Analysis of post-harvest deterioration in tuberous roots of

cassava (*Manihot esculenta* Grantz)

Jiang HUANG

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Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
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des namiddags om 13:30 uur in de Aula

Propositions

1. Postharvest physiological deterioration (PPD) is an inherent main constraint for cassava since at present wounding and mechanical damages cannot be prevented during the process of harvesting and handling of tuberous roots (this thesis).

2. The development a good of transformation system for cassava, the availability of genes that trigger or control the process of PPD and the public acceptance are three important pre-requisites to overcome the PPD problem by genetic modification (this thesis).

3. In plants, it is expected that programmed cell death (PCD) is necessary for growth and survival or in response to environmental stimuli and can occur on a local or large scale (Barlow, 1982. Science 260: 309-310).

4. The traditional baker's yeast *S. cerevisiae* is less suited for high productivity of heterologous proteins than more unconventional yeasts, like *K. lactis* (Gelissen, G. and Hollenberg, C.P., 1996. Gene 190: 87-97).

5. Understanding the history of life becomes less hypothetical when fossils are available (Allard, R.W., 1999. Principle of Plant Breeding, 2nd edition, pp3-12).

6. Sometimes, science is just like one of those riddles, where the answer is not only at the end of road, but on it as well.

7. Digital technology applications in daily life make people more enjoyable at home and easier reachable.

8. Experience is not only what happens to you, it could be what you deal with what happens to you (Aldous Huxley).

9. There is no story without coincidence.
   無巧不成書 (Chinese proverb).

10. The fact that more Chinese students are coming to Wageningen University will not automatically lead to Wageningen University becoming more popular in China.

Stellingen behorend bij het proefschrift: "Analysis of postharvest deterioration in tuberous roots of cassava (*Manihot esculenta* Grantz)" door Jiang HUANG. Wageningen, 3 december 2001.

謹 以 此 書 獻 給

敬 愛 的 父 母

可 愛 的 賢 妻

親 密 的 姐 姐

For my family

Bibliographic Abstract

This thesis deals with the analysis of postharvest physiological deterioration (PPD) in cassava tuberous roots at the physiological, biochemical and molecular level. By setting up a uniform visual system to monitor the induction and maintenance of PPD under the experimental conditions, amongst others physico-chemical properties of starch were characterized. Furthermore using this system, around 6,000 TDFs (transcript derived fragments) were screened via 100 primer combinations using the cDNA-AFLP technique. 70 TDFs showing an up-regulated, a down regulated or a transiently expression pattern were isolated following the first 72 hours of PPD. Based on the sequence information, a functional catalogue of these TDFs was established. By concentrating on enzymes possibly involved in oxidative stress, biochemical results indicated that PPD may be a peroxidase-mediated process. Using a reverse genetics approach, the putative cassava dad1 (defender against cell death gene 1) homologue was transformed into cassava FEC (friable embryogenic callus) lines. Transgenic plants were produced and characterized.

CONTENTS

Chapter 1

GENERAL INTRODUCTION

POSTHARVEST PHYSIOLOGICAL DETERIORATION IN CASSAVA TUBEROUS ROOTS

1

Cassava: an important crop in the tropical and sub-tropical regions

Cassava (*Manihot esculenta* Crantz) is a member of the family of Euphorbiaceae, and is one of the oldest cultivated crops by human beings although the early history of cassava is still a mystery (Gulick, *et al.*, 1983). It was reported that cassava has been cultivated in northern Amazonia more than 1000 years ago (Jones, 1959). Recently it was postulated that cassava is likely to be originated from wild *M. esculenta* populations along the southern region of the Amazon basin (Olsen, *et al.*, 1999 and 2001). Cassava is a perennial shrub, and in general the tuberous roots can be harvested from 6 months to a year after planting. However, the tuberous roots can remain under the ground for longer periods before harvesting (Wenham, 1995). Normally stem cuttings are used to vegetatively propagate cultivated cassava. Traditional superior genotypes have been maintained as clones and the majority of wild species seem to be seed propagated (Gulick, *et al.*, 1983). Cassava is an allotetraploid crop with 36 chromosomes (2n = 36) (Bai, 1987, Kalloo, *et al.*, 1993), and its genome has a relatively high G + C content (Wong, *et al.*, 1999).

Cassava is a very important tuberous root crop in the tropical and sub-tropical regions in the world. Cassava is the world's fourth largest source of calories after rice, sugar cane and maize, feeding more than 500 million people in Africa, Asia and Latin America (Cock, 1985a, Best and Henry, 1994). In these regions, cassava can be used as food for human beings and feed for animals as well as a raw material for industrial purposes. The production of cassava tuberous roots was calculated around 172.4 million metric tons in developing countries in 1993, and it is estimated that the yield will reach around 290.3 million metric tons by 2020, which value is worth about 13,937 million US$ (Scott, *et al.*, 2000a). Beside the tuberous roots, cassava leaves can also provide an important source of vitamin A in some of the developing countries. Cassava can produce more dry matter per hectare than other root and tuber crops like potatoes, sweet potatoes or yams (Scott, *et al.*, 2000a & 2000b). As one of the most efficient starch - producing crops in the world, the productivity of cassava can be achieved by using minimum inputs (Nestel and Cock, 1976). Cassava can grow under diverse conditions such as low soil fertility, high acidity soil and drought, where other crops like maize cannot grow. Especially, cassava can make use of limited resources in marginal agricultural land, which will attract small farmers' attention. With the rapid increase of the population in the 21st century, it is estimated that food production must be doubled by the year 2025, and

nearly tripled by 2050 in order to meet food needs in the future. Cassava, with its unique advantages, could contribute to the global food system to fit such enormous increases in food needs and to achieve food security in the 21st century.

Cassava: the most efficient producer of starch

The tuberous roots of cassava are rich in starch, which constitutes 85% of the root tissue based on the dry-weight content (Cock, 1985b). Cassava has higher starch contents than other tuber and root crops, and has a higher starch extraction ratio as well (Scott, *et al.,* 2000a). Generally speaking, a natural and good quality starch will include the following properties: absence of color and taste, high quality texture, ease of gelatinization, resistance to industrial treatments and high digestibility. Cassava starch qualifies for some of these properties such as high maximum viscosity (but lower than potato) and low gelatinization temperature. It is known in Europe and the USA that cassava is the term usually applied to the tuberous roots, whilst tapioca is the name used for the starch and other processed products from cassava tuberous roots. Cassava starch can be widely applied in many industrial uses such as food processing, paper, textile and adhesive manufacturing and in oil drilling (Kay, 1987). Starch can also be used as a raw material for producing many derived sugar products like glucose, fructose, maltodextrins and mannitol, which have different specific characteristics and uses in food, chemical or pharmaceutical industry (Balagopalan, *et al.,* 1988). Cassava starch can even be used as a source for baby foods in developing countries (Hamaker, *et al.*, 1991, Pardio Sedas and Waliszewski Kubiak, 1994).

There are many cassava varieties grown in the different parts of the world, but generally they fall into two main categories, named bitter and sweet cassava depending on their contents of cyanohydrin (Douglas and William, 1984). For industrial purposes bitter varieties are most frequently used, because of a generally higher starch content. Sweet cassava is preferred for food due to taste and its dough forming ability. Although cassava is one of the most efficient starch producers among tuber and root crops, cassava starch only occupies a small percentage of the amount of internationally traded starch (Wenham, 1995).

Postharvest deterioration: an inherent main constraint for cassava

One of the major problems limiting the possibilities of production, distribution and processing for cassava is the rapid deterioration in cassava tuberous roots after harvesting, which results in the problem that the tuberous roots have a very short storage life. Postharvest deterioration is an inherent constraint for cassava since wounding and mechanical damage cannot be prevented during the process of harvesting and handling of the tuberous roots. The final result of the rapid postharvest deterioration in the tuberous roots renders cassava totally unacceptable for human and animal consumption, as well as for industrial uses such as a processing resource for starch extraction. It is calculated that losses caused by postharvest deterioration are up to 25 percent of cassava tuberous roots after harvesting in the world (Jansen & Wheatlley, 1985, Wenham, 1995).

Description and observation of PPD (postharvest physiological deterioration)

Postharvest deterioration in cassava tuberous roots is made up of two distinct types of deterioration, namely primary deterioration and secondary deterioration (Booth, 1975 and 1976). Primary deterioration, which is a complex physiological and developmental process and also called postharvest physiological deterioration (PPD), is initiated within 24 hours to 48 hours after harvesting cassava (Wheatley, 1982, Plumbley & Rickard, 1991, Wenham, 1995). The process of PPD in cassava tuberous roots depends on the cassava genotype and environmental conditions under which it is grown (Booth, 1975, Wheatley, 1982, Wenham, 1995). Secondary deterioration is the consequence of the former process and involves the actions of micro-organisms (like bacteria and fungi), which starts from 5 to 7 days after harvesting (Booth, 1975 & 1976, Hirose & Data, 1984, Plumbley & Rickard, 1991). PPD in cassava tuberous roots is observed at the beginning as " vascular streaking ", which is described as a fine blue-black or brownish discoloration appearing as a ring around parenchymatous tissue in transverse sections (Averre, 1967, Mondaldo; 1973, Hirose, 1986). Afterwards the black color spreads rapidly on the whole surface of the tuberous roots, and this is called " vascular discoloration ". When checked under UV light, fresh cut cassava tuberous roots display a blue-violet fluorescence (Hirose and Data, 1984, Rickard, 1985). However, regions below the wounded tissue show a strong bright blue fluorescence, which increases in both intensity and size on the wound surface with time. The strong blue – yellow fluorescence develops in advance of the

appearance of visual symptoms of discoloration, but disappears in the areas where the discoloration has started (Hirose and Data, 1984, Rickard, 1985).

Traditional storage techniques for cassava tuberous roots

The best way to store cassava tuberous roots is to leave them in the ground for a long period (up to 3 years) until required, and then cassava may be harvested (Scott, *et al.,* 2000a). With regards to two essential requirements of any storage system, at least, it is necessary that the product should lose as little weight as possible during storage and it must be of acceptable quality after storage. There are several disadvantages, however, of leaving cassava tuberous roots in the ground. First, this may unnecessarily occupy a large field. It was estimated that it would cost three quarters of a million hectares of agricultural land to keep all cassava tuberous roots in the soil (Ingram and Humphries, 1972). Second, it will lead to extra losses due to increasing the possibility of pathogens attacking when the tuberous roots remain in the ground too long (Ingram and Humphries, 1972). Furthermore, although the tuberous roots may continue to increase in size, they become more fibrous and woody during the period of storage (Rickard and Coursey, 1981). Also cassava tuberous roots cooking time increases due to this storage (Wheatley *et al.,* 1985) and the content of extractable starch decreases (Booth and Coursey, 1974). Another simple storage method is to treat cassava tuberous roots with fungicide before packing them into plastic (polyethelene) bags, which can also delay the onset of PPD in cassava (Wheatley, 1989). However, this will cause environmental pollution and result in higher costs. Only in a few instances the successful storage of fresh cassava tuberous roots on a small scale has been recorded using high-cost systems, such as refrigeration and waxing (Singh and Marthur, 1953, IIT, 1973). Considering the conditions under which much of the world's cassava is grown, such techniques cannot be regarded as being generally widely applicable at present. Therefore, so far there is no good solution for the problem of PPD in cassava in the world.

Mechanisms of PPD

PPD is a complex developmental and physiological process accompanying the last phase of cassava tuberous root life cycle. The susceptibility of PPD is depending not only on a genetic component in cassava cultivars, but also on environmental effects such as soil conditions, relative humidity and temperature

where they are grown (Wheatley, 1982). Up to this date, however, the molecular mechanisms and biochemical pathways of the process of PPD in cassava tuberous roots are only poorly understood.

PPD is stimulated by wounding or mechanical damage in cassava tuberous roots. In most plants, wounding and/or damage of tissue leads to a series of complicated wound responses that finally result in recovery from a wound and return to more normal physiological status. Cassava tuberous roots can reveal such a typical wound - healing response only at conditions of relative humidity (RH) of around 80% to 90%. Under this condition PPD can be suppressed since a periderm layer will be formed in cassava tuberous roots. But this response is much slower compared with other tuber and root crops like sweet potato and yam (Passam, *et al.*, 1976). In environments with lower relative humidity (less than 80% RH), cassava tuberous roots can not develop this periderm layer and PPD initiates rapidly after harvesting. There are changes in respiratory rate during PPD in cassava tuberous roots and two respiratory rate peaks have been measured and observed in which the former peak is assumed due to the wounding, while the later peak is supposed to be related to PPD occurring in cassava tuberous roots (Hirose, 1986).

Previous research indicated that secondary metabolites like polyphenolic compounds, are related to the process of PPD in cassava tuberous roots (Plumbley and Rickard, 1991, Wheatley, 1982, Uritani, 1998 and 1999). It has been demonstrated that polyphenolics increased during the PPD process in cassava, notably scopoletin, scopolin and coumarins. Especially, the production of scopoletin and some of the scopolin related compounds seem to contribute to the increased fluorescence of discoloration during the process of PPD in cassava tuberous roots (Rickard, 1981, Buschmann, *et al.*, 2000a).

Several studies have shown that oxygen is necessary for initiation of the process of PPD (Averre, 1967, Booth, 1976 and 1977, Rickard, 1982). It has been assumed that oxidative stress is involved in PPD (Reilly, *et al.*, 1999). Other researchers even suggested that PPD in cassava is an oxidative, perhaps peroxidase - mediated process (Wheatley and Schwabe, 1985). Total peroxidase activity increased during PPD and novel peroxidases have been suggested to be induced following the process of PPD in cassava (Marriott, *et al.*, 1979 and 1980, Plumbley and Rickard, 1980).

There are many kinds of metabolism that are activated during PPD in cassava. It has been found that sugar contents increased and the amount of starch decreased during the process, while amylase activity was found to be present following the discoloration in cassava tuberous roots (Maini and Balagopal, 1978). It has also been shown that there are many changes in protein level which are not only caused by degradation and synthesis of existing proteins but also by *de novo* production of novel proteins (Beeching, *et al.,* 1994, Wenham, 1995, Uritani, 1998 and 1999). It was also reported that the amount and composition of membrane lipids were changing following the deterioration in cassava tuberous roots, which was hypothesized to be due to membrane disorganization or membrane degradation during the deterioration process in cassava tuberous roots (Tanaka, *et al.*, 1983, Lalaguna and Agudo, 1989).

There is little research carried out on the signalling pathway or possible involvement of hormones during PPD in cassava tuberous roots. It has been reported that ethylene production occurred following the process of PPD (Plumbley, *et al.,* 1981). But the results suggested that ethylene did not show a direct relation to the development of PPD in cassava (Wenham, 1995). It was also found that by pre-pruning treatments, which means leaving cassava stems of around 20 - 30 cm above the ground by cutting, PPD in tuberous roots can be delayed after harvesting two to four weeks later (Lazano, *et al.*, 1978, Wheatley *et al.,* 1985). This pruning treatment decreased dry matter (DM) and starch content in cassava tuberous roots (van Oirschot, *et al.*, 2000). However, it was also found that this pre-harvest treatment did not show significant effects on ethylene production following the process of PPD in cassava (Hirose, *et al.,* 1984).

Modern biotechnology: a potential powerful tool to improve quality traits for cassava

Previous results indicated that there is only a rather small proportion of the cassava varieties that did not show deterioration within one week after harvesting the tuberous roots (Wenham, 1995). Cassava, however, is a highly heterozygous allotetraploid crop, which makes it more difficult using traditional breeding methods to solve the PPD problem in cassava. Modern biotechnology, like genetic modification may provide a potential powerful tool to improve quality traits of this crop and/or create an improved new cassava genotype.

To develop a good transformation system and to isolate genes that trigger and/or control the process of PPD are two important pre-requirements for genetic modification. The first break-throughs for genetic transformation in cassava were reported in 1996 (Li, *et al.,* 1996, Raemakers, *et al.,* 1996, Schöpke *et al.,* 1996). The transgenic plants were produced either by microprojectile bombardment using friable embryogenic callus (FEC) lines (Taylor, *et al.,* 1996) or by transformation of cassava somatic embryos derived from cotyledons with *Agrobacterium tumefaciens* and later forming adventitious shoots. In our lab, the progress of optimal transformation bombardment protocols for cassava is still on going (Snepvangers, *et al.,* 1997, Muniyikwa, *et al.,* 1998, Raemakers, *et al.,* 2001). Very recently, another efficient and reproducible method, which was developed via *Agrobacterium* – mediated transformation using cassava FEC lines as starting materials, has become available as well (Schreuder, *et al.,* 2001).

The goal of this research

Our research strategy is to improve quality traits of cassava tuberous roots and/or create an improved new cassava variety using modern biotechnology. This could be beneficial to sustainable economic development for small farmers in developing countries. Also this will contribute to facilitate the broader acceptance of cassava as a safe food product in the global food system for human beings and animals, as well as a resource of high quality of raw materials for industrial purpose. The purpose of this project is to gain more insight in the molecular mechanism of PPD in cassava and identify important metabolic pathways during PPD by isolating and identifying genes directly or indirectly involved in this process, focusing on the early stages of PPD in cassava tuberous root. Finally using reverse genetics approaches, it is anticipated that transfer of key genes in an antisense orientation will delay or even prevent the process of PPD in cassava tuberous roots.

Outline of this thesis

The general introduction gives a broad description of the importance of cassava, which may contribute to the global food system in order to meet the enormous food needs in the future. It is emphasized that PPD in cassava tuberous roots is an inherent problem for cassava, which limits the possibilities of production, distribution and processing for cassava. However, the molecular mechanisms and

biochemical pathways of PPD in cassava tuberous roots are little understood (Chapter 1).

Chapter 2 describes how to set up a visual system to analyze PPD in cassava. In order to assess the quality of this system, several parameters like the contents of protein, starch, soluble sugars and nucleic acids were measured. Starch, the most abundant component in cassava tuberous roots, was investigated. Some of the physico-chemical properties of starch during the process of PPD in cassava were characterized.

A prerequisite for applying molecular tools such as genetic modification to cassava is the isolation and characterization of genes, which trigger and /or control the process of PPD. In Chapter 3 we have investigated differential gene expression in a systematic way during the first 72 hours of the process of PPD by using cDNA-AFLP. A functional catalogue of transcription derived fragments (TDFs) was established. Through the data analysis, molecular insight of the process of PPD in cassava has been gained.

The results of Chapter 3 indicated that genes involved in oxygen scavenging play an important role during the process of PPD in cassava tuberous roots. Furthermore in parallel with the molecular research, a biochemical approach was chosen by focusing on characterization of peroxidases during the process of PPD in cassava tuberous roots (Chapter 4).

In Chapter 5 by taking the reverse genetic approach, the putative defender against cell death 1 (dad1) homologue from cassava was selected for transfer into cassava in an antisense orientation using particle gun bombardment. Transgenic plants were selected and analysis has been started.

The final chapter is a general discussion, in which the results obtained in this thesis are discussed. Possible future developments are also discussed if finally an improved quality of cassava by delaying or preventing the process of PPD in cassava tuberous roots by genetic modification has been achieved.

Chapter 2

POSTHARVEST PHYSIOLOGICAL DETERIORATION IN CASSAVA: A
GERNERAL DESCRIPTION OF THE PROCESS AND ITS EFFECTS ON
STARCH PROPERTIES

Huang, J., Vermeesch, A., Suurs, L., Jacobsen, E., and Visser, R. G. F.

Laboratory of Plant Breeding, Department of Plant Sciences, The Graduate School of
Experimental Plant Science (EPS), Wageningen University, P.O.BOX 386, 6700AJ
Wageningen, The Netherlands.

Abstract

Tuberous roots of cassava are rich in starch which can be used for food, feed and industrial purposes. One of the major problems limiting the possibilities of production, distribution and processing for cassava is the rapid deterioration after harvesting, which leads to a fairly poor storage life of cassava tuberous roots. Postharvest deterioration is an inherent constraint for cassava, which consists of primary deterioration, called postharvest physiological deterioration (PPD) and secondary deterioration. PPD is a physiological process, while secondary deterioration is the result of the former process combined with microbial actions. The aim of this research was to study the process of PPD on different (physiological, molecular and biochemical) levels, especially focusing on the early stages of PPD in cassava. Cassava tuberous roots slices of two genotypes (*M.Col 22* and *Faroka*) were cut and incubated under controlled conditions for various times (from 0 hr to 17 days). From these different samples several parameters were investigated like protein, nucleic acid, soluble sugar and starch contents during the process of PPD. These studies showed that fresh weight of the cassava tuberous roots was reduced mainly due to water loss (physical change). DNA contents decreased as well as starch contents during the process, while protein content remained stable. The contents of soluble sugars increased during a period of 7 days. Starch, being the main component of cassava tuberous roots on dry weight basis, was isolated from the tuberous root slices at different time points and used to determine some structural and physico-chemical properties. It was clear from the results that, although visible changes occurred like discoloration of the starch samples, the overall effect of PPD on starch quantity and quality as well as the structure was modest. Differences in rheological properties were only observed with starch samples isolated from day 12 or later.

Introduction

Cassava is a highly versatile tuberous root crop in the tropical and subtropical regions in the world, and it can grow under conditions such as low fertility, high acidity soil and drought, where other crops like maize are not suitable. In these regions, cassava is an important food source for humans and animals. Cassava is the world's fourth largest source of calories as a crop after rice, sugar cane and maize (Ingram and Humphries, 1972, Cock, 1985a, Taylor, *et al.*, 1999). The tuberous roots of cassava are rich in starch, which can be widely applied in many food and industrial uses such as food processing, paper, textile and adhesive manufacturing and in the oil drilling field (Kay, 1987). Starch, also is a kind of raw material for producing many derived sugar products like glucose, fructose, maltodextrins and mannitol, which have different specific characteristics and uses in food, chemical or pharmaceutical industry (Balagopalan, *et al.*, 1988). Cassava starch can even be used as a source for baby foods in developing countries (Hamaker, *et al.*, 1991, Pardio Sedas and Waliszewski Kubiak, 1994) since native cassava starch is more suitable for digestion than other starches, like for instance potato starch (Blanshard, 1994). There are many cassava varieties in the different countries where cassava is grown, but they can be roughly divided into two main categories with regard to industrial application, named bitter and sweet cassava depending on their contents of cyanohydrin (Douglas and William, 1984). For industrial purposes bitter varieties are most frequently used because of a higher starch content. Sweet cassava is preferred for food due to its taste and its dough forming ability. This is, however not absolute. As one of the most efficient starch producers among crops, cassava starch (also called Tapioca, see Chapter 1) makes up 85% of the tuberous root storage tissue based on dry-matter contents. However, tapioca only occupies a small percentage of the amount of internationally traded starch (Wenham, 1995).

One of the major problems limiting the possibilities of production, distribution and processing for cassava, is the rapid deterioration after harvesting, which leads to a fairly poor storage life of cassava tuberous roots. It is generally assumed that the quality of starch in cassava tuberous roots decreases due to rapid deterioration (Maini and Balagopal, 1978, Wenham, 1995). Postharvest deterioration is an inherent constraint for cassava since mechanical damage and wounding can not be prevented during the harvesting and handling process of the tuberous roots. Postharvest deterioration in cassava consists of primary deterioration, and is later

followed by secondary deterioration (Booth, 1976 and 1977, Hirose and Data, 1984). The primary deterioration, also called postharvest physiological deterioration (PPD), starts from 24 hrs to 48 hrs after harvesting the tuberous roots depending on the cassava genotype and the environmental conditions where cassava is grown (Booth, 1976, Wenham, 1995). PPD is a complex physiological developmental process, in which many changes in gene expression stimulated by mechanical damage and/or loss of water are thought to be involved (Booth, 1976, Wenham, 1995). The secondary deterioration is a consequence of the former process, and is caused by microbial (bacterial and fungal) actions within 5-7 days (Plumbley and Rickard, 1991). However, up to date, the biochemical pathway and molecular mechanism of PPD in cassava tuberous roots are rather poorly understood.

In this paper a general description about the PPD process in our experimental system, and several possible negative effects of the process of PPD in cassava tuberous roots are given. Since starch is the most abundant component in cassava roots, special emphasis is made on changes in the quantity and quality of starch due to the process of PPD as exemplified by some physico-chemical characterisations and structural analyses.

Materials and Methods

Cassava tuberous roots harvesting

The ca. 1.5 year-old growing cassava tuberous roots attached to the stem of the cultivars of *M.Col 22* (from *Colombia*) and *Faroka* (from *Indonesia*) were harvested carefully from the tropical greenhouse of Wageningen University without causing any extra damage to the tuberous roots. The tuberous roots were washed with tap water, followed by incubation in 75% ethanol for 5 minutes and rinsing with sterile water again. The clean cassava tuberous roots were cut into slices of 10 mm thickness inside a fume hood. The cassava tuber slices were put into petri-dishes with the covers on and incubated at 30°C with a relative humidity (RH) around 50%. After incubation for 0h, 6h, 12h, 24h, 36h, 48h, 72h, 96h, 6 days, 7 days, 12 days and 17 days respectively, the samples of the different time points were frozen immediately in liquid nitrogen, and stored at -80°C for later analysis.

Isolation of DNA, protein, starch, and soluble sugars

The plant materials were ground to a fine powder in a pre-cooled pestle with a mortar containing liquid N_2. DNA was isolated from 100 mg of the different samples by using DNA Isolation Kits (*BIOzym, The Netherlands*) and the concentrations were measured by using a spectrophotometer (*Pharmacia Biotech, England*) at wavelengths of 260 nm and 280 nm. Starch was isolated from 150 mg of cassava tuberous root powder following the method described by Kuipers, *et al.*, (1994). The contents of soluble sugars (sucrose, glucose and fructose) were also determined using Saccharose/D-Glucose/D-Fructose kits purchased from *Boehringer Mannheim* (*Germany*). According to the starch assay provided with the *Boehringer Mannheim* (*Germany*) Starch kits, 50 μl out of the supernatant from the last step was used for determination of the total amount of protein by using a Protein Assay ESL kit (*Boehringer Mannheim, Germany*). All the data from the different samples were adjusted to the changes of the fresh weight of the cassava slices during incubation.

Amylose content and size and morphology of starch granules

The apparent amylose content of starch samples was measured according to the description by Hovenkamp-Hermelink *et al.* (1989). Starch granule morphology was checked by light microscopy after staining the granules with iodine. Starch granule average size and size distribution was determined by a Coulter counter multisizer IIe. For scanning electron microscopy (SEM), dried starch granule samples were critical-point dried, sputter coated, and observed using a JSM-6300F Scanning electron microscope.

Rheological determinations

Dynamic rheological properties of 8% starch suspensions (dry weight) at small deformations were determined by applying a small oscillating shear deformation using a Bohlin CVO controlled stress Rheometer. The rheometer was equipped with a concentric cylinder measuring geometry C25, which consists of a rotating bob (inner cylinder) located in a fixed cup (outer cylinder) with the sample contained in the annular gap between them. The pasting profile of 8% starch : water (w/v) suspension was obtained by heating the suspension to 90°C, where it was kept for 15 min followed by cooling to 20°C at a rate of 2°C/min and holding again for 15

min at 20°C. This process changing over a temperature range can be characteristic for the type of starch. The structural changes in gelatinization followed by re-crystallisation of amylose during cooling result in changes in the rheological properties, which can be determined by a Bohlin Rheometer (Flipse, 1995). Estimated is the storage modulus (G') which is a measure of the energy stored and released per cycle of deformation and per unit of volume.

**Differential Scanning Calorimetry (DSC)**

DSC was performed with a Perkin Elmer Pyris I with a Neslab RTE-140 waterbath-cooler. The instrument was calibrated with indium (mp = 156.6°C) and zinc (mp = 419.5°C) separately. 10 mg of starch (dry weight basis) was put into a stainless steel cup with 40 µl H_2O. The cup was hermetically sealed and equilibrated overnight before analysis. The suspension was heated from 20°C to 100°C at a scanning rate of 10°C/min. Another empty cup was used as a reference. For each endotherm, the melting enthalpy ΔH (J/g) and the onset To (°C) were computed automatically.

Results

Visualisation of PPD in cassava tuberous roots

In order to be able to analyse PPD in cassava, it is necessary to establish a uniform system, which shows the visible situation for initiation of PPD, and which allows spreading of the process of PPD in cassava tuberous roots under controlled conditions. After the cassava slices had been incubated at 30°C (RH around 50%) for 17 days, the experiment was terminated. No visible signs of bacterial/fungal infection could be discovered during these 17 days in the cassava tuberous root slices. The typical phenomenon "vascular streaking" (Averre, 1967; Mondaldo, 1973, Hirose and Data, 1984) of PPD in the cassava tuberous roots can be observed at 24 hrs and afterwards (Figure 1). The so-called "vascular discoloration" (Averre, 1967, Mondaldo, 1973, Hirose and Data, 1984) during PPD can be visualised around the 72 hrs time point and later (Figure 1). This intensified only in colour until 17 days when the experiment was terminated. When the cassava slices were observed under ultraviolet (UV) light, the slices at the time point of 0 hr showed a silver - bluish

Top Panel
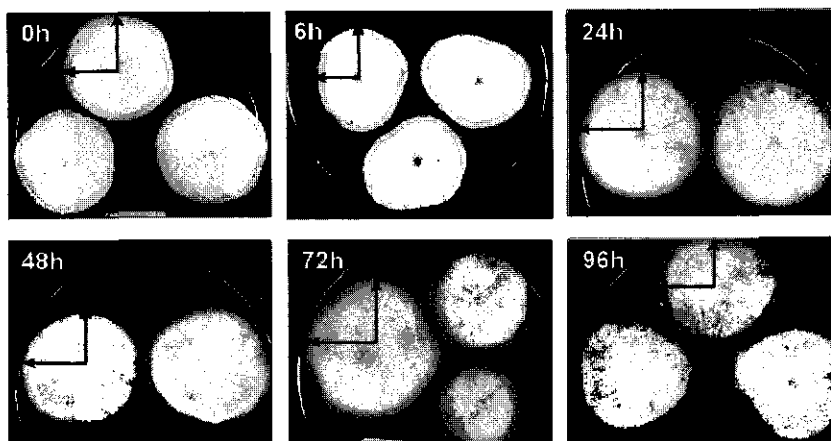
Lower Panel

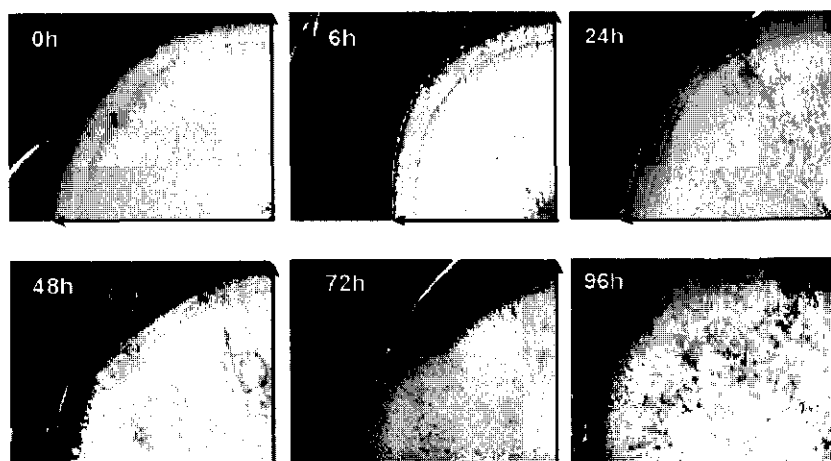**Figure 1.** Examples of postharvest physiological deterioration (PPD) in cassava tuberous root slices in the uniform system for phenotypic visualisation of PPD of *M.Col 22*. Hours indicate the time that the slices were kept under the experimental controlled conditions. Top panel reveals the whole surface of the slices, whilst lower panel shows the enlarged parts within the slices indicated by arrows.

fluorescence, while, strong blue-yellowish fluorescence developed at the 12 hrs time point and afterwards, and finally hardly any fluorescence could be seen for the slices at 96 hrs or later under UV light. These observations fit to the previous descriptions of the phenomenon of the process of PPD in cassava tuberous roots (Hirose and Data, 1984, Rickard, 1985). In all cases the cassava tuberous roots at the same time point showed the same visible phenomena of PPD. Figure 1 reveals that there is no visible sign for PPD in cassava tuberous roots at 0 hr and 6 hrs, while at 96 hrs, there is almost 100% visible PPD occurring in the cassava tuberous roots based on the scoring method of PPD as proposed by Wheatly (1982). No significant evidence for differences in the visible process of PPD could be found between the cassava varieties *M.Col 22* and *Faroka* under the controlled conditions employed in these experiments (data not shown). Fresh weight of cassava tuberous roots decreased during the process of PPD in cassava. At 17 days, the fresh weight loss was almost 40 percent compared to the fresh weight at the start of the experiment (Figure 2).

General changes in contents of nucleic acids, soluble sugars, protein and amylose during PPD

To monitor the system used for analysis of the process of PPD in cassava tuberous roots, several parameters were taken into account. During the process of PPD in cassava, the contents of DNA and protein from the samples of the different time points were measured and adjusted to the changes for fresh weight (Figure 3). After incubation for 12 days, the contents of DNA decreased (the DNA content at 12 days is half the amount of that at 0 hr), opposite to the protein content, which remained unaltered. The same analysis was done for starch as well as soluble sugars (Figure 4). Starch decreased from 260 µg/mg at 0h to 130 µg/mg at day 12 and the amount of sucrose increased 4 times at day 7 compared to 0 h, while the contents of hexose increased 2 times at the point of day 7. Amylose content from the different samples remained similar, which is around 21.2% ± SD 0.89.
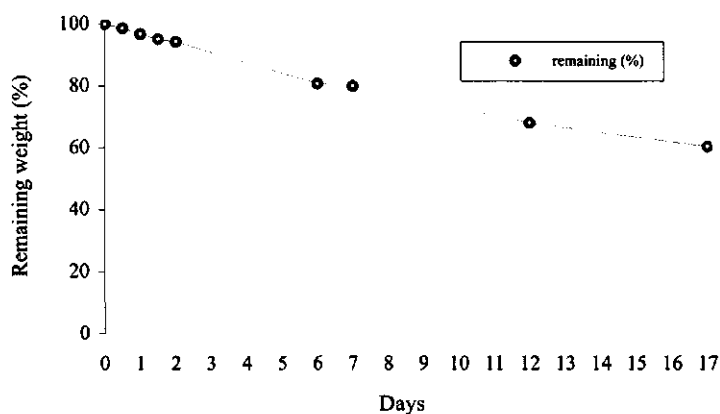
Figure 2. Fresh weight changes of cassava tuberous root slices of *M.Col 22* during incubation.
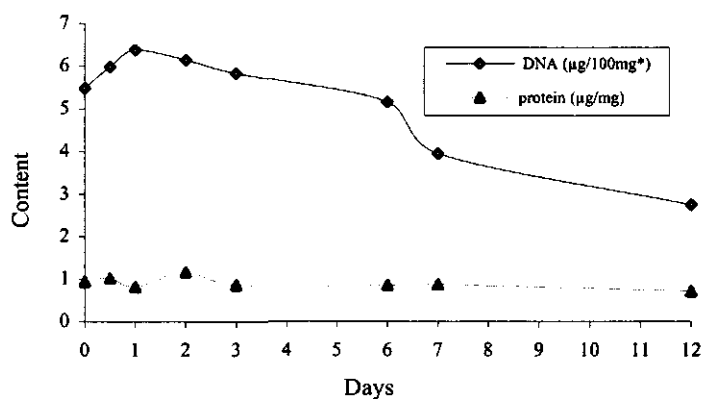
Figure 3. Changes in DNA and protein contents during the process of PPD in the cassava variety *M.Col 22* (the measurement based on the fresh weight).
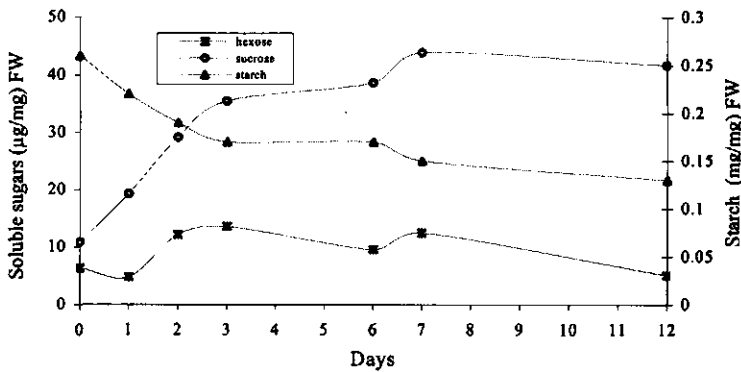
Figure 4. Changes in soluble sugars and starch contents during PPD in *M.Col 22*. Abbreviation: FW = fresh weight.
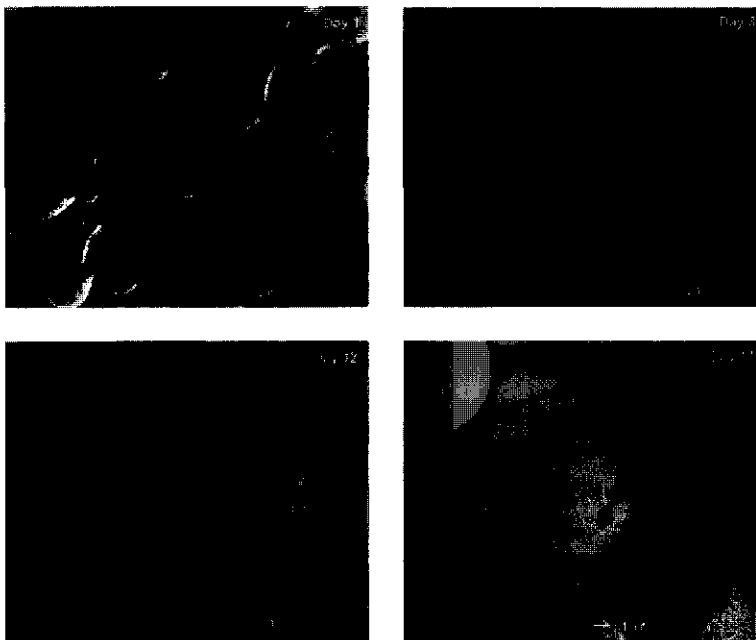
Figure 5. The morphology of starch granules of the cassava genotype *M.Col 22* during the process of PPD under scanning electron microscope (SEM). Arrows indicate damages of starch granules at day 12 and complete holes existing in the starch granules at day 17.

The size and morphology of starch granule during PPD

The average size of the starch granules was measured by a Coulter Counter. No big differences in the different samples were evident during the process of PPD in cassava tuberous roots. For all the samples, the average starch granule sizes were found to be around 16 µm, and there was no significant shift in the mean granule size for the various samples. No change in starch granule morphology could be discovered by light microscopy (data not shown). For SEM, there were no notable differences for size distribution and shapes of starch granules, however, it was clear that damages to the starch granules (pore – like structures) occurred at day 12, which resulted in holes existing on the starch granules at day 17 (Figure 5). No such damages were observed on the starch granules from the samples until day 12. The proportion of these damaged starch granules was around 1% at day 12, and did not increase dramatically at day 17.

Physico-chemical properties of starch-water suspensions
Bohlin analysis:

Starch undergoes a series of changes called gelatinization during heating. The drastic swelling occurs in all directions. This is not reversible and takes place nearly simultaneously with melting of the crystal structure and is combined with amylose leaching out of the starch granule. This process can be characterised for starch. The structural changes during gelatinization result in variations in the rheological properties of starch, which can be measured by a Bohlin VOR Rheometer. The 8% starch-suspensions (dry weight) from the samples of day 1, day 2, day 3 and day 12, were analysed by Bohlin measurements (Figure 6). The storage modulus (G') from all the samples increased quickly from the first 10 to 18 minutes, afterwards decreasing and then reaching a steady state level for all the samples during heating and cooling. It is clear, however, that changes in G' were highly different for the starch at day 1 compared with the starches at the other time points. The peak temperature in gelatinization of all the starch samples is around 66 ^{0}C. Hardly any differences were observed among the starch at day 2, day 3 and even day 12. G' of the starch at day 1 increased sharply at 61 ^{0}C, while the starches for the other samples increased at a slightly higher temperature of around 62 ^{0}C, which can also be shown by DSC measurement. The different samples reached the peak of viscosity almost at the same time around 18 minutes after heating (pasting temperature about 70 ^{0}C), but

the viscosity peak of day 1 was much higher compared to the peaks of the samples of the other time points.
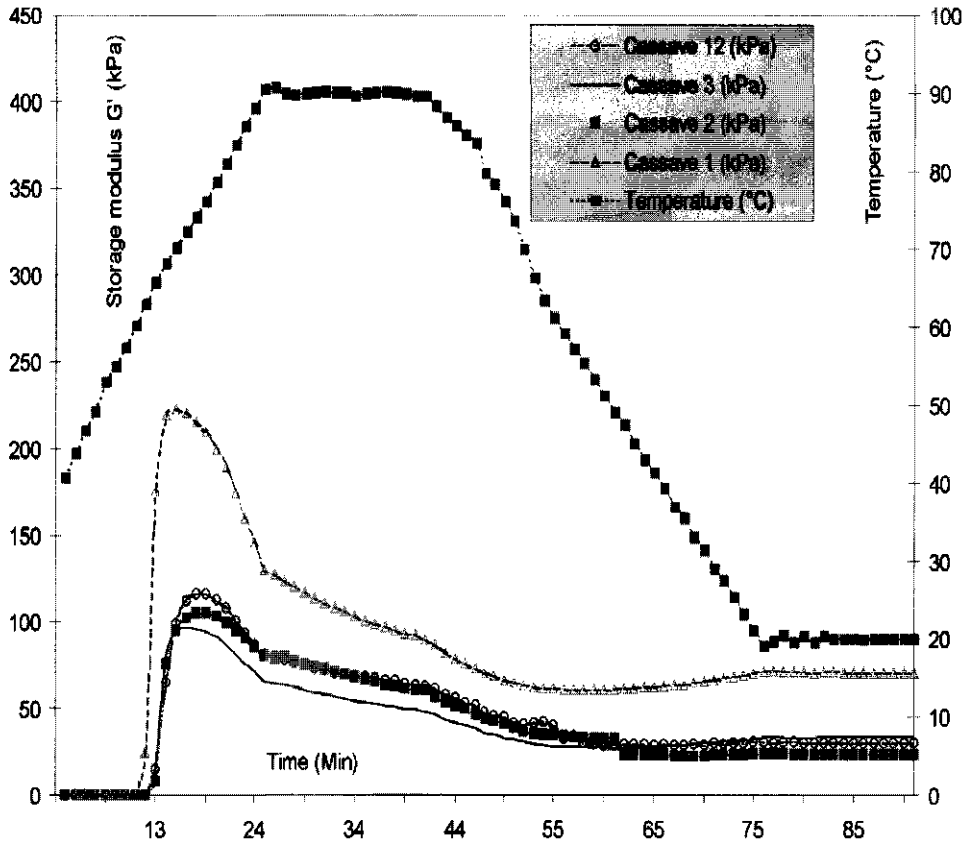
Figure 6. Bohlin gelatinization profile of 8% (dry weight) cassava starch suspensions during the process of PPD at day 1, day 2, day 3 and day 12 indicated in the figure as Cassava 1, 2, 3 and 12 respectively.

DSC analysis:

DSC is used for determination of phase transitions in many (in)organic, polymeric, food and biological materials. The measurements can be conducted dynamically as a function of temperature or isothermally as a function of time. Phase

transitions that can be investigated include crystallisation and melting of water, sugar, lipid, starch and other components, protein denaturation and retrogradation of starch. In the experiment, the energy (temperature) necessary for melting can be determined using DSC. The increase in the moduli coincides with the first stages of crystallite melting (see T_o), which is the best reference for DSC measurement. Table 1 shows the results of the DSC analysis which reveal that there are no differences until day 12 during the process of PPD in cassava tuberous roots as shown by the T_o values.

Table 1. DSC measurement for the 25% starch-water suspension. (T_o: temperature for onset of gelatinization, DM: dry matter, ΔH: enthalpy)

sample	DM (%)	T_o (^{0}C)	ΔH (KJ/g)
0	90.5	61.33	11.75
1 day	90.0	61.73	11.74
2 day	90.0	61.26	11.82
3 day	90.0	61.41	12.04
12 day	89.1	62.06	12.13
17 day	89.1	62.21	13.74

Discussion

PPD is a complex developmental physiological process during the last stage of the life cycle of cassava tuberous roots. From preliminary experiments, it was known that the process of PPD in cassava is influenced not only by environmental factors such as soil conditions, humidity, and temperature, but also by the cassava genetic background. It has been found that different cassava cultivars varied for the onset of PPD and the spread speed of the deterioration process (Booth, 1976). In our experimental facility, the cassava genotypes (*M.Col 22* and *Faroka*) were growing in the tropical greenhouse of Wageningen University, and there was hardly any effect based on the environmental conditions for PPD in contrast to when these cultivars would have been grown in their natural environment. By taking the same size of cassava tuberous roots from 4 - 5 different plants as starting material and cutting the

tuberous roots into the same thickness of slices (10 mm) with almost the same mechanical damage treatment (wounding), it was tried to set up a uniform visual system for initiating PPD and keeping the same spreading speed during the process of PPD in cassava. In our system, the visualised hallmark of PPD in cassava, 'vascular streaking' and 'vascular discoloration' (Averre, 1967, Mondaldo, 1973, Hirose and Data, 1984) could be observed starting at 24 hrs and 72 hrs respectively. Afterwards only the colour intensity increased until day 17 when the experiment was terminated. The cassava slices within the same time point showed the same phenomenon of PPD appearing (Figure 1). No visible signs of bacterial/fungal infection could be discovered in the cassava tuberous roots under our experimental conditions. In fact, since *M.Col 22* is highly sensitive to PPD, but fairly resistant to secondary deterioration (CIAT's Annual report, 1976), it confirms that only PPD occurs in *M.Col 22* under our controlled conditions especially at rather low relative humidity (40 - 50%). This was also confirmed by later using the cDNA-AFLP method (Chapter 3, Huang, *et al.*, 2001). The fresh weight decrease during the incubation (Figure 2) was mainly due to a physical change (namely water loss). Through the experiments, *Faroka* seems also sensitive to PPD.

Several parameters were measured from the samples in this uniform system to induce the PPD process in cassava. The reason that the DNA content decreased within 12 days (Figure 3) may be that some of the cells were dying and/or genomic DNA became degraded during the PPD process. Total protein content remained more or less unaffected (Figure 3) which suggested that, although changes of gene expression occurred during PPD in cassava, there is no influence of this on the protein level.

As the main component of cassava tuberous roots on a dry weight basis, starch properties during PPD were characterised. It was clearly shown in Figure 4 that starch content dropped during PPD as described before (Maini and Balagopal, 1978, Wenham, 1995). The content of soluble sugars (hexose and sucrose) increased. Sucrose, which is the major form of translocatable carbohydrate, and the most abundant of the sugar, had increased much more than with hexose (Figure 4). Because of the presence and production of high levels of phenolic compounds and viscous polysaccharides that can interfere with enzyme isolation and analysis, biochemical characterisation of PPD might be difficult. During starch isolation, the colour of the starch of time point 24hr and later time points was yellowish (not shown), which

indicated that the quality of the starch declined during PPD in cassava tuberous roots as well. Further studies on the rheological determinations of starch were performed. The viscosity of the sample at day 1 is notably more variable compared with that of the other samples analysed by a Bohlin Rheometer (Figure 6). There was no dramatic difference in the T_o value using DSC measurements (Table 1). Only the later time points of the process of PPD (from day 12) showed a slightly higher T_o value.

If differences do exist in the properties of starch, they must have a structural basis. In other words, the structure of the starch granules determines the properties of starch during the process of PPD in cassava. When using SEM to observe the morphology of starch granules for the different time points, damages were observed at day 12 and holes were found at day 17 on starch granule surfaces (Figure 5). Surface pores have been observed to exist on some corn, sorghum and millet natural starch granules (Fannon, *et al.*, 1992, Hall and Sayre, 1970), which were supposed to make the starch granule interior accessible (Fannon, *et al.*, 1993). No artifacts of processing or preparation of specimens for SEM were found to lead to these pores (Huber and BeMiller, 2000). However, such pores have never been discovered on natural cassava starch granules. These damages and holes on the starch granules are most probably caused by the process of PPD in cassava.

The general conclusion from the results of these measurements is that although phenotypical differences were observed in the colour of the starch (but not in the size) and morphology of the starch granule as well as in the Bohlin measurements, the overall impression is that starch quality and quantity are not as affected as one might expect based on general assumptions of the process of PPD in cassava tuberous roots.

Chapter 3

**MOLECULAR ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES
DURING POSTHARVEST DETERIORATION IN TUBEROUS ROOTS OF
CASSAVA (*MANIHOT ESCULENTA* CRANTZ)**

Huang, J., Bachem, C. W. B., Jacobsen, E., and Visser, R. G. F.

Laboratory of Plant Breeding, Department of Plant Sciences, The Graduate School of
Experimental Plant Science (EPS), Wageningen University, P. O. BOX 386, 6700 AJ
Wageningen, The Netherlands.

Abstract

One of the major problems for cassava is the rapid deterioration after harvesting cassava tuberous roots, which is limiting the possibilities for production and distribution of cassava in the world. Postharvest deterioration is an inherent constraint for cassava since wounding and mechanical damage of the tuberous roots can not be prevented during harvesting. Postharvest deterioration includes postharvest physiological deterioration (PPD) and secondary deterioration. To date, the molecular mechanism and biochemical pathways of PPD are poorly understood. The aim of this project, which is focusing on the early stages (first 72 hrs), is to gain molecular insight and identify important metabolic pathways during the process of PPD in cassava tuberous roots. Finally by using reverse genetic approaches it is attempted to delay or even prevent the process of PPD in cassava tuberous roots. By using a new RNA fingerprinting method, called cDNA-AFLP, we have screened more than 6,000 TDFs (transcript derived fragments) via up to 100 primer combinations during the early process of PPD in cassava. Only 10% of the TDFs are developmentally regulated, while the other 90% are expressed throughout the process of PPD in cassava tuberous roots. Furthermore, in order to set up a functional catalogue of differentially expressed genes during PPD, 70 TDFs were selected and isolated based on their expression patterns, which were either up-regulated, down-regulated or transiently induced. Around 40 of these TDFs were found to be similar to known genes in databases. The other 30 TDFs represent mostly genes without known function. Through data analysis, it is shown that important biochemical and physiological processes, such as oxygen stress, carbohydrate metabolism, protein metabolism and phenolic compounds synthesis, are involved in PPD in cassava tuberous roots.

Key words: *cassava, catalogue, cDNA-AFLP, differentially expressed genes, postharvest deterioration.*

Introduction

Cassava is an important crop in the tropical and sub-tropical regions. In these regions cassava is the fourth largest calorie resource as a crop for humans and animals after rice, sugar cane and maize (Ingram and Humphries, 1972, Taylor, *et al.*, 1999). Cassava can grow under conditions such as low fertility, high acidity soil and drought, where other crops like maize can not be grown. One of the major problems that limit the possibilities of production and distribution of cassava is the rapid deterioration after harvesting cassava tuberous roots, which results in a short storage life for cassava tuberous roots. Postharvest deterioration in cassava is an inherent constraint since wounding and mechanical damage of cassava tuberous roots can not be prevented during harvesting. In general, postharvest deterioration is divided into primary deterioration and secondary deterioration. Preliminary results indicated that primary deterioration is a complex physiological and developmental process, which starts from 24 hrs to 48 hrs after harvest depending on the cassava genotype (Plumbley & Rickard, 1991, Wenham, 1995). Secondary deterioration is the result of the former process and deterioration due to micro-organisms such as bacteria and fungi. Postharvest physiological deterioration (PPD) can be first observed as "vascular streaking" (Averre, 1967, Mondaldo, 1973 and Hirose, *et al.*, 1983 and 1984) in cassava tuberous roots around 24 hrs after harvesting. The colour spreading which occurs rapidly on the whole surface of cassava tuberous roots is called "vascular discoloration" (Averre, 1967, Mondaldo, 1973, Hirose, 1983 and 1984) and takes place from 96 hrs afterwards in our system under controlled conditions (Chapter 2, Huang, *et al.*, 1999). However, to date, little is known about the molecular mechanism and biochemical pathway leading to PPD in cassava. With the advance of biotechnology in agriculture, especially the break-through of developing a cassava regeneration system using friable embryogenic callus (FEC) lines (Taylor, *et al*, 1996) and/or through organogenesis based on adventitious shoot formation (Li, *et al*, 1996), cassava transformation was made possible (Li, *et al*, 1996, Schöpke *et al.*, 1996, Raemakers, *et al.*, 1996). This provides the possibility to make use of genetic engineering techniques to potentially solve the PPD problem in cassava. A prerequisite for applying molecular tools such as genetic modification to cassava is the isolation and characterisation of genes, which trigger and /or control the process of PPD. Our aim is to identify and isolate genes focusing on the early stages of primary deterioration, based on a RNA fingerprinting technique called cDNA-AFLP (Bachem

et al, 1996). Through the data analysis obtained with this method, it is shown that important biochemical and physiological processes during PPD in cassava tubers are switched on. Thus, more insight will be gained in the molecular basis of PPD in cassava tuberous roots.

Materials and Methods

Plant material preparation

The plant materials were prepared as described previously (Chapter 2, Huang, *et al.*, 1999). The samples from the different time points were collected and stored at -80°C for total RNA isolation.

cDNA-AFLP fingerprinting and TDF isolation

Around 2.5 g of plant material from the cassava tuberous root slices different time points and the control tissue (intact tuberous roots) was used for isolation of total RNA. Total RNA was also extracted from 1.5 g cassava tissues such as leaf, stem and petiole. The total RNA was isolated using the method as described previously (Bachem, *et al.*, 1996). Since high levels of phenolic compounds and viscous polysaccharides are present and produced during the process of PPD in cassava tuberous roots, and also because cassava tuberous roots are rich in starch, which can interfere with the quality of total RNA and even influence cDNA synthesis later, extra phenol : chloroform steps were taken and high-speed centrifugation during extraction of total RNA was necessary. According to the cDNA-AFLP protocol (Bachem *et al*, 1996), double stranded cDNA from different samples was synthesised. Two restriction enzymes TaqI (as a frequent cutter) and AseI (as a rare cutter) were chosen to cut the cDNA, which was used as the templates for the standard AFLP method (Vos *et al.*, 1995). TDFs were isolated from the 5% AFLP polyacrylamide gels according to their expression patterns, which revealed up-regulated, down-regulated or transient induced expression during the first 72hrs of the process of PPD in cassava tuberous roots.

Subcloning and sequencing of TDFs:

The isolated TDFs were amplified by PCR using the two primers without additional selective bases and the PCR products were directly subcloned into the vector system pGEM T-easy vector (*Promega, USA*). The sequencing was done by an automated sequencer. The sequences of the TDFs were compared to the sequences present in databases by using the BLAST 2.0 programs (Altschul, *et al*, 1997). If the BLAST programs did not find significant sequence similarity, then EST databases were searched as well.

Results

A uniform system for induction of the PPD process in cassava under controlled conditions

A uniform system, which initiated the visible signs of PPD and kept the same spreading speed during the process of PPD in cassava tuberous roots, was set up (Chapter 2, Huang, *et al.*, 1999). No visible signs of bacterial/fungal infection could be found in the cassava tuberous roots until 17 days after the initiation of the process of cassava PPD in this system. After that time, the experiment was terminated. No significant evidence for differences during the visible PPD process could be found between the cassava varieties *M.Col 22* and *Faroka* under the controlled conditions employed in these experiments (Chapter 2, Huang, *et al.*, 1999).

Expression profiling by cDNA-AFLP during PPD in cassava

cDNA-AFLP was used to analyse the expression profiling during PPD in cassava tuberous roots. The size arrangement of the expression patterns in the window of a 5% AFLP polyacrylamide gel ranged from around 1000 bp at the top to about 80 bp at the bottom. The total pattern inside the window of an AFLP gel includes around 60-70 bands. The typical expression patterns present in cDNA-AFLP during the process of PPD from 0h to 72h in cassava tuberous roots combined with the control tissue (T for the intact Tuber, P for Petiole, S for Stem, and L for Leaf) are displayed in Figure 1, in which a representative expression pattern is shown produced by choosing one primer combination (Ase I + selective extensions; AG, Taq I + selective extensions; AT). By using cDNA-AFLP, 4 different expression patterns can be detected during PPD in cassava tuberous roots; 1) induced expression (arrow A), 2)
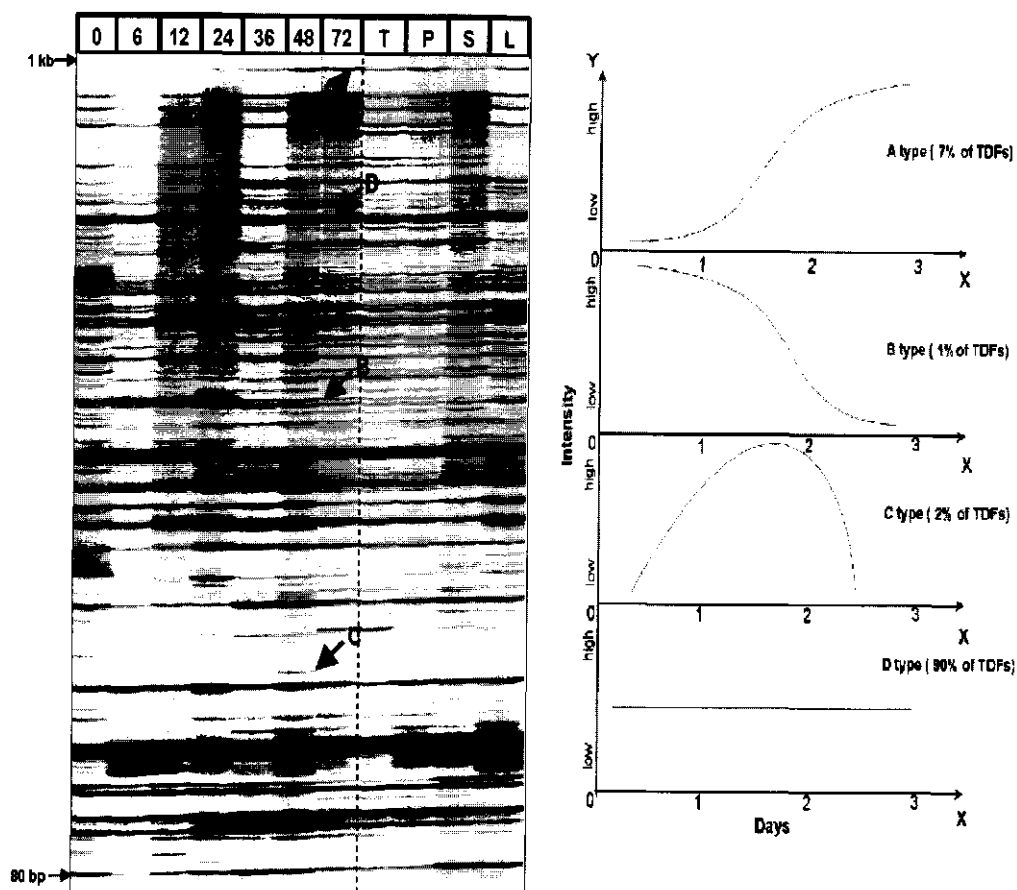
Figure 1 (left). cDNA-AFLP fingerprints of the templates prepared from cassava tuberous root slices from *M.Col 22* following PPD from 0h to 72 h at the controlled conditions plus control tissues; intact tuberous root (T), leaf (L), stem (S) and petiole (P) choosing one primer combination {Ase I + (selective extensions) AG; Taq I + (selective extensions) AT}.

Figure 2 (right). Schematic drawing of 4 different expressing patterns during PPD in cassava using cDNA-AFLP. A, B, C and D type indicating up-regulated, down-regulated, transiently induced and constitutive expression separately.

decreased expression (arrow B), 3) transient expression (arrow C), and 4) constitutive expression (arrow D). Through running around 100 different primer combinations (plus 2 additional selective bases), a total of about 6,000 TDFs were screened during the first 72 hrs of PPD. By counting the expression patterns for 10 AFLP gels (40 primer combinations), around 2165 TDFs showed a constitutive expression pattern which means almost 90% of the TDFs revealing constitutive expression (D type). Nearly 172 TDFs displayed an up-regulated expression pattern which indicates around 7% of the transcripts showing an up-regulated expression pattern (A type), more than half of them being induced at very early stages (first 48 hrs). About 59 of the TDFs are transiently induced, which accounts for ca. 2% of the transcripts (C type). Only 28 of the TDFs are showing a down-regulated expression pattern, which is about 1% of the TDFs (B type) in Figure 2. Therefore, extrapolating these results to the 100 primer combinations used means that in total around 5,400 TDFs show a constitutive expression pattern, nearly 420 TDFs reveal an up-regulated expression pattern, almost 120 TDFs are transiently expressed and only 60 TDFs display down-regulated expression patterns. These results suggest that only 10% (7% plus 2% plus 1%) of the genes are showing developmentally regulated expression patterns during the early stages (first 72hrs) of the primary deterioration process in cassava tuberous roots. Another interesting result from the cDNA-AFLP expression profile is that when TDFs show a constitutive expression pattern during PPD (D type), they also are expressed in all the control tissues (intact tuberous root, petiole, stem and leaf). When the TDFs display developmentally regulated expression patterns during PPD (A, B and C type), they are more variable expressed and sometimes even absent in some of the control tissues (intact tuberous root, petiole, stem and leaf) in Figure 1.

A functional catalogue of the TDFs

By running the different primer combinations plus 2 selective bases, around 70 TDFs were selected for further study based on their expression profile, which showed an up-regulated, a down-regulated or a transiently induced expression during the first 72 hrs of PPD in cassava tuberous roots. These 70 TDFs were not present in intact cassava tuberous roots (T), but they are present either at 0 hr after cutting as well as in the other control tissue (Stem, Petiole, Leaf) or not. After subcloning and sequencing, the sequences of the TDFs were analysed in the database of NCBI (eg. BLAST) against the latest releases of the public nucleotide and protein sequences,

along with available EST sequence data. About 40 out of the 70 TDFs share high amino acid similarity to putatively known genes by blast and EST searching in the database, including 3 of the 70 TDFs that match sequence similarities to ribosomal protein, while 30 out of 70 TDFs do not show any sequence similarity in databases. All the identified TDFs with the best similarity accession number are presented in Table 1 (E values less than 0.001 are considered to be not similar to known genes.). Through the data analysis, a further functional classification based on the function of the predicted gene products by means of sequence similarity of the TDFs could be established. This reveals that some of the important biochemical and physiological processes are possibly involved in PPD in cassava tuberous roots (Table 1). Clustering these sequences shows that 39% of the TDFs with a predicted gene function is expected to be related to stress responses. 34% of the TDFs are expected to be involved in metabolic processes, 19% of the TDFs may be included in signal transduction and 7% of the TDFs are thought to play a role in development. The system, which we set up to imitate the PPD process under controlled conditions monitors PPD in cassava tuberous roots, functions well since both genes involved in oxygen scavenging (like peroxidase, and cytochrome P450 monooxygenase) as well as genes related to the metabolism of flavonoid compounds (flavanol synthase) are induced during PPD. Also genes involved in wound response like WIZZ (transcription factor upon wounding), pectinacetylesterase, expansin and heat shock 70 proteins are either transiently induced or up regulated. Furthermore, genes related to protein metabolism like inosine 5'-phosphate dehydrogenase 1, isovalery-CoA dehydrogenase precursor, elongation factor 1-alpha, eukaryotic release factor 1 and translation factor EF-1 alpha like protein display an up-regulated or transiently induced expression during PPD in cassava tuberous roots.

Discussion

Primary postharvest deterioration is a complex physiological and developmental process accompanying the last phase of the cassava tuberous roots life cycle. It is not only dependent on the environmental conditions but also on the genetic background of the cassava variety (Booth, 1976 and 1977, Wenham, 1995). To gain a better understanding of PPD, we undertook a molecular approach to isolate and identify genes expressed during the process of PPD in cassava tuberous roots,

focusing on the early stages (first 72hrs) of primary deterioration. For this it was necessary to develop a system to monitor for the PPD process from the onset and to follow the spread of PPD in cassava in a synchronous way before applying the cDNA-AFLP technique. Herewith, we set up a phenotypic uniform test for inducing PPD in cassava tuberous roots (Huang, *et al.*, 1999). Using the cDNA-AFLP method it was shown that three different developmentally regulated expression patterns could be detected with different primer combinations, which reveal up–regulated, down–regulated and transiently induced expression patterns (Figure 1). Using the experimental test-system it was possible to reproduce the different expression patterns from experiment to experiment when using the same primer combination.

Using the cDNA-AFLP method, we have, for the first time been able to systematically screen a large number of genes expressed during PPD in cassava tuberous roots. In principle, if the appropriate sets of restriction enzyme combinations are chosen in a given template cDNAs pool during PPD, almost all virtually expressed genes including rare messages can be detected by cDNA-AFLP (Bachem, *et al.*, 1998). In plants, it has been estimated that a total of between 16,000 to 33,000 genes are encoded on the genome (Gilson and Somerville, 1993, Meyerowitz, 1994, Cooke, *et al.*, 1996). With recent rapid progress in genome sequencing and functional genomics, a more accurate estimation can be made. Results of the Arabidopsis chromosome sequencing progress shows that nearly 8,000 structural genes are present on Chromosome 2 and 4. The two chromosomes comprise around 30% of the genome in Arabidopsis (Li, *et al.*, 1999, Mayer, *et al.*, 1999). Thus approximately 25,000 genes are likely to be encoded by the whole Arabidopsis genome. Most recently it has been indicated that the Arabidopsis genome contains 25,498 functional genes (The Arabidopsis Initiative, 2000). By running up to 100 primer combinations, in total around 6,000 TDFs were screened during the early stages of PPD in cassava tuberous roots using the cDNA-AFLP technique.

It may be expected that a number of important processes occur during PPD. Amongst these are stress due to wounding, oxygen response and the deposition of various of phenolic compounds. Indeed several components of these processes were visualised and identified in the expression profile during PPD in cassava tuberous roots. For example, TDFs like A22, C7, C29 and C34 (Table 1) are thought to be involved in wounding response. The TDFs such as A8, C25 and C30 (shown in Table 1) are expected to be related to oxygen response and TDF A21 (Table 1) shows

sequence similarity to a gene involved in the production of phenolic compounds. Uritani (1998 and 1999) has shown that there are changes in protein level such as degradation and novel synthesis of proteins, which indicates that protein metabolism may be important for PPD in cassava. We also isolated and identified TDFs pathway by the cDNA-AFLP method like A10, A14, C9a, C22, C35 and C38 (see Table 1) that are part of the protein metabolism.

70 TDFs were isolated based on the expression patterns during the process of PPD. The TDFs can be functionally grouped based on the function of their predicted gene products (established by means of the sequence similarity). There are important processes involved in PPD, such as stress responses, carbohydrate, lipid, phenolic compounds and protein metabolism and signal transduction. Some of the TDFs may fit into two process classes or more. The majority of the isolated TDFs during PPD are the genes related to stress responses (39%) and metabolism (34%), followed by the genes involved in possible signal transduction pathways (19%) and the TDFs (7%) playing a role in development (see Table 1). Wounding of cassava tuberous roots leads to stress that, in turn switches on numerous signal transduction pathways. Thus, the classification and number of genes for the different categories are in line with what could be expected.

In general, TDFs, which are isolated by cDNA-AFLP, are within or near to the protein-coding region of genes (Bachem, *et al.,* 1998). However, nearly 43% of the TDFs reveal no match in databases. This might be due to similar genes in other organisms not being discovered yet or these types of genes with special functions, not being present in other organisms. With the rapid increase in sequence information available from yeast, *C. elegans* and Arabidopsis genome projects, the knowledge gap of the former possibility will be closed (Wambutt, *et al.,* 2000).

PPD is stimulated by mechanical damage and wounding in cassava tuberous roots. In a high humidity environmental condition (RH around 80%-90%), PPD in cassava tuberous roots can be prevented by forming a wound-healing periderm (Booth, 1976). However, the speed of the wound healing response in cassava is notably slower compared with other root crops like yam (Passam, *et al.,* 1976). This indicates that cell wall metabolism may play a role as well during PPD in cassava tuberous roots. The TDFs such as A8, A21, A22, C7 and C25 (in Table 1), which are thought to be related to cell wall metabolism, indeed point into this direction.

Older research indicated that oxygen is required for primary deterioration in cassava (Averre, 1967, Booth, 1976 and 1977, Rickard, 1982). Furthermore, it has been shown that oxidative stress is involved in the process of PPD in cassava (Reilly, *et al.*, 1999). There is evidence showing that oxidative stress may cause programmed cell death (PCD) in animals and plants (Amor, *et al.,* 1998, Jabs, 1999, Piffanelli, *et al.*, 1999). Three TDFs (A8, C25 and A12 in Table 1) were isolated with high sequence similarity to tobacco peroxidase (accession No. AB027753, Hiraga, *et al.*, 1999), peà cytochrome P450 monooxygenase (accession No. U29335, Frank, *et al.,* 1996)) and citrus defender against cell death 1 (dad1) (accession No. AB011798, Takaya, *et al.*, 2000). These TDFs show either an up-regulated or a transiently induced expression during the first 72 hrs of PPD in cassava tuberous roots. It is known that cytochrome P450 monoxygenase mediates a wide range of oxidative responses that are related to the biosynthesis of plant secondary metabolites including phenylpropanoids and phytoalexins. Dad1 is thought to be an evolutionarily conserved PCD inhibitor in animals and plants (Gallois, *et al.*, 1997, Tanaka, *et al.*, 1997, Takaya, *et al.,* 2000). Another antioxidant enzyme, catalase was also isolated from a cassava tuberous root cDNA library at 48hrs of primary postharvest deterioration. (Reilly, *et al.,* 1999). It is believed that catalase plays an essential role in the defence against oxidative stress (Rocha, *et al.*, 1996, Zámocký and Koller, 1999). Catalase can not only quickly eliminate hydrogen peroxide (H_2O_2) and other small organic peroxides to avoid toxicity, but also protect other cellular enzymes like superoxide dismutases (Michiels, *et al.*, 1994). Catalases are supposed to prevent superoxide dismutases from inactivation by higher concentrations of H_2O_2 (Fridovich, 1995). These findings strongly suggest that PCD may occur in the process of PPD in cassava tuberous roots.

Currently, experiments are being done to check whether DNA laddering occurs during the process of PPD in cassava tuberous roots, which is the hallmark of PCD in animals and plants (Greenberg, 1996, Ryerson and Heath, 1996, Wang, *et al.*, 1996).

Based on the function of putative gene products by means of sequence similarity of the TDFs, and combined with the expression profile as well as the important processes to be expected to occur during PPD in cassava tuberous roots, several candidate genes are selected for further study by transforming them into cassava in an antisense approach.

Table 1. Characterisation and functional classification of TDFs during PPD in cassava by cDNA-AFLP

TDF	P.C.*	Size	Putative identity	Best similarity accession No.	E value	Str.	Met.	Sig.	Dev.	Expression pattern
A10	44/73	226.	(EST) inosine 5'-phosphate dehydrogenase 1	house mouse NP_035959	4e-4	*				
A12	44/77	423.	DAD1 (defender against apoptotic death 1)	Citrus AB011798	3e-42	*			*	
A14	45/64	254.	isovaleryl-CoA dehydrogenase precursor	Arabidopsis AF160729	9e-23	*				
A21	45/70	228.	flavonol synthase	Citrus AB011796	2e-06	*				
A22	45/71	338.	similar to expansin	rough lemon AF015782	e-13	*				
A5	44/68	308.	allergen like protein	Arabidopsis Z97342	2e-08	*				
A7	44/69	197.	C2H2-type zinc finger protein	petunia AB006601	4e-06	*		*		
A8	44/71	407.	peroxidase	tobacco AB027753	4e-32	*				
B25	45/72	210.	40s ribosomal protein	Arabidopsis thaliana AC007047	2e-19	*		*		
B26	45/73	179.	CPRD 12 protein	cowpea D88121	2e-05	*				
B3	44/66	119.	EST similar to hypothetical protein	Arabidopsis AC004705	2e-04	*				
B9	44/73	351.	Ca2+-binding protein	Arabidopsis AF009228	1e-15	*		*		
C12	46/74	261.	no apical meristem like protein (NAM)	Arabidopsis AC006233	4e-11	*				
C14	46/76	203.	beta-COP like protein	Arabidopsis CAB45907	4e-11	*				
C15	46/77	383.	40s ribosomal protein	Arabidopsis thaliana AC007047	3e-08	*				
C16	46/78	569.	similar to peptide transport proteins	Arabidopsis AC011809	2e-31	*				
C16b	46/78	474.	HVA22 homologs	Arabidopsis AF141659	1 e-22	*		*		
C17	46/78	365.	similar to peptide transport proteins	Arabidopsis AC011809	3e-31	*				
C18	46/78	169.	(EST) putative glucosyltransferase	Arabidopsis AC002333	2e-07	*				
C19a	46/79	173.	(EST) putative 21kD protein precursor	Medicago sativa Y11553	1e-33	*				
C2	46/64	161.	R2R3-MYB transcription factor	Arabidopsis AL132978	9e-19	*		*		
C22	47/72	168.	elongation factor 1-alpha	cassava AF041463	6e-04	*		*		
C24a	47/73	250.	ZPT2-14 zinc-finger protein	petunia x hybrid AB006601	7e-05	*		*		
C25	47/73	218.	cytochrome P450 monoxygenase	pea U29335	6e-08	*			*	
C26	47/75	287.	UDP-glucose pyrophosphorylase	popato U20345	4e-19	*		*	*	
C27	47/75	225.	putative serine/threonine kinase	Arabidopsis AC005850	2e-18	*		*		
C28	47/78	251.	serine/threonine-specific kinase like protein	Arabidopsis AL078470	9e-12	*		*		
C29	47/77	402.	WIZZ (transcription factor upon wounding)	Nicotiana tabacum AB028022	7e-36	*		*		
C30	47/74	429.	chlorophyllase	Citrus sinensis AF160869	1e-22	*		*		
C31	47/76	487.	cytochrome P450	Nicotiana tabacum AB015762	1e-20	*			*	
C34	49/67	358.	heat shock 70 protein	tomato P27322	3e-33	*				

						Str	Met	Sig	Dev
C35	49/67	138.	eukaryotic release factor 1	Arabidopsis U40217	5e - 10			*	
C36	48/76	380.	putative receptor-like protein kinase	Arabidopsis AC005312	9e - 44				*
C37	49/67	269.	26S ribosomal protein	Citrus limon X05910	e - 113				
C38	48/77	288.	Golgi associated protein se-wap41	maize U89897	9e - 30		*		
C4	46/66	210.	ATP Synthase Delta chain	sweet potato P22778	4e - 15		*		
C6b	46/66	298.	Ca2+-binding protein	Arabidopsis AF009228	2e - 15			*	
C7	46/67	174.	putative pectinacetylesterase protein	Arabidopsis AL022580	3e - 13		*		
C7b	46/67	297.	Ca2+ - binding protein	Arabidopsis AF009228	2e - 15			*	
C9a	46/69	302.	(EST) Translation factor EF-1 alpha - like protein	Arabidopsis AL021635	0.001		*		

Note

P. C.*: Primer combination. A list of primers is available on http://www.dpw.wau.nl/pv/.

* Str.; Stress responses.

* Met.; Carbohydrate metabolism, protein metabolism and phenolic compounds production.

* Sig.; Signal transduction.

* Dev.; Development.

Chapter 4

**POSTHARVEST PHYSIOLOGICAL DETERIORATION IN CASSAVA
TUBEROUS ROOTS: A PEROXIDASE - MEDIATED OXIDATIVE
PROCESS?**

Huang, J., Hendriks, T., Suurs, L., Jacobsen, E., and Visser, R. G. F.

Laboratory of Plant Breeding, Department of Plant Sciences, The Graduate School of
Experimental Plant Science (EPS), Wageningen University, P.O.BOX 386, 6700AJ
Wageningen, The Netherlands.

Abstract

Progress has been made in molecular analysis of postharvest physiological deterioration (PPD) in cassava tuberous roots, focusing on the early stages. By using the cDNA-AFLP technique, a number of TDFs (transcript derived fragments) have been identified and isolated from the AFLP gels, which have shown an up - regulated, a down - regulated or a transiently induced expression pattern during the first 72 hours of the PPD process in cassava tuberous roots. Through the functional catalogue, genes possibly important in oxidative stress like peroxidase and cytochrome P450 monooxygenase, have been identified. To further assess the possible involvement of peroxidase (EC 1.11.1.7) in PPD, changes in peroxidase activity, isoenzyme pattern, and localization during PPD were determined. Both total and specific peroxidase activity increased during PPD. It was shown that the specific activity of peroxidase increased up to day 7 in line with the phenotypically developmental symptoms of the PPD process in cassava tuberous roots. At least 7 peroxidase isoenzymes in cassava tuberous roots could be observed on a native polyacrylamide gel and novel peroxidase isoenzymes were induced during the process of PPD. Using tissue printing, it was shown that the peroxidase activity is localized in the epidermal layers, parenchyma tissue and vascular bundles in cassava tuberous roots during the process of PPD. The results obtained indicate that indeed peroxidase may be involved in PPD in cassava.

Key words; *cassava, postharvest physiological deterioration, peroxidases, enzyme activity, tissue printing.*

Introduction

Cassava is an important tuberous root crop used for both food and feeding purposes, as well as a source for starch for many different applications, like in food processing, the manufacturing of paper, textile and adhesives, and even in oil drilling (Kay, 1987). Unfortunately, the production, distribution and processing of the cassava tuberous roots are severely hampered by a poor storage life caused by a rapid deterioration after harvest. Apparently cassava tuberous roots, unlike other underground storage organs such as yam and potato tubers, lack a rapid wound healing mechanism to protect the organ from deterioration (Passam, *et al.,* 1976, Wenham, 1995). To solve the cassava storage problem, either by traditional breeding or biotechnological approaches, a complete understanding of the postharvest deterioration process is a prerequisite.

In the process of postharvest deterioration of cassava roots, two stages can be distinguished: the primary or postharvest physiological (PPD) deterioration, and the secondary deterioration, which results from microbial infections following the former process (Booth, 1975 and 1976). PPD in cassava tuberous roots is a physiological developmental process that is initiated and/or accelerated by mechanical damage and/or water loss, and starts within 24-48 hours after harvest (Wheatley, 1982, Wenham, 1995). Changes in many metabolic pathways accompany PPD, like in carbohydrate, protein, lipid metabolism (Maini and Balagopal, 1978, Tanaka, *et al.,* 1983, Lalaguna and Agulo, 1989, Uritani, 1998 & 1999, Huang, *et al.,* 1999), and the synthesis of polyphenolic compounds (Wheatley, 1982). The molecular mechanisms underlying the initiation of PPD, however, are poorly understood.

Recently, we initiated molecular analysis of PPD by a systematic survey of the changes in gene expression that accompany the early stages of PPD in cassava tuberous roots. Thick cassava root slices perpendicular to the root axis were used as a model system for this study. In the slices the first visible sign of PPD, a brown discoloration at the site of the vascular bundles called 'vascular streaking' (Mondaldo, 1973, Averre, 1976, Hirose, 1986), appears about 24 hours after harvesting. Around 72 hours after harvesting this browning rapidly spreads over the whole surface of the slices, a process referred to as 'vascular discoloration' (Hirose and Data, 1984, Rickard, 1985). Under the experimental conditions, PPD seems to develop uniformly in all slices, as indicated by the simultaneous appearance of both vascular streaking and discoloration (Chapter 2, Huang, *et al.,* 1999). Using this model system, changes

in the gene expression during the first 72 hours of PPD were studied by an RNA fingerprinting technique called cDNA-AFLP (Bachem *et al.*, 1996). From AFLP gels 70 TDFs (Transcripts Derived Fragments) were selected that represented genes from which the expression was either up-regulated, down-regulated, or transiently induced. After sequencing, 30 TDFs showed no homology to transcripts from genes with a known function, whereas 40 TDFs were shown to be derived from transcripts encoding enzymes most likely involved in processes that occur during PPD, like carbohydrate and protein metabolism. In addition, several TDFs were found to represent genes that may be involved in stress responses, like wounding and oxidative stress, and in programmed cell death (Chapter 3, Huang, *et al.*, 2001).

In this paper the detailed analysis of two TDFs, TDFc25 and TDFa8, derived from transcripts encoding a putative cytochrome P450 monooxygenase and a peroxidase, respectively, are presented. As compared to apparently constitutively expressed genes, the expression of the cassava monooxygenase gene was up-regulated during PPD, whereas the expression of the peroxidase gene was transiently induced. For both genes the level of transcripts was highest 6 hours after the onset of PPD, but very low or absent in all other organs tested, including in intact cassava roots. In an attempt to further assess the possible involvement of peroxidase in PPD, changes in peroxidase activity, isoenzyme pattern, and localization during PPD were determined.

Materials and Methods

Plant material preparation

Tuberous roots were harvested from about 1.5-year old, greenhouse-grown cassava plants (*M. Col 22*). A uniform system for the onset of PPD in cassava was established as described previously (Chapter 2 and Chapter 3, Huang, *et al.*, 1999 and 2001) by cutting 1 cm thick slices perpendicular to the root axis and keeping them under controlled conditions. At different time points of deterioration (day 0, day 1, day 2, day 3, and day 7) slices were collected. For peroxidase activity determinations the samples were divided into two batches; one for peroxidase activity measurements and another for tissue printing. Cassava root slices for activity measurements were peeled. Subsequently the enzyme extracts were stored at - 20°C for later analysis after

freezing immediately in liquid nitrogen. Another batch of the slices was used for tissue printing without peeling.

The cDNA-AFLP technique and the sequencing of TDFs

The cDNA-AFLP method was performed during the process of PPD in cassava tuberous roots, and the TDFs were isolated, cloned and sequenced as described previously (Chapter 3, Huang, *et al.*, 2001). The sequences of the TDFs were compared to the latest sequences present in public databases by using the BLAST 2.0 programs (Altschul, *et al*, 1997).

Extraction and measurement of protein content and peroxidase activity

5 grams of the tuberous root material were crushed in 5 ml TBS (0.02 M Tris-NaCl, pH = 7.5) in a Warring Blender, and centrifuged at 13,000g for 4 minutes. The supernatant was used for the measurement of protein content and peroxidase activity.

Protein content was determined using the ESL-kit (*Roche, Switerland*) in an Ultrospec 2000 spectrophotometer (*Amersham - Pharmacia Biotech*). BSA (bovine serum albumin) was used as a standard reference for protein measuring. The extract was diluted 5 times with TBS buffer (0.02 M Tris-NaCl, pH = 7.5) before measuring.

Peroxidase activity was determined according to Vallejos (1983) and Hendriks, *et al.*, (1991) The reaction mix (1 ml) contained 50 mM NaAc buffer (pH = 5), 4 mM guaiacol and 2.2 mM H_2O_2. After adding 10 μl of the tuberous root extract, the increase of the absorbance at 470 nm was followed for 1 min in an Ultrospec 2000. Peroxidase activities were expressed as the increase in the absorption per min per fresh weight of tissue or protein content, respectively. Peroxidase activities per fresh weight of tissue were corrected for the changes in the fresh weight of the slices during the process of PPD (Chapter 2, Huang, *et al.*, 1999).

Electrophoresis and detection of peroxidase isoenzymes

Electrophoresis was carried out on a precast polyacrylamide IEF (isoelectrofocusing) PhastGel (43 X 50 X 0.45 mm) in a pH range from 3 to 9 as described in Application File No. 100 from Pharmacia. Samples were applied on the gel in the middle using a 4 μl comb containing 6 wells. The program for the running

conditions was established on the control unit from the PhastSystem. The temperature was kept at 15 °C and the migration time was about 30 min. Peroxidase activity was detected by submerging the gel in the guaiacol reaction mix described above. After approximately 20 min of staining the gels were washed and photographed.

Tissue printing and detection of peroxidase activity

Freshly cut slices were prepared for the prints by slicing the samples into around 4-5 mm thick transverse segments from the upper surface. The protocol for tissue printing in cassava was according to Spruce, et al., (1987), Hendriks and van Loon (1990) and Gabriela, *et al.*, (1996) with some modifications with respect to print time (about 1 min) and applied pressure (about 10 kg/slice). PVDF (polyvinylidene fluoride) transfer membranes from PolyScreen (*NEW*[TM] *Life Science Products, Inc., USA*) were used for tissue printing. Peroxidase activity was detected by submerging the prints in the guaiacol reaction mix described above. After approximately 20 minutes of staining the prints were washed and photographed.

Results

Two TDFs reveal differential expression of cytochrome p450 monooxygenase and peroxidase genes during PPD

Using the cassava PPD model system described previously (Chapter 2, Huang, *et al.*, 1999), changes in the gene expression during the first 72 h of PPD were studied by cDNA-AFLP (Chapter 3, Huang, *et al.*, 2001). From AFLP gels 70 TDFs (transcripts derived fragments) were selected that represented genes from which the expression was either up-regulated, down-regulated, or transiently induced. After isolation from gels, subcloning and sequencing, the sequences of the TDFs were compared to sequences in public databases. From this catalogue two TDFs, TDFc25 and TDFa8, were selected for detailed analysis.

When compared to TDFs derived from ever-present transcripts, supposedly representing constitutively expressed genes, the genes represented by TDFc25 and TDFa8 showed an up-regulated and a transiently induced expression pattern during the 72 hours after the induction of PPD (Figure 1). For both genes transcript levels

were very low or absent in tuberous roots at day 0, as well as in other organs, but reached their highest levels already 6 hours after the onset of PPD (Figure 1). Transcript levels of the gene represented by TDFa8 were very low or absent again 72 hours after the onset of PPD.
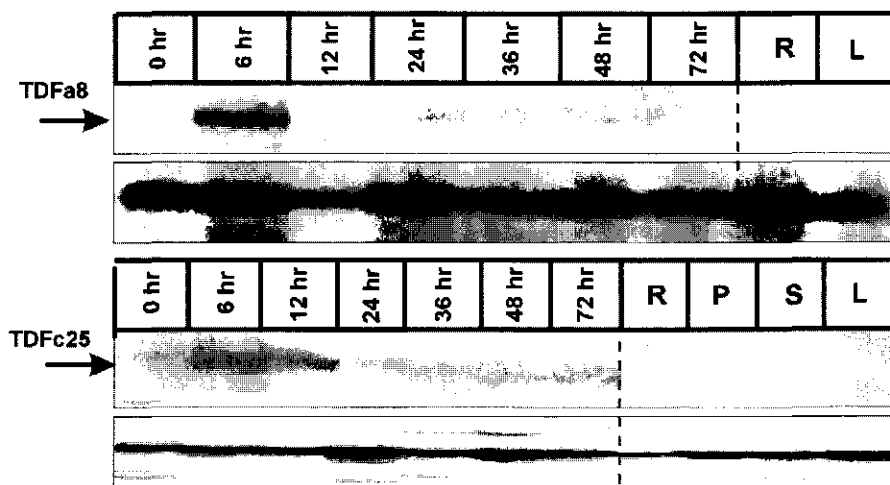
Figure 1. Expression pattern of genes important to oxygen scavenging (such as TDFa8 & TDFc25) during the early stages of PPD in cassava tuberous roots using cDNA-AFLP. Numbers indicate the hours after the start of incubation of the cassava slices under the experimental controlled conditions. Note that the 12-hour lane in the top panel was a drop out. As a control two constitutive bands are shown in both panels. Abbreviations: R = cassava intact tuberous roots, L = leaves, P = petioles, and S = stems.

The 410 bp TDFa8 sequence revealed a 231 bp open reading frame (ORF), followed by a TGA stop codon, and a 130 bp untranslated region (UTR). The 77 amino acid sequence deduced from the open reading frame showed a high sequence similarity with several plant peroxidases (EC 1.11.1.7), like from tobacco, peanut, tomato, and *Arabidopsis thaliana* (Figure 2). Apparently TDFa8 was derived from a transcript encoding a cassava peroxidase.
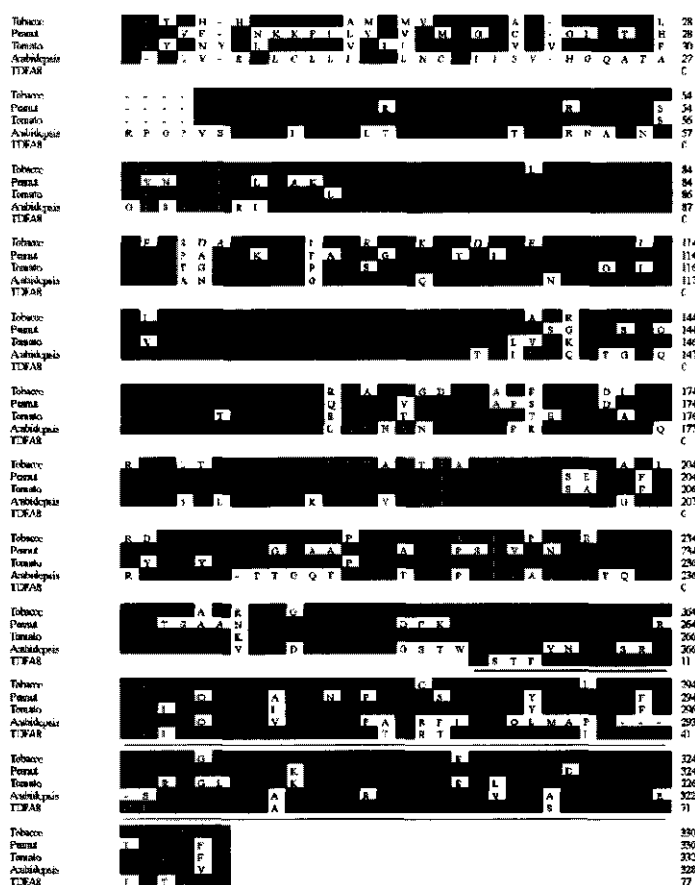
Figure 2. An alignment of the derived amino acid sequence of TDFa8 (underlined) with other peroxidases sequences from tobacco (accession number:AN027753), peanut(accession number: M37637), tomato (accession number: X94943), and Arabidopsis (accession number: X90997). Identical amino acids are highlighted by a solid box.

Sequencing of the 230 bp TDFc25 revealed a 105 bp open reading frame (ORF), followed by a TGA stop codon, and a 57 bp untranslated region (UTR). The open reading frame encoded a putative 36 amino acids-long peptide that showed most high sequence similarity with cytochrome p450 monooxygenase in pea (accession no.

U20335, E value of 1e-07, Frank, *et al.*, 1996), and also a high similarity (82%) with spring vetch. It is therefore highly likely that TDFc25 was derived from a transcript encoding a cassava cytochrome p450 monooxygenase.

From the results above it could be concluded that TDFc25 and TDFa8 represent a cytochrome p450 monooxygenase gene and a peroxidase gene that are differentially expressed during the process of PPD. The expression of both genes is quickly elevated after the onset of PPD, i.e within 6 hours, suggesting at least that their respective products may have a role in the early stages of PPD.

Cassava peroxidases may be involved in the process of PPD in tuberous roots

In other plant species multiple-copy gene families encode both cytochrome p450 monooxygenases and peroxidases. For example, in *Arabidopsis* there may be as many as 273 different cytochrome p450 monooxygenase genes and in *Arabidopsis* and rice more than 40 peroxidase genes (Yamamoto and Sasaki, 1997, Østergaard, *et al.*, 1998). At the protein level this possibly results in the presence of highly homologous members of the respective enzymes, and may be a complicating factor in the assessment of the possible function in PPD of the cytochrome p450 monooxygenase and peroxidase encoded by the genes represented by TDFc25 and TDFa8, respectively. As compared to cytochrome p450 monooxygenases, however, plant peroxidase isoenzymes are easy to detect after electrophoresis and staining for enzyme activity. In a first attempt to at least assess a possible role of peroxidase in PPD, it was decided to study changes in peroxidase activity during this process in cassava tuberous roots, in particular with respect to localization and isoenzyme pattern.

Tissue printing was used to study the distribution of soluble peroxidase activity in the different root tissues. The tissue printing experiments showed that at day 0 peroxidase activity was hardly detectable in the slices (Figure 3). At day 1, however, peroxidase activity was detected in nearly all tissues, but most prominently in or near the epidermis and the vascular bundles. A very similar pattern was observed at day 2, though total activity seemed a little lower. This decrease in activity seemed to continue, because the level of activity was lower again at day 3 (Figure 3) and at day 7 had become almost entirely undetectable (not shown).

The tissue print results suggested that there is a considerable increase in peroxidase activity during the first 24 hrs of PPD and particularly in or near the epidermis and vascular bundles. The localization of peroxidase activity in the vascular bundles seems to correlate with the occurrence of vascular streaking, the first visible sign of PPD (Mondaldo, 1973, Averre, 1976, Hirose, 1986). The distribution of the phenomenon of vascular streaking in cassava suggests that PPD starts in the internal tissues of the tuberous root. In the next experiments attention was focussed on the changes in peroxidase activity in the internal root tissues, i.e. in root slices from which the peel had been removed.
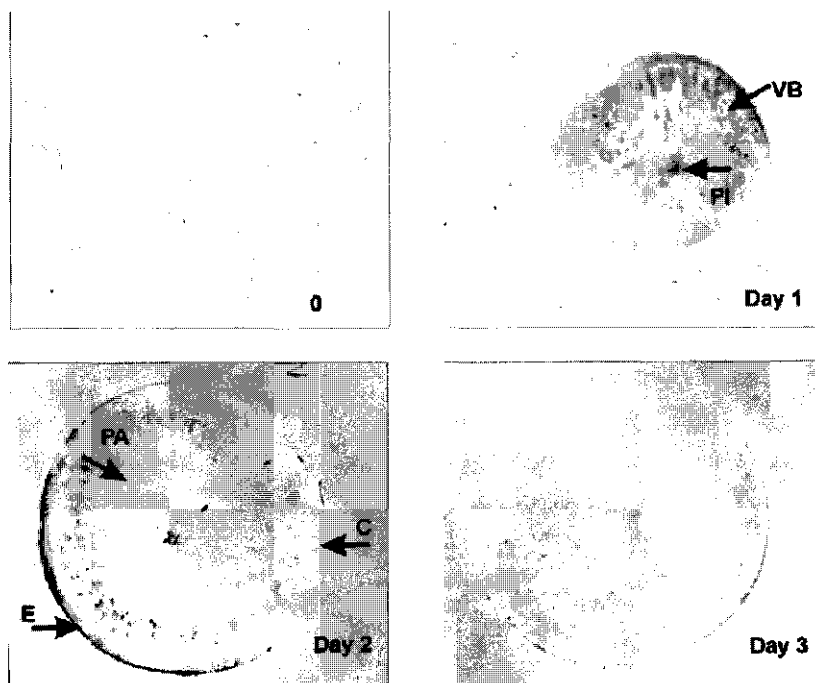
Figure 3. Tissue printing of cassava peroxidase isoenzymes during the PPD process in cassava tuberous roots. Prints were prepared by fresh transverse cuts from slices of day 0, 1, 2, & 3. Abbreviations: C = cortex region; E = epidermal layers; PA = parenchyma tissue; PI = pith; VB = vascular bundles.

As shown in Figure 4, in the internal root tissues both total and specific activity of peroxidase increased almost in a linear fashion during the first 7 days of PPD up to a level 20 times higher than at day 0. In accordance with previous observations (Chapter 2, Huang, *et al.*, 1999), total protein content of the slices remained virtually constant during PPD, only showing a slight decrease after day 4.
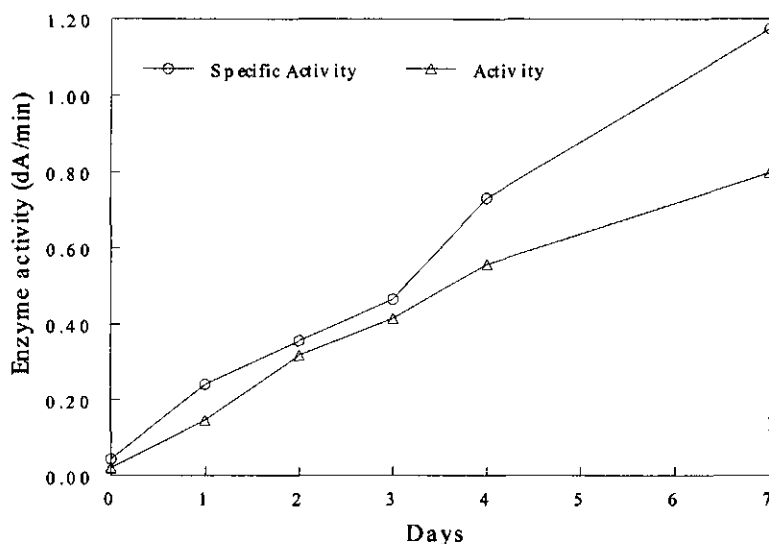
Figure 4. Peroxidase activity during the process of PPD in cassava tuberous roots. Shown are both the activity per minute per gram of fresh weight and the specific activity per minute per microgram of protein.

The continuous increase of peroxidase activity in the internal root tissues seemed in contrast to the results obtained by tissue printing (Figure 3), which suggested a decrease in peroxidase activity after day 1. In fact, the decrease in peroxidase activity in the tissue prints was rather puzzling because it suggested peroxidase(s) being degraded or inactivated, whereas peroxidases are known to be very stable. However, during PPD experiments, there is a considerable loss of water from the root slices (Chapter 2, Huang, *et al.*, 1999). Upon progress of PPD the loss of water may cause a hampering of the transfer of proteins from the root slices

towards the filter, and thus resulting in a decrease in the detection of peroxidase activity. Furthermore, the peroxidase activity detected upon tissue printing may reflect only part of the total peroxidase activity measured in the extracts. The tissue printing technique has proven particular useful in the detection of soluble peroxidases present in the apoplast (Hendriks and Van Loon, 1990, Reid, *et al.*, 1992, Gabriela, *et al.*, 1996). For the detection of peroxidases in the cell, e.g. in the vacuole, this technique might lack sensitivity because the transfer is limited to peroxidases released by single damaged cells at the surface of the slices only.
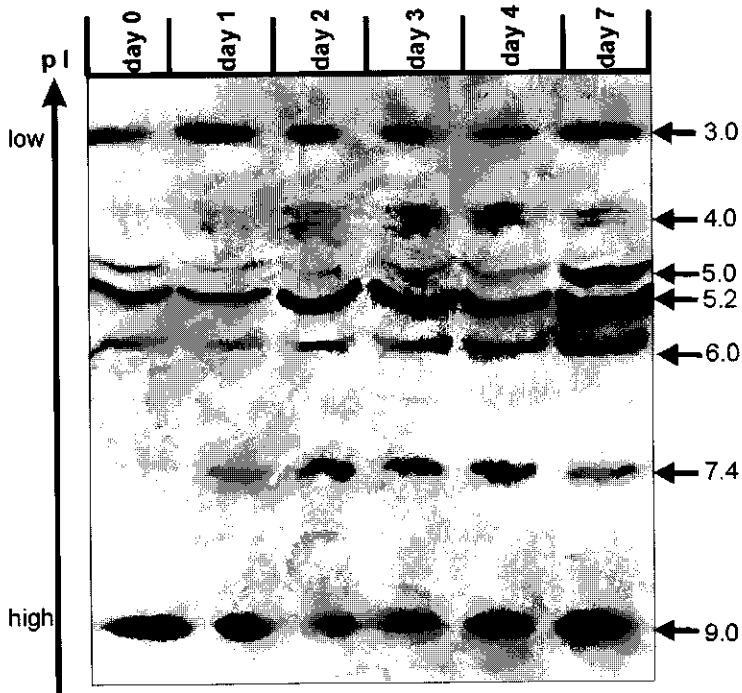
Figure 5. Cassava peroxidase isoenzymes measurement during the process of PPD in cassava tuberous roots by IEF electrophoresis after enzyme activity staining. pI values are indicated at the right.

IEF electrophoresis (pH range from 3 to 9) was used to analyze peroxidase isoenzyme composition in cassava inner root tissues during PPD. As shown in Figure 5, at least 7 peroxidase isoenzymes were detected in the extracts upon electrophoresis and staining for peroxidase activity. Most of the peroxidase isoenzymes were present already at day 0 (e.g. bands at pI 3.0, 5.0, 5.2, 6.0 & 9.0), and some of them showed an increase in activity during PPD (e.g. bands at pI 5.2, pI 6.0 and pI 9.0). Other peroxidase isoenzymes, e.g. bands at pI 4.0 and pI 7.4, seemed to be produced *de novo* within 24 h after the onset of PPD in cassava. None of the peroxidase isoenzymes showed a decrease in activity, which further strengthens the notion that the decrease in peroxidase activity during PPD observed by tissue printing is an artifact.

The increase in the activity of the peroxidase isoenzymes during PPD seemed to last up to day 7, suggesting that their production continued at least up to that day. The activity of the peroxidase isoenzymes with pI 4.0 and pI 7.4, however, hardly changed after the day they became visible, i.e. day 1 (Figure 5). When compared with the level of transcript of the peroxidase gene represented by TDFa8 (Figure 1), it seems that the *de novo* synthesized isoenzymes are the most likely candidates to be encoded by this gene.

Discussion

PPD is a complex physiological and developmental process, which is the final phase in the cassava tuberous root life cycle. Using the cDNA-AFLP method, a large number of differentially expressed genes during the PPD process in cassava tuberous roots were studied (Chapter 3, Huang. *et al.*, 2001). TDFa8 and TDFc25, representing genes that were differentially expressed during the first 72 hours of PPD, were isolated from cassava tuberous roots. Their expression pattern revealed that the genes represented by TDFa8 and TDFc25 were expressed only during the PPD process and not in control tissues, such as intact tuberous roots and leaves. Based on sequence analysis, TDFa8 and TDFc25 are most likely obtained from transcripts of genes encoding peroxidase and cytochrome p450 monooxygenase, respectively. The localization of peroxidase activity was shown to be mainly restricted to vascular bundles and parenchyma tissues in cassava tuberous roots following the process of

PPD. Both total and specific activity of cassava peroxidases in the internal tissues increased during the process of PPD.

Cytochrome P450 monoxygenases mediate as terminal oxidases a wide range of oxidative responses that are related to the biosynthesis of plant secondary metabolites, including phenylpropanoids and phytoalexins (Frank, *et al.,* 1996). It has been shown that the phenolic compounds, i.e. scopoletin and scopolin, might be associated with visible signs of PPD in cassava (Rickard, 1981 and 1985, Wheatley and Schwabe, 1985).

Plant peroxidases are supposed to play a role in development, defense responses and even signal transduction pathways (Hiraga, *et al.,* 2001). Most plants possess a large number of peroxidases, and their expression profile is influenced by environmental stimuli and is often developmentally regulated and tissue specific (Cassab and Warner, 1988). More than 40 independent ESTs (expressed sequence tags) of different peroxidases are expressed in Arabidopsis and rice (Yamamoto and Sasaki, 1997, Østergaard, *et al.*, 1998). Furthermore, 21 peroxidase genes have been identified in rice recently and all these peroxidase genes revealed unique expression profiles (Hiraga, *et al.*, 2001). Different peroxidases are expected to have different functions in various physiological processes. These functions include scavenging of peroxide, lignification, suberization, cross-linking of cell wall polysaccharides and/or proteins, phenol oxidation and hormonal signaling (Østergaard, *et al.*, 1998).

PPD is accompanied by an increase in peroxidase activity, particularly in epidermis and vascular bundles. This increase is caused by the enhancement of the activity of peroxidase isoenzymes already present, as well as by newly synthesized isoenzymes. Together with the observed expression pattern of the peroxidase gene represented by TDFa8, this suggests that peroxidases are involved in PPD in cassava. Especially the rapid appearance of peroxidase activity in the vascular bundles is interesting since during PPD, both "vascular streaking" and "vascular discoloration" are visualized in vascular bundles and around parenchyma regions in the tuberous roots. In transgenic tobacco, enhanced activity of a soluble apoplastic peroxidase caused an increase in wound – induced browning (Lagrimini, 1990). These results suggest that peroxidases may be associated with the occurrence of "vascular streaking" and "vascular discoloration" during PPD in cassava tuberous roots. The peroxidase gene represented by TDFa8 might code for one of the peroxidase

Abstract

In this paper the isolation of a TDF (transcript derived fragment), named TDFa12, is described, which exhibits a differential expression pattern during the first 72 hours of PPD in cassava. TDFa12 showed most sequence similarity with some of the representative dad1 (defender against apoptotic death 1) homologues from other species like Arabidopsis, apple, rice, human, mouse and *C. elegans*. Southern hybridisation results suggest that dad1 has a low copy number in the cassava genome. Transformants were prepared using particle bombardment of cassava FEC (friable embryogenic callus) lines with DNA carrying the putative cassava dad1 homologue in an antisense orientation under the control of the CaMV 35s promoter. Two strategies were taken for the production of transgenic plants: one based on luciferase screening only and one on kanamycin plus luciferase selection. It was proven that cassava FEC lines could be bombarded with a mixture of two different isolated plasmids and that co-transformation occurred at a high frequency for the first time. In total 94 transgenic lines, carrying the dad1 gene, were obtained. However, only 6 of them regenerated into plants. This low percentage was caused by an intrinsic low capacity of the used FEC lines to regenerate into plants. The transgenic nature of the plants was confirmed by Southern blotting using the luciferase gene as a probe. One transgenic line showed new phenotypic changes i.e. early senescence in leaves, which might be attributed to the expression of the introduced dad1 gene. The possible reasons for this alternative morphology in the transgenic plant are given and discussed.

Introduction

One of the approaches to investigate the process of PPD is based on the biochemical characterization of enzymes possibly crucial to oxidative stress in cassava tuberous roots. It is assumed that PPD is a peroxidase – mediated oxidative process (Chapter 4). Reactive oxygen species (ROS) are induced rapidly following oxidative stress, and are involved in many processes such as programmed cell death (PCD), regulation of gene expression and signal transduction (Nose, 2000, Bethke and Jones, 2001). It is indicated that oxidative stress can also influence the cell cycle progress in plants (Reichheld, *et al.*, 1994). Plenty of evidence shows that oxidative stress leads to PCD in animals and plants (Amor, *et al.*, 1998 & 2000, Jabs, 1999, Piffanelli, *et al.*, 1999). In cassava, two TDFs (transcript derived fragments) important to oxidative stress, which share the best sequence similarities to tobacco peroxidase (accession No. AB027752 and E value = 4e-32, Hiraga, *et al.*, 1999) and pea cytochrome P450 monooxygenase (accession No. U20335 and E value = 6e-8, Frank, *et al.*, 1996), have been identified and isolated through a systematical analysis of changes in gene expression during the first 72 hours of the process of PPD (Chapter 4). Catalase, another gene playing an essential role in defense against oxidative stress (Rocha, *et al.*, 1996), was isolated from a cassava tuberous root cDNA library 48 hrs after the start of PPD (Reilly, *et al.*, 1999). H_2O_2, one of the ROS, which is known to trigger PCD response in animals and plants (Tenhaken, *et al.*, 1995, Jabs, 1999, Bethke and Jones, 2001), was found to be produced at early stages of PPD in cassava tuberous roots (Buschmann, *et al.*, 2000b). Furthermore, a TDF (named TDFa12) sharing a high sequence similarity with defender against apoptotic death 1 (dad1) cDNA was also putatively identified and isolated during the process of PPD in cassava using the cDNA-AFLP technique. It has been suggested that PCD may be involved in the process of PPD in cassava (Chapter 3, Huang, *et al.*, 2001).

PCD is a broader term related to an active physiological process of cell death under genetically controlled mechanism(s) (Ellis, *et al.*, 1991, Greenberg, 1996). PCD is expected to be an integral essential part of normal developmental stages to selectively remove unwanted cells or harmful cells in response to a range of intrinsic or extrinsic stimuli (Willams and Smith, 1993). Considerable research indicates that PCD also occurs in the plant kingdom just like in the animal kingdom, while the mechanism(s) of PCD in plants are less well understood (Havel and Durzan, 1996, Pennel and Lamb, 1997, Lam, *et al.*, 1999). In plants, PCD is thought to play a role in

many developmental processes such as senescence, xylogenesis, aleurone deletion, the death of the root cap cells, and somatic embryogenesis. PCD also takes place during the interaction between plants and environmental factors (Pennel and Lamb, 1997). Apoptosis is a kind of PCD with striking events which include a set of distinct morphological and biochemical changes like condensation of the nucleus and chromatin, fragmentation of nuclear DNA at internucleosomal regions, membrane blebbing and the formation of apoptotic bodies (Schwartzman and Cidlowski, 1993). DNA laddering is believed to be one of the hallmarks of apoptosis in animal and plant cells. However, not all PCD can be discovered these features just like in apoptosis (Schwartz, et al., 1993).

The dad1 gene was first isolated from human beings and mapped the human chromosome (Apte, et al., 1995), and later isolated from C. elegans. It has been shown to "rescue" a temperature-sensitive hamster cell line mutant from apoptosis after expression (Nakashima, et al., 1993, Sugimoto, et al., 1995). It was also shown that dad1 is a subunit of an oligosaccharyltransferase that is located in the endoplasmic reticulum (ER) membrane (Kelleher and Gilmore, 1997) and supposed to be functioning in N-linked glycosylation down-regulation of apoptosis (Sanjay, et al., 1998). However, over-expression of dad1 in mice did not have an effect on apoptosis rather than to influence cell division (Hong, et al., 1999). Intriguingly, expression of the dad1 homologues from Arabidopsis and rice also showed the same "rescue" function as the ones from human and C.elegans as apoptosis suppressor (Gallois, et al., 1997, Tanaka, et al., 1997). More recently, more plant dad1 homologues have been isolated from pea, apple and citrus (Orzáez and Granell, 1997, Dong, et al., 1998, Moriguchi, et al., 2000).

There is no information on the characterisation of dad1 gene(s) in cassava. Our main interest is to delay the process of PPD in cassava tuberous roots after harvesting through regulation of the process of PPD and/or PCD by creating transgenic cassava.

At the moment there are different protocols available for the transformation of cassava. One method is based on organogenesis (Li, et al., 1996). The other methods are based on the use of FEC (friable embryogenic callus). FEC consists of small, spherical shaped, pre-globular units and transgenic plants were produced via particle bombardment (Raemakers, et al., 1996, Schöpke, et al., 1996, Munyikwa, et al., 1998). Schöpke, et al., (1996) used kanamycin as a selective marker, and

Raemakers, *et al.*, (1996) and Munyikwa, *et al.*, (1998) used luciferase activity for the selection of transgenic tissue. Kanamycin based selection yielded a much higher number of transgenic lines per bombarded dish than luciferase selection. On the other hand almost all tissues obtained from luciferase selection were capable to regenerate into transgenic plants, whereas with kanamycin selection only a fraction of the tissues yielded transgenic plants (Schöpke, *et al.*, 1996).

Here, the isolation of TDFa12 that exhibits a differential expression pattern during the early stages of the PPD process in cassava tuberous roots is reported. It shares a high sequence similarity to dad1 homologues in the public databases. To try to unravel a possible link between PCD and PPD in cassava, a reverse genetic approach was chosen to select the putative cassava dad1 as a candidate gene. The transformation of cassava FEC via particle bombardment using luciferase screening and kanamycin plus luciferase selection was described. The transgenic plants, carrying the putative cassava dad1 homologue in antisense, were investigated in this chapter.

Materials and Methods

Establishing an uniform system for the induction of the PPD process in cassava tuberous roots

Tuberous roots from around 1.5-years old cassava plants (*M.Col 22* and *Faroka*) were harvested from the greenhouse of Wageningen University. The plant materials were prepared and an uniform system for the induction and maintenance of the same speed spreading during the process of PPD in cassava was set up as described previously (Chapter 2, Huang, *et al.*, 1999). The different samples of various time points of deterioration were collected and stored at $-80\,^{\circ}C$ for analysis after freezing immediately in liquid nitrogen.

Plant tissues and culture media

FEC lines of the genotype TMS 60444 were initiated as described by Taylor, *et al.*, (1996). Totally 6 lines were collected (line A, B, C, D, E, F), which all were used in the experiments. The media used were as follows; solid FEC proliferation medium (GD6) consisting of Gresshoff and Doy (1972) salts and vitamins, 60 g/l

sucrose, 10 g/l micro agar and 10 mg/l picloram. Liquid proliferation medium (SH6) made up of Schenk and Hildebrandt (1972) salts and vitamins, 60 g/l sucrose and 10 mg/l picloram. FEC maturation medium containing Murashige and Skoog (1962) salts and vitamins, 10 g/l micro agar, 20 g/l sucrose (MS2) plus 1 mg/l picloram. Torpedo-shape somatic embryos were transferred for further maturation to MS2 supplemented with 0.1 mg/l BA. Green somatic embryos were cultured for plant formation on cotton plugs saturated with liquid MS2 and 1.0 mg/l BA. Shoots were rooted on MS2 medium. In all media the pH was adjusted to 5.7-5.8 before autoclaving and plant tissues were cultured in a growth chamber with a temperature of 30 °C, photoperiod of 12 hours and an irradiance of 40 µmol/s m^2 PAR.

Construction of TDFa12 in an antisense orientation and other plasmids

The TDFa12 was subcloned into the vector pJIT 125 (Guerineau and Mullineaux, 1993) by a PCR-aided cloning approach. The vector pJIT 125 kindly provided by Dr. J.F. Guerineau, John Innes Institute, UK), contains the genes coding for luciferase (Luc) and β-glucuronidase (GUS) both driven by the CaMV 35s promoter and terminated by the CaMV 35s polyA region. The TDFa12 was cloned into the vector pJIT 125 to replace the GUS gene at the BamH I sites. The two primers used for cloning were designed as follows: upper one: 5' - CGATTGACGAT*GGATCC*TGACC - 3' and lower one: 5' - TATTTACATATCAAGT*GGATCC*TAAAAA - 3'. The PCR product, which only included the putative coding sequence, was around 200 bp containing two restriction enzyme BamH I sites (*GATTCC*). This PCR fragment was first digested with restriction/ enzyme BamH I and then directly cloned into the pJIT 125 vector in the BamH I site, which leads to the 35s-dad1-polyA, 35s-luciferase-polyA construct (pCasdad1). The construct possessed the antibiotic resistant gene (ampicillin) for selection of the bacteria carrying the pCasdad1 vector. The antisense TDFa12 constructs were identified by the PCR analysis using different primer combinations: one primer from the CaMV 35s region (5'-TGCCCAGCTATCTGTCACTTTA –3') and two primers from the TDFa12 internal regions (upper one: 5'- GCGGTTTTCACTGCTCTGATT- 3' & lower one: 5'- TTGCTGATTTTGTCCTCTGC- 3'). The prepared construct is shown in Figure 1.
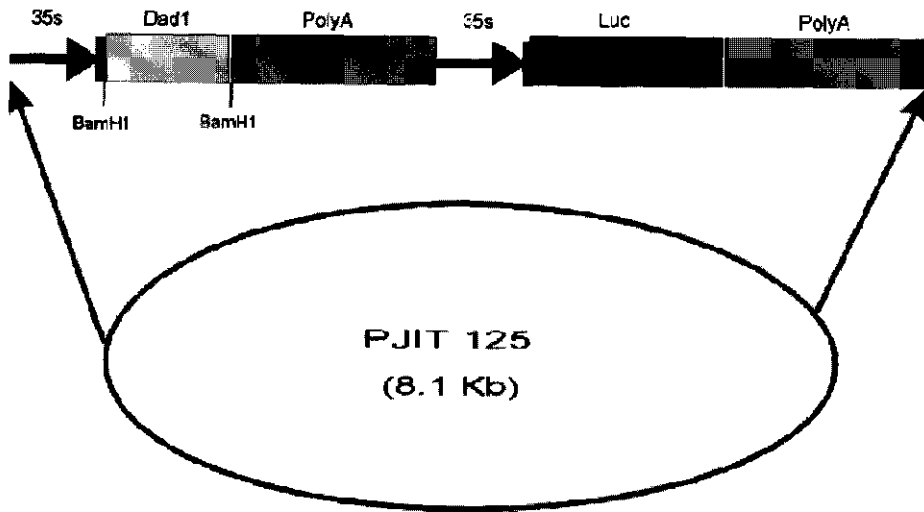
Figure 1. Schematic representation of the plasmid pCasdad1 containing the putative cassava dad1 homologue in antisense within the vector pJIT 125 in the BamH I sites.

Besides the pCasdad1 plasmid, the vectors pJIT64 and pDC2 have been used. pJIT64 (Guerineau & Mullineaux, 1993) was also kindly provided by Dr. J.F. Guerineau (John Innes Institute, UK) and pDC2 was kindly provided by Plant Genetic Systems, Belgium. pJIT64 also contains the luciferase gene flanked by the double CaMV 35S promoter and the CaMV 35S terminator. pDC2 contains the ß-glucuronidase gene flanked by the *Agrobacterium* dual TR2' (Velten, *et al.*, 1984) and the CaMV 35S terminator and the neomycin phosphotransferase gene flanked by the TR 1' promoter (Velten, *et al.*, 1984) and the nos terminator.

Particle bombardment

20 µg of plasmid DNA (in the case of co-transformation 10 µg of each plasmid) was coated onto 10 µg of gold particles (1.6 µm) by using the coating protocol as described by Raemakers, *et al.* (1996).

In the first experiment 6 different FEC lines of TMS 60444 (18 months old) were bombarded with particles coated only with DNA of the pCasdad1 plasmid. Around 100 mg FEC was used per bombardment. After bombardment, the FEC was transferred to liquid SH6 medium. Two weeks later the FEC was collected on solid GD6 medium and assayed for luciferase activity. Each luciferase (luc) spot plus the tissues in a radius of 0.5-1.0 cm around the luc spots were subcultured as an individual line in liquid SH6 medium. Two weeks later, the FEC was assayed again for luciferase activity. Lines without luciferase activity were discarded and lines with 4 or more luc spots were used for subclump division (Raemakers, et al., 1999). Subclump division started with subculturing the tissues around 0.5 to 1.0 cm diameter per luc positive spot. The tissue was divided as fine as possible on GD6 medium. Two weeks later the Petri dish was covered with small clumps of FEC tissue. Only the luc positive clumps were subcultured. For this the clumps were divided in subclumps and cultured on GD6 medium. This selection procedure was repeated 2-3 rounds or more before the positive tissue was cultured for plant regeneration.

The best responding line of the first experiment was also used in the second experiment. In this experiment the FEC was either bombarded with DNA of the plasmid pCasdad1 or with a mixture of pCasdad1/pDC2. The pCasdad1 bombarded FEC was treated as in the first experiment. The pCasdad1/pDC2 callus was first cultured in liquid SH6 medium and two weeks later it was divided very finely over a nylon sheet cultured on GD6+10 mg/l kanamycin. Three weeks later the nylon sheets with on top the callus were transferred to fresh medium. After 6 weeks of culture the number of clumps per bombarded dish was recorded and luc positive clumps were cultured for plant regeneration.

In the third experiment FEC of one line was bombarded with either pCasdad1/pDC2 or with pJIT64/pDC2 as a control. The aim of this experiment was to determine if bombardment with DNA of the putative cassava dad1 homologue has a negative effect on plant regeneration of transgenic lines. The FEC was cultured as described for the second experiment.

Assays for reporter gene activity

Plant tissue was sprayed with 0.15 mg ml^{-1} firefly luciferin. Luciferase activity was measured using the luminometer (VIM intensified CD camera and Argus-50 photon counting image processor of Hamamatsu Phototronic Systems).

A modified histochemical GUS assay of Jefferson, *et al.,* (1987) was made. The assay solution included 0.2 M Na_2HPO_4, 0.2 M NaH_2PO_4, 10% triton X100, 10 mM Na_2EDTA, 0.5 mM potassium ferrocyanide, 20% hydrogenperoxide and 0.96 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexylamine salt (overnight incubation at 37 °C in the dark).

Total RNA isolation and the cDNA-AFLP method

The total RNA from the samples of different time points during the process of PPD in cassava and from control tissues (intact tuberous roots and leaf) were isolated as described previously (Chapter 3, Huang, *et al.,* 2001). The templates used for cDNA-AFLP were prepared and the cDNA-AFLP procedure was performed based on the method developed by Bachem, *et al.* (1996).

TDFA12 isolation, subcloning and sequencing

According to the differential expression patterns during the process of PPD, TDFa12 was isolated from the polyacrylamide AFLP gel and amplified using the non-selective extension primer combination (Ase I and Taq I anchor). The PCR product was directly cloned into the pGME – T easy vector (*Promega, USA*). The sequencing was done by an automated sequencer and the sequence of TDFa12 was compared to the latest sequences present in the public databases by using the BLAST 2.0 program (Altschul, *et al.,* 1997).

Genomic DNA isolation and Southern blot

The plant materials were ground to a fine powder in a pre-cooled pestle with a mortar containing liquid N_2. Genomic DNA was isolated from the cassava leaf using the DNA Isolation Kit (*BIOzym, the Netherlands*). The DNA was digested with different restriction enzymes and electrophoresed on 1% agarose gels. Then DNA was transferred to Hybond N^+ filter in 0.4 M NaOH/0.6 M NaCl solution. Hybridisation and washing steps were performed according the Hybond N manual. ^{32}P - labelled DNA probes were obtained by random priming using the *redi*primer™ II Kits (*Amersham Pharmacio Biotech, UK*).

Confirmation of the transgenic plants by Southern blot

The transgenic plants were confirmed by Southern blot using the luciferase gene as a probe. This probe was made by PCR using the primers; upper one: 5'- ACG CCTTTGGAATGTTTACAT 3' and lower one: 5'- CGGTTGTTACTTGACGGCGAC 3', which resulted in a PCR product of around 800 bp. The PCR reaction conditions were as follows: denaturation at 94 °C for 4 minutes, then 30 cycles at the condition of 94 °C for 30 seconds, 62 °C for 1.5 minutes, 72 °C for 1 minute, and finally followed by 4 minutes extension at 72 °C.

Results

TDFa12 showing a differential expression during the process of PPD in cassava tuberous roots reveals a high sequence similarity to dad1 homologues.

A molecular approach was taken to analyse changes of gene expression patterns in cassava focusing on the early stages (first 72 hours) of the PPD process. It was possible to systematically screen a large number of expressed genes during the process of PPD in cassava by using the cDNA-AFLP technique in our uniform system for initiating and maintaining the speed of the process of PPD in cassava tuberous roots (Chapter 2 & 3, Huang *et al.*, 1999 and 2001). In principle, genes that reveal an up-regulated, a down-regulated or a transiently induced expression pattern during the PPD process are attractive. Based on the cDNA-AFLP technique, a TDF (named TDFa12) fulfilled these requirements (Figure 2). Figure 2 shows that TDFa12 exhibits an up-regulated expression pattern during the first 72 hours of PPD in cassava by choosing the primer combination No.44 (Ase I anchor plus selective extension AA) and No.77 (Taq I anchor plus selective extension TC). The size is around 430 bp in the polyacrylamide AFLP gel. After isolation, subcloning and sequencing, it was found that TDFA12 shares a high sequence similarity with dad1 cDNA (best sequence similarity accession No. AB011798, E value of 3e-42, Takaya, *et al.*, 2000) in the public databases.

By searching the latest release sequence information in the public databases, it was found that dad1 genes from different origins have very high conserved sequences. The full length (encoding plus 3' & 5' untranslated region) of dad1 cDNA is around 600-700 bp, encoding a protein of about 115 amino acids with a predicted
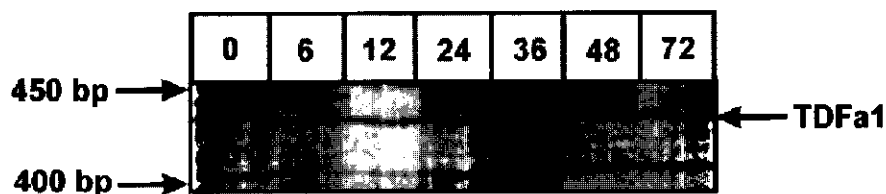
Figure 2. Expression pattern of TDFa12 during the process of PPD using the primer combination No.44 and No.77 in cDNA-AFLP (http://www.DPW.WAU.NL/PV/). Numbers indicate the hours of incubating the cassava slices under the controlled conditions.
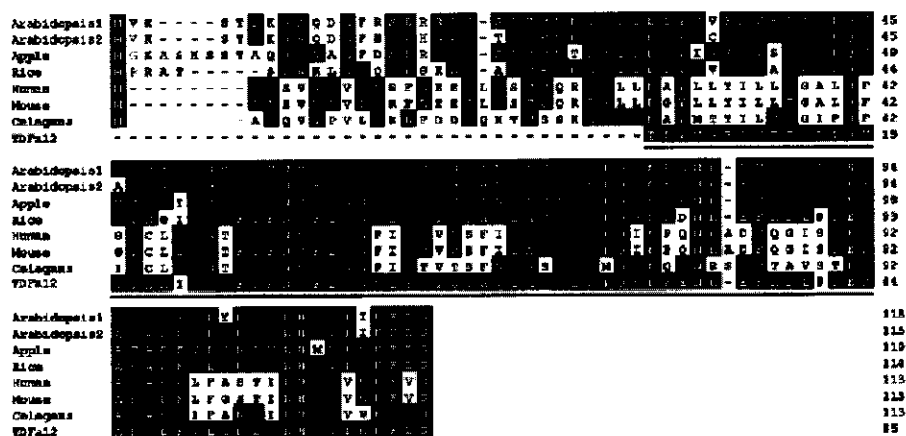
Figure 3. Alignment of the amino acid sequence derived from TDFa12 (underlined) and 7 other dad1 sequences from various origins. Arabidopsis (accession number AF030172 and AC005314), apple (accession number U68560), rice (accession number D89726), human (accession number A54437), mouse (accession number U22107), C. elegans (accession number; S59116). Homologous amino acids are indicated by a solid box.

molecular mass of around 12.5 kDa. Further sequence analysis using the DNASTAR 4.2 software packages (*DNASTAR Inc., USA*), showed that TDFa12 is around 430 bp in length including 85 amino acid residues in the coding region along with a TAA stop codon plus about 140-nucleotides in the 3' UTR (untranslated region), and that it shares a high sequence homology with some of the representative dad1 genes from other species such as Arabidopsis, apple, rice, human, mouse and *C. elegans*. In Figure 3, sequence similarities of TDFa12 with other dad1 homologues from various species are displayed.

The organisation of the putative dad1 homologue in the cassava genome

High quality genomic DNA was isolated from cassava to check the potential copy number of dad1 gene(s) in the cassava genome. The cassava genomic DNA was digested with various restriction enzymes such as BamH 1, EcoR I, EcoR V, Hind III, Nco I, and the digested DNA fragments were hybridized with the TDFa12 fragment as a probe. The Southern blot shows that dad1 is present as a low copy number gene in the cassava genome (Figure 4A).

Selection of transgenic lines

Particle bombardment was used to introduce the putative cassava dad1 homologue into the cassava genotype TMS60444 using FEC lines. In the first set of experiments selection of transgenic tissue was based on the activity of the *luciferase* gene (Chia, *et al.*, 1994). Transient expression was observed 2 days after bombardment of FEC. The highest luc activity observed was around 11,500 photons/15 seconds on one Petri-dish. Six FEC lines of TMS60444 were bombarded with pCasdad1. Two weeks after bombardment the Petri dishes were assayed again for luc activity. The amount of emitted photons had decreased with at least 90% compared to the first measurement at 2 days after bombardment. Luc activity was only seen as small spots. There is a large difference between the lines with respect to the number of luc spots (Table 1). The B line had the lowest number and the C line possessed the highest number of luc spots. A maximum number of 17 spots were observed in one Petri dish. Almost 300 spots were subcultured as individual lines. Three weeks later 106 lines still contained luc positive tissue. However, only in 52 lines, 4 or more luc spots per Petri dish were detected. Further subculture resulted in 34 stably transformed lines: 32 were derived from the C line and 2 from the D line.

The overall efficiency in this experiment was around 0.4 transgenic line per bombarded dish. In the next set of experiments the overall efficiency of subclump division was compared with kanamycin selection.

Table 1: Number of luciferase positive lines obtained after bombardment of different FEC lines with pCasdad1 (around 100 mg FEC units used per line, expressed as the means of 15 bombarded dishes per line)

FEC line	luciferase spots (2 weeks after bombardment)	luciferase positive lines (5 weeks after bombardment)	luciferase positive lines (9 weeks after bombardment)
A	1.5	0	0
B	1	0	0
C	10.5	3.2	2.1
D	1.6	0.2	0.1
E	3.5	0	0
F	1.1	0	0

Because pCasdad1 does not contain the nptII gene, the FEC was bombarded with a mixture of pCasdad1/pDC2. Based on the results of Table 1, line C was chosen as starting material. Two weeks after bombardment the FEC was divided finely over nylon filters and cultured on GD medium supplemented with 10 mg/l kanamycin. After about 4 weeks of culture small colonies of FEC became visible. Two weeks later all the colonies were tested for luc activity. The luc negative colonies were also tested for gus activity. The luc positive colonies were first cultured as individual lines and 3 weeks later a small part of each individual line was tested for gus activity. The results of both the luc and gus assays are shown in Table 2.

In total 150 clumps were selected. 32 percent of these clumps were luc negative and gus positive suggesting that only DNA from pDC2 was integrated into these cells. Sixty eight percent of the colonies were both luc and gus positive, suggesting that integration of DNA of both pDC2 and pCasdad1 had occurred.

Table 2. Number of vigorously growing colonies on the kanamycin containing medium 6 weeks after bombardment.

Plasmids	Average colonies # (per bombarded dish)	Nature of transgenic colonies after kanamycin selection *			
		Luc $^+$/gus $^+$	Luc $^-$/gus $^+$	Luc $^+$/gus $^-$	Luc $^-$/gus $^-$
pCasdad1/pDC2	4.7	68%	32%	0%	0%
pJIT64/pDC2	5.6	57%	38%	0%	0%

(*: In total 20 dishes with pCasdad1/pDC2 and 10 dishes with pJIT64/pDC2 were bombarded, a total of 150 colonies were evaluated for luciferase and gus activity.)

Plant regeneration from the putative dad1 positive FEC lines

34 luciferase positive lines derived from subclump division (bombardment with pCasdad1) and 60 from kanamycin selection (bombardment with pCasdad1/pDC2) were cultured for maturation. Most of the luc positive lines were derived from FEC line C; only 2 lines were derived from line D. After 4 weeks of culture on maturation medium both luc positive lines derived from the D line yielded the first somatic embryos. For the transgenic lines derived from the C line this was 3-5 weeks later. In total 56 somatic embryos were isolated from line D. Only 19 of the 94 cultured transgenic lines derived from line C, yielded somatic embryos and the number of somatic embryos per line varied from 1 to 20. The ability to form mature somatic embryos was not restricted to a specific selection regime. Raemakers, *et al.*, (1996) had shown that the number of somatic embryos of a particular line could be increased by culturing green somatic embryos for secondary somatic embryogenesis. In this way, plants could be regenerated from transgenic lines, which yielded only a low number of somatic embryos. This technique was also applied here. Secondary somatic embryogenic cultures were obtained from somatic embryos of 12 luciferase positive lines.

In the next step of the regeneration process somatic embryos were cultured for germination into plants. Somatic embryos of in total 12 lines were cultured and plants were obtained from in total 6 lines. In the other 6 lines the somatic embryos failed to germinate properly. Two plants were obtained from FEC bombarded with both pCasdad1/pDC2 grown on the kanamycin containing medium followed selection

for luc activity (line 5 and 34) and 4 plants from pCasdad1 selected only by luc activity (line 1, 4, 11 and 12).

Low capability of plant regeneration for the FEC lines

In the experiments described here only 6 out of 94 lines germinated into plants. The reason for this low efficiency might be due to the introduction of the putative cassava dad1 gene, which might have a negative effect on plant regeneration or the FEC line itself having a low capability to develop into plants. This was investigated. First the same FEC line was bombarded with a mixture of pJIT64/pDC2 DNA. The number of luc positive lines produced with the pJIT64/pDC2 mixture was slightly higher than after bombardment with pCasdad1/pDC2. The capability of luciferase positive cultures to regenerate into mature somatic embryos was almost the same for both. To test the second hypothesis non-transformed FEC of different lines were cultured for regeneration. The results of different lines for both maturation and germination are shown (Table 3). Four of the 6 tested lines yielded more than 1000 somatic embryos per 100 mg cultured FEC. The first somatic embryos appeared after 3 weeks of culture. Two lines, B and C, yielded less than 100 somatic embryos and the first appeared after 4-5 weeks of culture. The C-line gave the lowest number of somatic embryos. Also germination of somatic embryos in this line was considerably lower than in the other tested lines.

Table 3. Capability of different FEC lines to form mature somatic embryos and subsequently plants.

line	Mature somatic embryos (SE)	SE which germinate into a plant (%)
A	>1000	73
B	84.7	54
C	29	12
D	>1000	46
E	>1000	65
F	>1000	58

(Data are the means of 2 replications; 100 mg FEC per replication was cultured for 9 weeks.)

The morphology of the transgenic plants

In total 6 transgenic lines derived from FEC lines regenerated into plants. No dramatic phenotypic differences were observed compared with the normal control when these transgenic plants were grown in vitro except for transgenic line 5. Senescence in the leaves of transgenic line 5 seemed to occur earlier compared with that of the control plants in vitro (Figure 5A). Whether this also exists in the greenhouse will have to be assessed. So far attempts to transfer line 5 to the greenhouse failed. The other lines, which were transferred to the greenhouse, were all growing slower and showed a different leaf shape (Figure 5B).
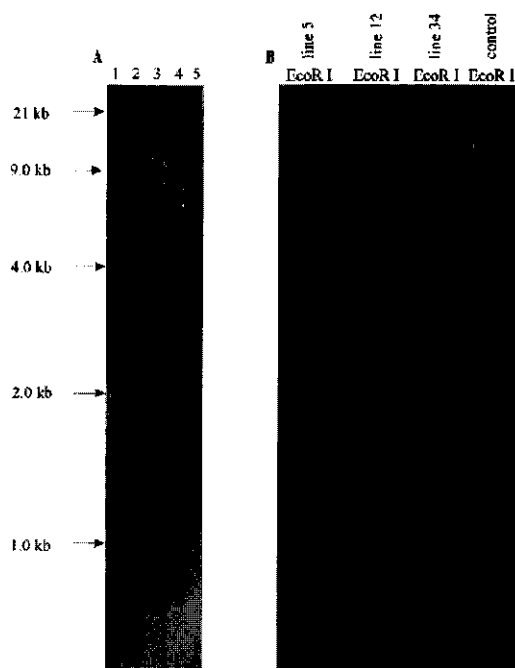
Figure 4A. Southern blot of high quality genomic DNA of cassava digested with enzymes BamH I (lane 1), EcoR I (lane 2), EcoR V (lane 3), Hind III (lane 4) and Nco I (lane 5). TDFa12 from cassava was used as a probe indicating a low copy number of dad1existing in the cassava genome. **4B.** Southern hybridization of cassava genomic DNA from transgenic plants 5, 12 and 34, bombarded with pCasdad1 or pCasdad1/pDC2 plasmids, carrying the putative cassava dad1 gene in an antisense orientation. A PCR–amplified coding sequence of the *luciferase* gene was used as a probe.

Confirmation of the transgenic nature by Southern blot

Since the size of the putative cassava dad1 TDF is rather small, the PCR product from the luciferase gene was used as a probe to check the transgenic nature of the transformants. It was shown that 3 of the transgenic lines carried the luciferase gene (line 5, 12 and 34), which means that these 3 transgenic plants probably also carried the putative dad1 gene (Figure 4B). The transgene copy number integrated into the chromosomes seemed to vary between the different transformants based on the intensity of the bands.
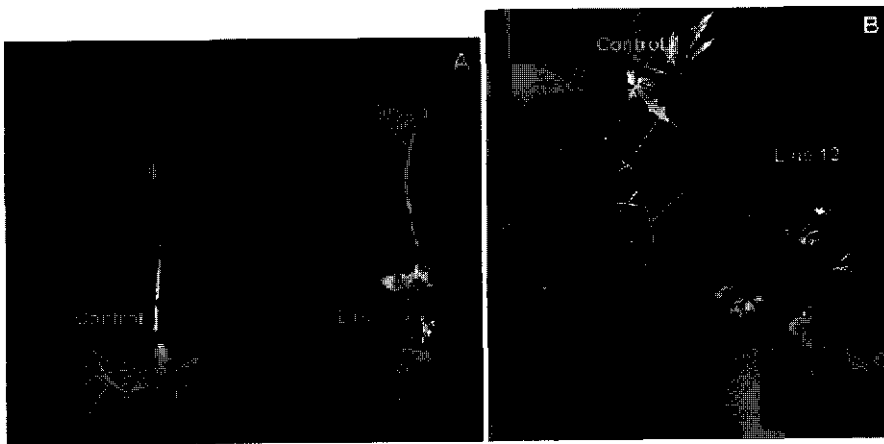
Figure 5A. In vitro phenotypes of a non-transgenic control cassava plant after tissue culture regeneration and the transgenic line 5 carrying the putative cassava dad1 homologue in an antisense orientation driven by the CaMV 35s promoter. **5B.** Greenhouse grown non-transgenic control cassava plant after tissue culture regeneration and the transgenic line 12 with the putative cassava dad1 homologue in an antisense orientation driven by the CaMV 35s promoter.

Discussion

A large number of expressed genes have been systematically screened during the first 72 hours of the PPD process in cassava tuberous roots, which is a complex physiological process (Chapter 3, Huang, *et al.*, 2001). Using the cDNA- AFLP method, it is reported here that TDFa12, which exhibited a differential expression

pattern during the process of PPD in cassava, was isolated. The result obtained from Southern blot analysis suggested that dad1 may have a low copy number in the cassava genome like in apple (Dong, *et al.*, 1998) and in pea (Orzáez and Granell, 1997). Very recent results suggested that the functional part of the dad1 gene might be within the C - terminal region (Maskshima, *et al.* 2000). The data indicated that the dad1 gene lacking 20 amino acid residues from the N-terminal part still exhibited the ability to complement the Chinese hamster cell line mutation, while a dad1 gene only missing 4 amino acid residues from the C-terminal region, had no "rescue" function at all. TDFa12 (about 430 bp) is a part of the transcribed fragment of the full-length dad1 cDNA (around 600-700 bp) including the C-terminal part. DNA laddering is one of the hallmarks of apoptosis. However, we could not detect DNA laddering during the process of PPD in cassava tuberous roots, which suggests that cell death may be due to a different mechanism like PCD existing in the barley aleurone layer (Fath, *et al.*, 1999) (data not shown).

It is expected that physiological deterioration only occurs after harvesting of cassava tuberous roots, which results in loss of its quality. This suggests that after harvest cassava tuberous roots shift to a kind of senescence situation due to PPD. Here the isolated putative dad1 homologue from cassava showed an up-regulated expression during the process of PPD, which is in contrast with the expression pattern of the full-length dad1 homologue from pea which was down-regulated during senescence of flower petals in pea (Orzáez and Granell, 1997). Considering the results from Southern blot analysis of dad1 genes, this contrast could be explained since different homologues from the dad1 gene family in cassava could have diverse functions and show variable expression patterns. A similar expression pattern as in cassava occurred in apple, which also showed an up regulated expression pattern in leaf, flower and during fruit ripening as well (Dong, *et al.*, 1998). Moreover, the apple dad1 mRNA seemed to be highly expressed in vascular bundles of flowers. If a similar expression pattern of the dad1 gene could also be observed in vascular tissues following PPD in cassava tuberous roots, which is in line with the location of the visual symptoms of "vascular streaking" and "vascular discoloration", it would indicate that the putative dad1 homologue from cassava plays a role in PPD in cassava.

Here the antisense approach was taken in order to unravel the potential function of dad1 in PPD of cassava, since it is expected that down regulation of the

expression level of the dad1 gene will accelerate the process of PPD in cassava. Also generally speaking the antisense effect can be more easily observed compared with the sense approach. Previously cassava FEC lines have been used to produce transgenic plants (Raemakers, *et al.*, 1996, Schöpke, *et al.*, 1996, Munyikwa, *et al.*, 1998). In general FEC used for genetic modification yields non-chimeric transformed plants since FEC units have a single cell origin (Taylor, *et al.*, 1996). In addition somatic embryos are easy for DNA delivery since the origin of proliferating embryogenic tissue is present at or near the surface of the older embryos (Hansen and Wright, 1999). When the selection was based on the activity of the luciferase gene, around 50% of the transgenic lines regenerated plants (Raemakers, *et al.*, 1996, Munyikwa, *et al.*, 1998). Here less than 1% of the lines regenerated into plants (Table 3). It was shown that this low percentage was mainly due to the used FEC line which itself has a very low ability to regenerate plants compared to other lines. This makes it painstakingly clear that selection of FEC lines for particle bombardment should not only be based on its ability to produce transgenic callus lines, but also on its capability to regenerate into plants.

The results described here show for the first time, that cassava FEC lines can be bombarded with mixtures of DNA from two different plasmids and that co-transformation occurs at relatively high frequencies (Table 2). This may open the road for multiple gene transformation in cassava using simultaneously more than one plasmid.

It was known that delivery of foreign DNA by particle bombardment under suitable selection conditions is able to stably integrate into the chromosomes of the nucleus of transgenic plants. Southern blot analysis confirmed that some of the transgenic plants were carrying the target gene (Figure 4B). Since equal amounts of DNA were loaded, the band intensities are an indication for the copy numbers of the integrated transgenes in the plants. Particle bombardment for direct delivery of DNA generally could lead to complicated patterns of target DNA integration in plants. It was shown that indeed the copy number of the transgene was different between the transgenic cassava plants (Figure 4B). It is reported that the use of particle bombardment to deliver only a so-called "minimal gene cassette" (promoter, open reading frame, plus terminator without the vector backbone sequence) into plants can result in low transgene copy numbers, simple expression patterns and high expression (Fu, *et al.*, 2000). Figure 5B showed that the transgenic plants were growing slower

than the control plants, which might be due to somaclonal variation or the antisense effects of the target gene. It was reported that old FEC lines have a high chance of somaclonal variation (Raemakers, *et al.,* 1999). The FEC lines used in the experiments described here were 1.5 years old, which is not that old, however, it is important to use very young FEC lines for bombardment in the future to minimise occurrence of somaclonal variation. Transgenic line 5 expressed earlier senescence in leaves in comparison with the control plants (Figure 5A). This phenomenon has never been observed in non transformed plants derived from FEC lines or transformed plants containing marker genes, or other genes (Raemakers, personal communication), thus indicating possible effects of the dad1 gene. It is known that senescence is an oxidative process that is considered as general deterioration of cellular metabolism (Pastori and Río, 1997). In plants, the best-studied example of senescence is leaf tissue. Leaf senescence is thought to represent a modified plant form of PCD (Jones and Dangl, 1996). The senescence syndrome includes damage of chlorophyll, proteins, lipids and nucleic acids, which leads to yellow leaf, and finally cell death (Gan and Amasino, 1997). This transgenic line will be moved into the greenhouse for further studies.

More transgenic plants carrying the putative cassava dad1 homologue have to be produced so that further investigation can be performed on these transgenic plants in the future. This will shed more light on the function of the cassava dad1 gene during the process of PPD in the tuberous roots.

Acknowledgement

Chapter 6

GENERAL DISCUSSION

Cassava (*Manihot esculenta* Crantz) is the fourth largest source of calories after rice, sugar cane and maize in the world, which feeds more than 500 million people in the tropical and sub-tropical regions. Cassava, one of the most efficient carbohydrate-producing crops, is rich in starch in its tuberous roots which can be widely applied for food and non-food purposes. In spite of being one of the oldest cultivated crops by human beings (Gulick, *et al.*, 1983), cassava breeding is less advanced than other root and tuber crops like potato. Cassava is a highly heterozygous allotetraploid and vegetatively propagated crop. Furthermore, cassava has an irregular flowering time and low seed production. These natural characteristics result in the fact that a traditional breeding approach is more difficult and time consuming for cassava. These, *vice versa*, offer opportunities for cassava to become a more suitable crop by genetic modification to improve its agricultural quality traits. In order to provide more and better cassava as a food integrated into the global food system for the world's rapid population growth in the future (Scott, *et al.*, 2000a and 2000b), genetic modification becomes a potential powerful technology to improve valuable quality traits for cassava using existing cultivars. The work described in this thesis is the result from the analysis of postharvest physiological deterioration (PPD) in cassava tuberous roots at different (physiological, biochemical and molecular) levels. More insight into the mechanisms of PPD in cassava has been gained. Finally, the use of genetic modification as a tool to potentially improve root quality by prolonging the shelf life of cassava tuberous roots after harvesting is further discussed. This could be of benefit to sustainable economic development for small farmers in developing countries (http://books.nap.edu/html/transgic).

An efficient transformation system and isolated genes available are two essential pre-requisites for genetic modification. Especially the development of a good transformation system is necessary before gene technology can be used efficiently. In addition, transgenic plants as an assay system can also make it possible to study gene function and regulation of, for example, developmental processes in plants. It was first reported in 1996 that several laboratories had achieved the break-through for transformation of cassava (Li, *et al.*, 1996, Raemakers, *et al.*, 1996, Schöpke *et al.*, 1996) either by microprojectile bombardment using friable embryogenic callus (FEC) lines (Taylor, *et al.*, 1996) or by transformation of cassava somatic embryos derived from cotyledons with *Agrobacterium tumefaciens* and later forming adventitious shoots. Since then advances for transformation of cassava have

been made (Snepvangers, *et al.*, 1997, González, *et al.*, 1998, Li., *et al.*, 1998, Muniyikwa, *et al.*, 1998). However, practical and routine transformation protocols are still required to be established (Sarria, *et al.*, 2000, Zhang, *et al.*, 2000a, Raemakers, *et al.*, 2001, Schreuder, *et al.*, 2001). It was also reported that transgenic cassava plants could be obtained by electroporation using protoplasts isolated from FEC (Sofiari, *et al.*, 1998). The selection used for the cassava transgenic tissues can roughly be classified into chemical selection using antibiotics such as paromycin, geneticin or hygromycin as a selective marker (Li, *et al.*, 1996 and 1998, Schreuder, *et al.*, 2001), or herbicides such as Basta as a selective agent (Snepvangers, *et al.*, 1997, Sarria, *et al.*, 2000). Non-invasive selection by the visual luciferase activity (Raemakers, *et al.*, 1996), or a combination of chemical selection with a visual screen marker (luciferase) (Muniyikwa, *et al.*, 1998) are also used. In the future, one of the plant transformation approaches is to eliminate selectable markers due to public concerns raised about the use of antibiotic genes for selection. This can be achieved by crossing the selective marker(s) afterwards through meiotic recombination or employing non-antibiotic marker systems. For cassava the former approach is not very likely due to the natural properties of cassava cultivars. More recently an antibiotic-free selection system, a so called positive system using mannose as the selection agent was developed in cassava transformation (Zhang, *et al.*, 2000b, Zhang and Puonti-Kaerlas, 2000). This positive selection system is based on either xylose (Haldrup, *et al.*, 1998a and 1998b) or mannose (Joersbo, *et al.*, 1998 and 1999) and has also been applied to potato, tomato and sugar beet. The principle for this selection is based on the fact that plants cannot make use of mannose (or xylose) directly. The uptake of mannose results in starvation and prevents growing due to the accumulation of mannose-6-phosphate. If plants can synthesize the *E.coli* phosphomannose isomerase, which can convert mannose-6-phosphate to easily metabolisable fructose-6-phosphate, then plants will be able to deploy mannose as a sole carbon source. The mannose positive selection for cassava FEC suspension is dependent on the sucrose content in the medium and the low regeneration capacity has still to be resolved (Zhang, *et al.*, 2000b).

Postharvest deterioration is an inherent constraint for cassava after harvesting which leads to a very poor storage life of the harvested tuberous roots. Postharvest deterioration, which is one of the major problems limiting the further possibilities of production, distribution and processing of cassava, includes primary deterioration and

secondary deterioration. Primary deterioration, so called postharvest physiological deterioration (PPD) is a complex physiological process depending on the environmental situation and the cassava genotype. Secondary deterioration is the result of the former deterioration and involves the action of micro-organisms such as bacteria and fungi. To analyze PPD in cassava tuberous roots, a system to mimic the process of PPD in cassava tuberous roots is required. For this purpose, a phenotypical visualized uniform system for the onset and maintaining the same spreading speed during the process of PPD in cassava under experimental conditions was developed and several parameters were investigated in this system (Chapter 2). However, starch, the most abundant component in the tuberous roots, seems to be less influenced during the PPD process as was expected. This could be due to the fact that environmental aspects have a more pronounced effect on PPD in cassava tuberous roots than the genetic make-up of the cassava genotype. Making use of this uniform system to better understand the molecular mechanism of PPD in cassava, a systematic survey of gene expression at the early stages of PPD in cassava focusing on the first 72 hours was carried out (Chapter 3). Using cDNA-AFLP (Bachem, *et al.*, 1996), several expression patterns could be detected at the first 72 hours following the process of PPD in cassava tuberous roots (Figure 1). 70 TDFs (transcript derived fragments) exhibiting an up-regulated, a down-regulated or a transient expression pattern during the first 72 hours of the PPD process in cassava, were isolated after using around 100 AFLP primer combinations. Based on the sequence information, a functional catalogue of these TDFs was established in this thesis. Based on the molecular analysis of PPD in cassava and other research about PPD, a biochemical approach focusing on the enzymes potentially important to oxidative stress, like peroxidase (EC 1.11.1.7), was made. The specific activity and localization as well as the expression of peroxidase during the process of PPD was investigated in cassava tuberous roots (Chapter 4). Results were obtained indicating that PPD might be a peroxidase-mediated oxidative process.

The most important application of cDNA-AFLP in this study was to identify differentially expressed genes. Especially the cDNA-AFLP technique is an effective method for a large-scale identification and isolation of genes, which are differentially expressed in specific tissues during a particular developmental stage or in response to

certain stress (Bachem, *et al.*, 2000, Durrant, *et al.*, 2000). Compared with the microarray-based technologies including cDNA and oligonucleotide microarrays, the cDNA-AFLP technique does not require sequence information in advance and is highly sensitive as well as relatively low in costs. Recently, it was reported that the EST (expression sequence tags) project from cassava has been started (Suarez, *et al.*, 2000). A programme package called GenEST that establishes a potential bi-directional link between ESTs data and the expression profiles produced by cDNA-AFLP has become available (Qin, *et al.*, 2001). Furthermore the cDNA-AFLP technique can be adapted so that each TDF band in the AFLP gel is really a response to one particular cDNA (Breyne and Zabeau, 2001). Accumulating information on ESTs from cassava combined with cDNA-AFLP expression profile data may provide a more powerful tool for gene discovery and functional genomics in cassava.
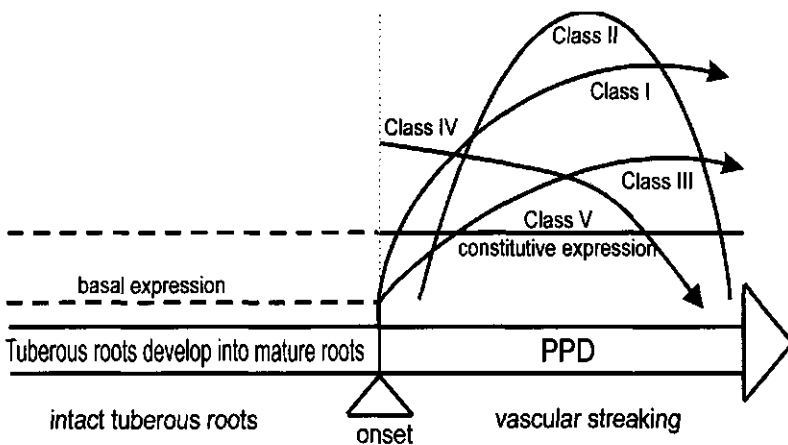
Figure 1. Schematic presentation of differential gene expression patterns during the process of PPD in cassava tuberous roots. Class I and Class III reveal an up-regulated expression pattern but Class III shows a low basal level of expression during cassava tuberous root development and this level increases during the process of PPD. Class II exhibits a transient expression mode during PPD. Class IV displays a down- regulated expression pattern during the PPD process. Class V shows a constitutive expression during the process at the same level as during cassava tuberous root development.

The molecular genetic map of cassava, mainly based on RFLP (restriction fragment length polymorphism) markers, was constructed in 1997 which included 20 linkage groups covering around 60% of the cassava genome (Fregene, *et al.*, 1997). Afterwards, cassava microsatellites were added to further saturate the cassava genetic map (Chavariaga-Aguirre, *et al.*, 1998). Recently, an investigation on SSR (simple sequence repeat) markers in the cassava genome was performed in order to set up an SSR - based molecular genetic map of cassava, which can generally be integrated into the existing RFLP framework of genetic maps (Liu, *et al.*, 1996, Taramino and Tingey, 1996). These efforts provide a possibility to make use of marker-assisted genetic analysis and selection of important agronomic traits in cassava breeding. Further research could be made to map these TDFs in a F1 population of a cassava cross population in which PPD has been phenotypically evaluated using a candidate gene approach.

There are two dimensions to further confirm expression profiling of the isolated TDFs direcly to the PPD process using our phenotypically uniform system for PPD in cassava tuberous roots. One is based on using a different cassava variety which shows a diverse sensitivity to PPD. Another is based on choosing the same cassava clone, but with a pre-pruning treatment which shows delayed symptoms of PPD. A small number of cassava clones less sensitive to deterioration exists, which means that PPD in the tuberous roots starts only after one week after harvest (Wenham, 1995). It is known that the cultivar of *M.Col 22* used in our experiments is highly sensitive to PPD. If another cassava cultivar with low susceptibility of PPD would be taken, the expression patterns of these isolated TDFs could have been further investigated using cDNA micro-array. The same approach could also be executed using the same variety but with pre-prunning treatment. The confirmed TDFs can be used as diagnostic molecular markers for indicating PPD in cassava tuberous roots at different stages.

Based on a systematical survey of changes in gene expression during PPD and a biochemical analysis of enzymes important to oxidative stress by focusing on peroxidase, it was strongly suggested that PCD (programmed cell death) might be involved in the process of PPD in cassava tuberous roots (Chapter 3 and 5). The main purpose of the project was to create transgenic cassava to influence /or delay the process of PPD in the tuberous roots after harvesting. Using a genetic transformation strategy, an attempt was made to down-regulate the levels of enzymes/genes involved

in the process of PPD in cassava by employing a reverse genetic approach. Therefore, the putative cassava dad1 homologue was introduced into cassava FEC lines using the luciferase gene as a selective marker by particle bombardment. The transgenic cassava plants carrying the putative dad1 (defender against cell death 1) homologue in an antisense orientation were investigated (Chapter 5) in order to answer questions like: What is the function of the putative cassava dad1 gene during the process of PPD in cassava tuberous roots? What kind of possible link is present between PPD and PCD in cassava? If PCD plays a crucial role in PPD in cassava tuberous roots, it is important to determine the function of gene(s) that might regulate PCD. The first transgenic cassava plants have been obtained, but it is clear that more cassava transgenic plants with the putative dad1 homologue have to be produced for further analysis.

To produce transgenic cassava with a delayed process of PPD in the tuberous roots after harvesting, the current effort focused on the modification of expression levels of genes possibly involved in this process (Chapter 5). Higher expression levels can possibly be achieved by taking a strong or process-induced promoter, selecting only "one copy" transgenic plants to prevent the problem of gene silencing, using a matrix attachment region (MAR) sequence flanking the transgene, etc. A root-specific promoter or, more preferably, a PPD-induced promoter is theoretically the most accessible approach for cassava. Further research will go into this direction.

PPD in cassava tuberous roots is a complex physiological process. There are many metabolic pathways involved in this process in cassava (Chapter 3). It has become clear that many of the desired agronomic traits under development will require the simultaneous engineering of multiple genes or an entire pathway (Miflin, 2000). Therefore, transfer of a single gene or several genes individually into cassava can possibly not solve the PPD problem entirely. Further improvement of cassava transformation systems will be required, including methods for the introduction of multiple genes into cassava.

Finally, it can be expected that transgenic cassava plants which may exhibit less or even no PPD in the tuberous roots will facilitate the broader acceptance of cassava as a food product for human beings and animals as well as industrial applications in the future.

Summary

One of the major problems limiting the further expanding possibilities of production, processing and distribution for cassava (*Manihot esculanta* Crantz.) is the rapid deterioration, which results in very short storage life of the tuberous roots after harvesting. Postharvest deterioration in cassava is an inherent constraint since wounding and mechanic damages cannot be prevented during the process of harvesting and handling of the tuberous roots. General speaking, postharvest deterioration in cassava tuberous roots is made up of primary deterioration and secondary deterioration. Primary deterioration is a complicated physiological deterioration, called postharvest physiological deterioration (PPD), which starts from 24 hours to 48 hours after harvest depending on environmental conditions and the cassava genotype. Secondary deterioration is the result of the former process combined with the activity of microorganisms like bacteria and fungi. It is generally initiated from day 5 to day 7.

A system mimicking PPD in the tuberous roots of cassava was set up under pre-set experimental conditions with tuberous root slices, revealing the same phenotypical symptoms for the onset of PPD and maintaining the same spreading speed. In this uniform system, so called "vascular streaking" was first observed around 24 hours, whilst "vascular discoloration" appeared after about 72 hours. Afterwards only the color intensity increased until the experiment was terminated. No presence of bacteria and fungi could be observed in this experimental system. To monitor the system, several parameters were investigated like the amount of nuclear acids, protein, starch, and soluble sugars, etc. Starch, as the most abundant component in cassava tuberous roots received more attention. Emphasis was placed on characteristics of some of the physico-chemical properties of starch. The general conclusion from the results is that not only the quantity but also the quality of starch decreased following PPD in cassava, as shown by the differences of the morphology of the starch granules and the Bohlin profile. However, the overall impression is that these changes of the starch properties are not as much affected as one might expect based on general assumptions of the process of PPD in cassava tuberous roots.

There was little information available about genes related to the process of PPD in cassava at the start of the project. In order to understand the complex

operations involved in PPD, it is essential to identify the potentially important components of its genetic program, even including those that might not be directly involved in PPD in cassava. Research has focused on the early stages of the process of PPD in the tuberous roots. One strategy is to isolate transcripts that are differentially expressed during PPD in cassava. A systematic survey of changes in gene expression was performed using the cDNA-AFLP technique. In total about 6,000 TDFs (transcripts derived fragments) were screened and 70 TDFs showing an up-regulated, a down-regulated or a transiently expression pattern during the first 72 hours of PPD were isolated using around 100 AFLP primer combinations. A functional catalogue of these TDFs was established based on comparing their sequence information in the databases. This yielded molecular insight into the process of PPD in cassava. The data analysis suggested that several processes were switched on during the complex PPD process in cassava tuberous roots, such as protein metabolism, carbohydrate metabolism, phenolic biosynthesis, and cell wall metabolism.

Through the functional catalogue of the TDFs based on the sequence information, a set of the TDFs was grouped into a set of putative genes possibly crucial to oxygen scavenging, like peroxidase and cytochrome p450 monooxygenase. In parallel to the molecular analysis of PPD, a biochemical approach was taken to characterize enzymes probably important to oxidative stress focusing on peroxidase (EC 1. 11.1.7) during PPD in cassava. It was shown that the specific peroxidase activity increased and this increasing accompanied the development of the phenotypical symptoms of PPD in cassava. Novel peroxidase isoforms seemed to be synthesized and the activity was mainly localized in the parenchyma tissue, the vascular bundles and the pith during PPD in the tuberous roots. These results indicated that PPD might be a peroxidase-mediated oxidative process.

The data obtained strongly indicated that PCD (programmed cell death) is possibly involved in the process of PPD in cassava (Chapter 3 and Chapter 4). A TDF with a high sequence similarity to the putative dad1 (defender against cell death 1) homologue revealing a differential expression pattern was isolated in the first 72 hours of PPD in cassava. Southern hybridization results indicated that dad1 has a low-copy number in the cassava genome. Transformation was achieved using particle bombardment of cassava FEC (friable embryogenic callus) lines with DNA carrying the putative dad1 homologue from cassava driven by the CaMV 35s promoter. Two strategies were taken for the production of transgenic plants: one was based on

luciferase screening only and the other one on kanamycin plus luciferase selection. It was shown that cassava FEC lines could be bombarded with a mixture of two different DNA plasmids and that co-transformation occurred at a high frequency. In total 94 lines carrying the putative dad1 gene in an antisense orientation were obtained; however, only 6 of them regenerated into plants. This low percentage was caused by an intrinsic low capacity of the used FEC lines to regenerate into plants. The transgenic nature of the plants was confirmed by Southern blot analysis. One transgenic line showed earlier senescence in leaf, which might be attributed to the expression of the introduced dad1 gene. Leaf senescence is a modified PCD in plants. More transformants carrying the putative cassava dad1 homologue will be produced in the future, which will shed light on the function of the putative dad1 homologue from cassava during the process of PPD in the tuberous roots.

Cassava is a vegetatively propagated and highly heterozygous allotetraploid crop. These natural properties lead cassava to become a difficult crop through traditional breeding since inheritance of desirable characters is difficult to be predicted. These, *vice versa*, offer an opportunity for cassava to become a more suitable crop by using genetic modification to improve its agronomic traits, for example to create transgenic cassava, in which the process of PPD in the tuberous roots is delayed or less. This thesis is a first step into the unraveling of the problem of PPD in cassava tuberous roots. More research is needed by a combination of molecular genetic and physiological analysis in order to create more progress in this field. Also we can make use of marker-assisted genetic analysis of PPD to map the isolated TDFs in a F1 cross population in which phenotypical characterization has been evaluated. The mapped TDFs could also be employed as diagnostic molecular markers to further improve screening for PPD efficiency in the cassava germplasm pool.

Samenvatting

Een van de grootste problemen bij het grootschalig verspreiden en gebruiken van het tropische gewas cassave (*Manihot esculenta* Crantz) is de snelle verrotting van de geoogste wortelknollen. Vanwege dit probleem, bekend onder de engelse naam Postharvest Deterioration (PD), is de bewaarbaarheid van vers geoogste wortelknollen zeer beperkt. De wortelknollen dienen binnen enkele dagen na de oogst verwerkt te worden waardoor de mogelijkheden voor alternatieve toepassingen van de wortelknollen, waarbij bewaarbaarheid een rol speelt, maar moeizaam van de grond komen. PD is een complex fysiologisch proces dat in een primaire en een secundaire fase kan worden verdeeld. Het primaire proces, ook wel bekend onder de naam Postharvest Physiological Deterioration (PPD), start relatief kort na de oogst en duurt, afhankelijk van het cassave genotype, 3 tot 5 dagen. Het secundaire proces is het gevolg van een combinatie van PPD en de activiteit van micro-organismen, zoals bacteriën en schimmels, en begint rond dag 5 na de oogst.

In dit onderzoek werd een experimenteel systeem opgezet dat het volgen van de ontwikkeling van het proces van PPD in wortelknolweefsel en in de tijd mogelijk maakte. Dit uniforme systeem, uitgetest in twee verschillende cassave genotypen MCOL 22 en Faroka, toonde de reeds eerder beschreven biochemische processen bekend onder de naam 'vascular streaking' en ' vascular discoloration' na respectievelijk 24 uur en 72 uur. Vascular streaking is een proces waarbij vanuit de vaatbundels een donkere kleur zichtbaar wordt. Vascular discoloration is het proces waarbij de donkere kleur zich door de hele knol uitbreidt. Beide processen zijn het gevolg van de vorming van schadelijke fenol verbindingen. Aanwezigheid van bacteriën en schimmels kon worden uitgesloten waarmee dus duidelijk alleen het primaire proces PPD bestudeerd kon worden middels dit experimentele systeem. Om de invloed van PPD op allerlei basale processen in de knol te onderzoeken werden de hoeveelheden suikers, zetmeel, eiwit en nucleine zuren op verschillende tijdstippen bepaald. Aangezien de wortelknol naast water veel zetmeel bevat werden ook allerlei fysisch chemische en rheologische eigenschappen van het zetmeel onderzocht. De algehele conclusie uit dit deel van het onderzoek was dat niet alleen de hoeveelheid maar ook de kwaliteit van het zetmeel achteruit ging tijdens PPD. De veranderingen in het zetmeel waren echter lang niet zo dramatisch als dat men algemeen in de literatuur veronderstelde.

Bij het begin van dit project was er absoluut geen informatie voorhanden over de aard en aantallen genen die een beslissende rol bij PPD zouden kunnen spelen. Het opgezette

experimentele uniforme systeem om PPD te initiëren en te volgen in de tijd leende zich uitstekend om met behulp van een RNA vingerafdruk techniek op zoek te gaan naar zulke genen. Een systematische analyse van genexpressie gedurende de eerste 72 uur van PPD werd uitgevoerd gebruikmakend van de techniek cDNA-AFLP. In totaal werden 6000 expressiebanden zichtbaar gemaakt door 100 primercombinaties te gebruiken. Van deze 6000 banden vertoonden er 70 een patroon dat duidde op achtereenvolgens een toenemende expressie, een afnemende expressie of een transiente expressie. De 70 banden, van grootte variërend van 100 tot 500 basen, werden geïsoleerd en hun basenvolgorde werd bepaald. Door de basenvolgorde te vergelijken met basenvolgorden van bekende genen in zogenaamde databestanden was het mogelijk om aan circa 60% van deze banden een bekende functie toe te schrijven. De datavergelijking liet zien dat bij PPD zeer veel verschillende processen een rol spelen zoals eiwit metabolisme, suiker metabolisme en celwand metabolisme. Een meer gedetailleerdere analyse liet zien dat er ook banden geïsoleerd waren die coderen voor enzymen zoals peroxidase, catalase en cytochroom P540 monooxygenase. Deze enzymen spelen een rol bij het wegwerken van zuurstof radicalen, die schade aanrichten aan de cellen. Een gedetailleerde analyse van het optreden en de rol van verschillende perioxidases tijdens het proces van PPD, zowel via activiteitsbepaling als via in situ weefsel hybridisatie, wijst in de richting dat PPD een peroxidase afhankelijk oxidatief proces is. Daarnaast werden aanwijzingen verkregen, onder andere door de isolatie van een band die sterke homologie vertoonde met het zogenaamde DAD-gen (Defender Against cell Death), dat geprogrammeerde celdood een rol speelt bij PPD in cassave.

Cassave is een vegetatief vermeerderd zeer heterozygoot allotetraploid gewas. Deze natuurlijke eigenschappen maken dat cassave een moeilijk gewas is in de veredeling. De overerving van gewenste eigenschappen is slecht te voorspellen en de periode die nodig is om gewenste eigenschappen in het gewas te introduceren zeer lang. Omgekeerd maakt dit dat cassave in potentie een zeer geschikt gewas is om met behulp van genetische modificatie te veredelen.

Door transformatie met behulp van 'particle bombardment', een methode waarbij DNA op kogeltjes gecoat in plantenweefsel wordt geschoten, werd getracht om getransformeerde cassave planten te maken waarin PPD vertraagd of zelfs helemaal afwezig zou zijn. Speciaal cassave weefsel, Friable Embryogeen Callus (FEC) werd gebruikt voor de transformatie en regeneratie. Het DAD-gen werd in antisense oriëntatie samen met een reporter of selectie gen, het luciferase of het kanamycine resistentie gen, ingebracht. In totaal werden 94 FEC lijnen verkregen die het DAD gen bevatten. Echter slechts 6 lijnen konden

geregenereerd worden tot planten. Dit extreem lage percentage werd veroorzaakt door een zeer lage regeneratiecapaciteit van de gebruikte lijn. Deze lijn was geselecteerd voor zijn hoge transiente transformatie efficiëntie maar bleek naderhand een zeer slechte regeneratie efficiëntie te bezitten. Het transgene karakter werd vastgesteld in een aantal planten waarvan er één een zeer snelle veroudering liet zien in bladeren. Aangezien bladveroudering een speciale vorm van geprogrammeerde celdood is zou dit erop kunnen duiden dat DAD inderdaad een ingang zou kunnen zijn om iets aan PPD in cassave te doen. Meer onafhankelijke transformanten moeten gemaakt en bestudeerd worden teneinde dit te bevestigen.

Dit proefschrift is een eerste stap op weg naar de moleculaire ontrafeling van het probleem van PPD. Meer onderzoek is nodig, onder andere door een combinatie van moleculair genetische en fenotypsiche data uit splitsende populaties, om vooruitgang te boeken op dit belangrijke terrein.

中文摘要

木薯 (*Manihot esculenta* Grantz) 是一种非常重要的 (亚) 热带块根 (tuberous roots) 作物，可加工成食品，动物的饲料，也可以作为某些工业产品的原料。在亚洲，非洲和拉丁美洲，木薯是 5 亿多人口的能量来源。作为一种最有效的淀粉生产作物，木薯能以较少的投入，获得较大的回报。该作物可以生长在酸性及贫瘠的土壤里，并能抗旱。因其能在耕地的边缘地段生长，可充分地利用有限的土地资源。但是限制木薯进一步推广和深加工的主要因素之一是在木薯收获后，其块根很容易腐败 (post-harvest deterioration)，导致木薯块根收获后难以储存。一般来说，收获后的木薯块根腐败可分为初级腐败 (primary deterioration) 和次级腐败 (secondary deterioration) 两个阶段。初级腐败是一种复杂的生理过程，又称为生理腐败 (post-harvest physiological deterioration)。根据木薯品种差异和生长环境不同，生理腐败一般发生在收获后 24-48 小时，次级腐败是生理腐败过程的结果，并有微生物 (细菌和真菌) 参与，一般发生在收获后 5-7 天。本论文从生理，生化和分子生物学的角度，研究木薯收获后其根茎块生理腐败的过程。

首先，我们在实验室条件下建立了一个模拟木薯生理腐败过程的同步系统。在此条件下，木薯块根表现出一致的生理腐败的早期及后期表征 (第二章)。在这个模拟系统里，大约 24 小时后，可以观察到典型的生理腐败的表型征兆 --- 维管束条纹化 ” ('vascular streaking')，而 “ 维管束变色 ”('vascular discoloration') 的现象出现在约 72 小时之后。此后只有木薯维管组织颜色的加深，没有观察到任何细菌和真菌感染的迹象。为了检测此系统，我们测定了核苷酸，蛋白质，淀粉和可溶性糖的含量等相关参数，并对木薯块根中含量最丰富的淀粉物理化学特性进行了重点研究。淀粉粒的形态学观察和 Bohlin 图谱结果表明，虽然在木薯生理腐败过程中，淀粉的数量和质量都有所下降，但总的来说，淀粉性质的改变并不像以前人们想象的那么大。

在本研究工作开始之前，很少木薯的基因被分离到。为了进一步了解复杂的木薯生理腐败过程，很有必要找到与此过程有关的遗传因子。本研究重点集中在分离木薯生理腐败的早期过程 (头 72 小时) 中差异表达的基因 (第三章)。使用 cNDA-AFLP 技术和约 100 对 AFLP 引物组合，我们系统的研究了在木薯生理腐败开始的前 72 小时期间表达的 6，000 TDFs (transcript derived fragments，转录子片段)。由此得到了 70 个表达加强，减弱，或者瞬间诱导表达的 TDFs。根据与基因信息库 (Genebank) 序列比较，我们将这些 TDFs 按功能分类，探索了木薯生理腐败分子机理。结果表明了好几种代谢途径参与了复杂的木薯生理腐败过程，如蛋白质和碳水化合物的代谢，多酚类的合成，及细胞壁的降解等。

基因序列比较表明多个与氧化反应有关的 TDFs 在木薯生理腐败早期呈现出差异表达，例如过氧化物酶和细胞色素 P450 单氧化物酶。在此基础上，我们利用了生

物化学的手段，对过氧化物酶 (EC 1.11.1.7) 在木薯生理腐败过程作用进行了进一步的研究。结果表明，随著木薯生理腐败的进行，过氧化物酶的特异性活性升高，新的过氧化物同工酶也被合成。该酶的活性主要分布在木薯块根的维管组织和分生组织。此结果表明木薯生理腐败极有可能是一个过氧化物酶参与的氧化过程 (第四章)。

在我们的研究过程中还发现 PCD (programmed cell death，细胞凋亡) 极有能参与了木薯生理腐败的过程 (第三章 & 第四章)。在木薯生理腐败的头 72 小时里分离到的其中一 TDF (TDFa12) 的基因序列与 dad1 (defender against apoptotic death 1, 抗细胞凋亡基因 1) 具有高度的同源性 (第五章)。Southern 杂交结果表明在木薯的基因组中，dad1 基因只有较低的拷贝数目。我们利用基因枪技术 (particle bombardment) 将带有 CaMV 35S 启动子调控之下木薯 dad1 基因的外源质粒 (plasmid) DNA 导入木薯的 FEC (friable embryogenic callus, 粉末型胚性愈伤组织) 系，然后通过组织培养的方法来获得了转基因木薯。我们利用了两种方法来筛选转基因木薯，一是只用 luciferase (荧光酶) 作为报告基因 (reporter gene)，二是利用共转化, 采用 kanamycin (卡那霉素) 和 luciferase (荧光酶) 一起作为报告基因。实验结果表明木薯的 FEC 系能用基因枪同时导入两个不同的质粒 DNA，且协同转化的高频率很高。但是从筛选出的 94 个转基因愈伤组织系中只获得了 6 个转基因植株。这是由于所用的 FEC 系本身再生能力较差的原因。Southern 杂交验证了转基因整合到受体的基因组。其中一转基因植株叶子出现早期衰老 (senescence) 的现象。这一观察与前人提出的叶子的衰老是一种细胞凋亡的过程的结论相吻合。

木薯是一种通过营养繁殖的高度杂合的异源四倍体作物。该属性使木薯很难用传统的育种方式进行改良。现代生物工程技术为该作物的遗传改造，特别是与农业经济作用相关的一些重要性状改良提供了新的手段。例如我们可利用遗传改良 (genetic modification) 创造新的转基因木薯品系，来减缓收获后其块根生理腐败过程。本论文的研究是揭示木薯生理腐败机理的第一步。今后需要结合分子遗传学和木薯腐败的生理学进一步探索。同时我们也可以利用分子标记作为辅助育种 (marker-assisted) 手段，寻找与生理腐败直接相关的 TDFs。反过来，这些标记的 TDFs 也能作为诊断工具去从全球木薯资源库中筛选可能存在的无生理腐败的木薯品系及与之有关的分子标记。

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Acknowledgements

Curriculum Vitae

(Michael) Jiang HUANG (黃 江) was born in Wuhan (China) on the 16th of December 1966. From 1973 to 1984, he finished his fundamental study from primary school, junior high school and senior high school. He went to Wuhan University for his academic education in 1984, and he received bachelor degree in 1988. Afterwards he had been working in Wuhan Institute for Biological Products, Ministry of Public Health for 5 years, and he had stayed in the Sino-Danish Postgraduate Medical Training Centre (Beijing), Ministry of Public Health for one year (1992-1993).

In March 1994, he came to the Netherlands for his MSc study on Biotechnology in Wageningen University (WU). He started his PhD study in the Lab of Plant Breeding, WU in June 1996. The results of the PhD research were described in this thesis.

He continued his scientific career as a postdoc researcher in Prof. Ab van Ooyen's group (DSM and WU) in the Lab of Microbiology at Department of Agrotechnology and Food Sciences, WU.