAAGAGAAAGAGGAAAGAT  
 CGGGTGAAGGTGACAGGCGCAGAGTGTCTCAGGAGCGCCGCGGAGAAAGGAGGAGGAG  
 CAAGTCAAGGTCGCTCGGCCAAGAAATCCAGGTCGGCAGAGAGAAAGGAGGCGGAGAA  
 AGTGTATTGTGAAGAGGCCAAGAAAATTAAGGAGCAAGAAAAGAGAAAGGAGGAGGAA  
 \* \* \* \* \*

AF263611.1| Populus balsamifer

1630 1640 1650 1660 1670 1680  
 GAAGTTTGTAGTATAGAGGGATAGAATACG-----ACAAGGCCAAATTCGTGAAGTTT

## R2

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AY515506.1| Triticum aestivum      GAGGTGCTGGTCGTGGAGGGGATCGAGATCGCCGACCATTTCAACAAGTTCGTCAAAGTTC
AJ309175.1| Ipomoea batatas pa    GAAATCTTGCCTTAGAAGGCGTTGAACTGG-----AAATTGACAAGTACGCCAAGTTC
Z11702.1| V.faba mRNA for poly   GAGGTTTTAGTGATTGAGGGGATTGACTTTT-----ATATGAATATAGCCATTAAAGTTT
Clustal Consensus                 ** * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

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                                1690      1700      1710      1720      1730      1740
AF263611.1| Populus balsamifer    GACGTGTTCCATAAAATGA---TGACGTTGAGATGCCTTCTAAAACGAGAAAATACAGAG---
AY515506.1| Triticum aestivum    GACGTGTTGGTGAACGAGCCCGAGGGCGGAGTGGGCAGCACCGGCGCAGGGCGACGGGG
AJ309175.1| Ipomoea batatas pa    GACGTTTACCTGAACGA---CTGGGACGACCCGAGCGGGCGGCAAGGCGGGAG---
Z11702.1| V.faba mRNA for poly   GATGTTTATATTAATGA---TGAAGATGATAAGG---TTGGGGCAGGGAAATCTGAG---
Clustal Consensus                 ** * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

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                                1750      1760      1770      1780      1790      1800
AF263611.1| Populus balsamifer    ---TTTGTGGGAGCTTTGTTAATGTTTCTCA---T---AAGCATGCGAAGAAGTCCA
AY515506.1| Triticum aestivum    TACTGCGCTGGGAGCTTCGCGCATCGCCGCATGGTCCGGCCGAGGAGATGAGGAAAG
AJ309175.1| Ipomoea batatas pa    ---TACGCCGGGAGCTCTGCGCATCTCCCCCA-----CAAGCAAAAGGGGATG
Z11702.1| V.faba mRNA for poly   ---TTTGTGGAAGCTTTGTGAATATCCCTCA-----TTCCGCACATGCAACAAGAAACA
Clustal Consensus                 * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

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                                1810      1820      1830      1840      1850      1860
AF263611.1| Populus balsamifer    AGAC AAGA-----TTGATATTGGGGATTAAGAAATTTGGGAAGACTTAGAAAAGT
AY515506.1| Triticum aestivum    GGACCGETCAAGACGGTGGCGAGGTTGGCGTGTGCGACCTGATGGACGACATCGGGGCG
AJ309175.1| Ipomoea batatas pa    AAGAAAGTCAAGACTACTCTCAGCCTGGGCTGAATGAGCCGCTCGAGGATTTGGGCGCC
Z11702.1| V.faba mRNA for poly   AGAA GATTATTACTTCTTTAAGAATTGGTATAACTGATTTGTTGGAAGATTTGCATGTC
Clustal Consensus                 * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

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## R1

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                                1870      1880      1890      1900      1910      1920
AF263611.1| Populus balsamifer    GATGGTGTGATAGCATTGTTGGTGGCTTGGTGCCCAAGTCTAATAGTGTAGTGATCCT
AY515506.1| Triticum aestivum    GACGACGACCGACGCGTGGTGGTGTGCTCGTACCCCAAGT-----GCGGCGGTGAGCTG
AJ309175.1| Ipomoea batatas pa    GAGGACGACGACACTATTCGGTGGCTTGGCTCCAAAAG-----TTGGCGGCGGTGTT
Z11702.1| V.faba mRNA for poly   GAAGGCGATGATAATATTGTTGTTACATTGGTTCCAAAAT-----GTGGGAGTGGACAA
Clustal Consensus                 * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

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## תקציר

קטכולאמינים (דופמין, נוראפינפרין ואפינפרין) הם קבוצה של אמינים ביוגניים המעורבים בהעברת אימפולסים עצביים במערכות עצבים של בעלי חיים שונים. האנזים טירוזין הידרוקסילז (TH) הוא האנזים הקובע את קצב הייצור של קטכולאמינים בבעלי חיים ומביא ליצרת L-דופה אשר משמש כסובסטרט של דופמין וקטכולאמינים אחרים.

דופמין נמצא בכמויות גדולות בקליפה של בננה ובכמויות נמוכות יותר בציפה. גם התפקיד וגם הסינתזה של דופמין בצמחים אינה ידועה. שני מסלולים לביוסינתזה של דופמין הוצעו בצמחים; האחד דרך ייצור של L-דופה בדומה לקיים בבעלי חיים, והאחר דרך ייצור הטיירמין על ידי האנזים הראשון בתהליך המבצע דקרבוקסילציה של טירוזין ואח"כ אוקסידציה נוספת כנראה על ידי אחד מהאנזימים של PPO-פוליפנול אוקסידז.

לתכולת שני הפרקורסורים של דופמין: טרמין ו L-דופה, יש השפעה מרעעת על בריאות האדם, משום שמוצרים עשירים ב L-דופה עשויים להחליף את הטיפולים המכילים L-דופה, כנגד מחלת הפרקינסון. בנוסף, רמת טירמין גבוהה עלולה לגרום לנזק חמור לאנשים הלוקחים את החומר MAOI (מעכב של מונואמין אוקסידז), כטפול נגד דיכאון.

מטרתה של עבודה זו היא לבדוד את הגנים עבור האנזימים המעורבים בביוסינתזה של דופמין בבננה. העבודה שלנו מבססת את הממצאים שרמה גבוהה יותר של דופמין קיימת בקליפה בהשוואה לציפה. רצפים חלקיים המקודדים לאנזימים טירוזין דקרבוקסילז ופוליפנול אוקסידז בודדו מהזן הנפוץ של בננה מקבוצת הקבנדיש "גרנד ניין" והביטוי של הגנים נלמד בהשוואה לפרמטרים של הבשלת הפרי. בזן הזה ובעוד שישה זנים שונים של בננה, בדרך כלל, הביטוי של שני הגנים היה גבוה יותר בקליפה בהשוואה לציפה. השיבוט של הגן המקודד לטיירוזין הידרוקסילז מ"גרנד ניין" לא הצליח, גם כאשר השתמשנו בזנים האחרים או בזן של שעועית "מוקונה", בה קיימת רמה גבוהה של L דופה. יחד עם זאת, באמצעות שימוש בנוגדנים כנגד טירוזין הידרוקסילז אנימלי, נמצא חלבון המגיב לנוגדנים בקליפה של בננה. הגן לטיירוזין דקרבוקסילז הראה רמה גבוהה במיוחד בפירות ירוקים, בעוד רמת הפוליפנול אוקסידז היתה הגבוהה ביותר בשלב הפיק הקלימקטרי של ייצור האתילן. עדיין לא ברור אם ביטוי הגן של פוליפנול אוקסידז אכן מבטא את פעילותו, משום שהגן שייך למשפחה של גנים, כפי שנמצא בצמחים אחרים. התוצאות שלנו מראות שיתכן וכל האנזימים הללו משתתפים בתהליך ייצור הדופמין בבננה משום שבכולם היתה רמה גבוהה יותר בקליפה בהשוואה לציפה, ובכולם נמצאה ירידה בשלב שלאחר השיא הקלימקטרי, מה שנמצא במתאם לרמת ייצור הדופמין. העבודה הזאת מניחה את הבסיס להכנסת שינויים ברמת הקטקולאמינים בפירות הבננה ובמיוחד ברמת ה L-דופה והטיירמין, שלרמתם חשיבות רבה במזון.