INSTITUTO POTOSINO DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA, A.C.

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“Proteomic analysis of amaranth under abiotic stress”

Tesis que presenta

José Ángel Huerta Ocampo

Para obtener el grado de

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Directora de la Tesis:

Dra. Ana Paulina Barba de la Rosa

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José Angel Huerta Ocampo

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Proteomic analysis of amaranth under abiotic stress

que se desarrolló bajo la dirección de

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Al Comité Tutoral

Contenido

Tables

Figures

Annexes

Abbreviations

2-DE	Two-dimensional electrophoresis
3D	Three-dimensional
BSA	Bovine serum albumin
cDNA	complementary DNA
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
ds	Decisiemens
DTT	Dithiothreitol
DW	Dry weight
EC	Electrical conductivity
ESI	Electrospray ionization
EST	Espressed sequence tag
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
kVh	Kilo volt hour
LC-MS/MS	Liquid chromatography tandem mass spectrometry
m/z	mass-to-charge ratio
mA	milli-amps
mRNA	Messenger RNA
MS	Mass spectrometry
nrNCBI	Non-redundant National Center for Biotechnology Information
ORF	open reading frame
PMSF	Phenylmethylsulfonyl fluoride
PTM	Posttranslational modification
PVPP	Polyvinylpyrrolidone
RF	Radio frequency
RNA	Ribonucleic acid
RNAi	RNA interference
SAGE	Serial analysis of gene expression
SDS	Sodium dodecyl sulfate

T-DNA	Transfer DNA
TOF	Time of flight
Tris	Hydroxymethyl aminomethane
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
V	Volt

Resumen

Análisis proteómico de amaranto bajo condiciones de estrés abiótico

Las especies comerciales de amaranto de grano *Amaranthus hypochondriacus, A. cruentus* y *A. caudatus* son nativas de América y están distribuidas en los cinco continentes. El amaranto es un pseudocereal altamente nutritivo y no alergénico con notables propiedades nutracéuticas, capaz de adaptarse a diversas condiciones de cultivo tales como suelos pobres en nutrientes y moderadamente salinos, condiciones de poca agua y altas temperaturas. Estas características lo convierten en un buen modelo para estudiar la tolerancia a condiciones de estrés abiótico como la sequía y el estrés salino.

El estrés abiótico es uno de los principales factores que limitan la productividad agrícola mundial y genera la reducción de hasta el 50% en el rendimiento potencial de los cultivos. La exposición de las plantas a condiciones de estrés abiótico, desencadena cambios fisiológicos que se rigen por la expresión de diferentes grupos de genes y proteínas cuya actividad permite a las plantas tolerar condiciones ambientales adversas. El empleo cada vez más frecuente de las tecnologías “omicas” permite la identificación de genes y proteínas asociados con la tolerancia al estrés abiótico.

Como parte del proyecto *Amaranth-Future-Food*, en este trabajo se evaluó mediante un enfoque proteómico la respuesta del amaranto a condiciones de estrés abiótico como la sequía y el estrés salino. Para lo cual se obtuvieron los patrones de proteínas mediante Electroforesis Bidimensional (2-DE) y se identificaron las proteínas diferencialmente acumuladas mediante Espectrometría de Masas en Tándem. Algunas de estas proteínas pueden considerarse como asociadas con la tolerancia al estrés abiótico. Sin embargo, es necesaria una caracterización funcional de estas proteínas a fin de demostrar su importancia en la respuesta de las plantas ante el estrés.

Algunas alternativas para demostrar el papel que juegan estas proteínas diferencialmente acumuladas en la respuesta ante el estrés abiótico son: la complementación funcional de levaduras sensibles a componentes del estrés abiótico como el estrés osmótico, el silenciamiento génico, la expresión de dichas proteínas en líneas insercionales así como la evaluación de la acumulación diferencial de estas proteínas candidatas en híbridos que mantengan los rasgos deseados. Estos acercamientos nos darían una idea más clara del papel que desempeñan tales proteínas en los mecanismos de respuesta al estrés abiótico.

PALABRAS CLAVE: Amaranto, electroforesis bidimensional, espectrometría de masas, estrés salino, sequía.

Abstract

Proteomic analysis of amaranth under abiotic stress

Commercial amaranth species such as *Amaranthus hypochondriacus, A. cruentus and A. caudatus* are native to America and are worldwide distributed. Amaranth is a highly nutritious and non-allergenic pseudo-cereal crop with remarkable nutraceutical properties. Amaranth is also a promising species often grown under semiarid conditions prone to both drought and salinity. For the above described amaranth is a good model to study tolerance to abiotic stress conditions such as drought and salt stress.

Abiotic stress is one of the major factors limiting crop productivity worldwide and responsible for the reduction of up to 50% in the potential yield of crops. Plant exposure to abiotic stress triggers physiological changes ruled by the expression of different sets of genes and proteins whose activities enable plants to tolerate stressful environmental conditions. The application of 'omics' technologies has allowed the identification of genes and proteins related to abiotic stress tolerance.

As part of the *Amaranth-Future-Food* project, in the present thesis, 2-DE gel coupled with LC-MS/MS approach was applied in order to analyze the changes in amaranth protein accumulation in response to abiotic stress conditions such as drought and salt stress. Some of the differentially accumulated proteins in response to stress may be considered associated to abiotic stress tolerance. However, functional characterization of these proteins in order to demonstrate its importance in plant response to abiotic stress is needed.

Functional complementation by heterologous expression in yeast, gene silencing, heterologous expression in insertional lines and evaluation of differential accumulation of these proteins in hybrids from cultivars with contrasting tolerance to abiotic stress that preserve the desired trait are among the alternatives to demonstrate the role played by such proteins in plant responsive mechanisms to cope with abiotic stresses.

KEY WORDS: Amaranth, drought, mass spectrometry, salt stress, two-dimensional electrophoresis.

1. Introduction

Amaranth is a dicotyledonous plant with high plasticity and adapted to different soils and climates. It is drought tolerant and moderately tolerant to salt stress, it grows rapidly during hot weather and requires less water than conventional crops such as maize, rice and wheat.

Amaranth belong to the group of plants that carry on photosynthesis by the specialized C4 pathway. It is also one of the few C4 crop species that are not grasses. Amaranth grain is considered as a pseudo-cereal because it shares some features with grains of monocots. Two of the most important characteristics of amaranth seed are its high protein content and the high biological quality of these proteins.

Amaranth was an important crop for antique cultures of America and it was also linked to their religious ceremonies. During the last decades, a considerable amount of research has been done on nutritional and nutraceutical properties of this plant.

The objective of the project Amaranth Future Food (www.amaranth-future-food.net) involving 11 countries including Mexico was to provide the tools for an extensive and sustainable exploitation of this underutilized crop. As part of this project, in the Laboratory of Proteomics and Molecular Biomedicine at IPICYT, we are interested in the identification of differentially expressed genes and proteins in amaranth plants under abiotic stress conditions such as drought and salt stress.

In the present thesis, differential accumulation of proteins in amaranth plants under abiotic stress conditions such as drought and salt stress was analyzed using a proteomic approach. Some of the differentially accumulated proteins can be considered as potential candidates associated with tolerance to abiotic stress. However, functional characterization of these prospective candidates is necessary in order to demonstrate their importance in plant response to abiotic stress.

In addition to the proteomic analysis of amaranth under abiotic stress, an analysis of the ripening process in papaya fruits using the proteomic approach was also carried out.

1.1 Amaranth

1.1.1 Amaranth"s origin

The species for production of Amaranth grain are native to America, while species commonly used as vegetables have originated in Asia (Grubben & Sloten, 1981). However, some authors suggest that the production of grain species of this genus have been cultivated since ancient times in South Asia and may be originating from that place (Singh, 1961).

Archaeological evidence found in Mexico, confirmed the American origin of the species of amaranth grown for grain, since both its leaves and seeds were used by prehistoric inhabitants of America long before the process of domestication of these plants (Sauer, 1967). Excavations by McNeish (1964) indicate that amaranth domestication took place at the same time as that of corn (5200 to 3400 BC).

The three most important species for the production of grain amaranth *A. cruentus*, *A. hypochondriacus* and *A. caudatus* are of American origin. The first two are widely distributed in Mexico, the most important center for diversity (Espitia-Rangel et al., 2010).

1.1.2 Historical aspects of amaranth cultivation

As a result of archaeo-botanical studies conducted in Mexico in the caves of the Valley of Tehuacán, Puebla, it was confirmed that amaranth was widely cultivated long before the Spanish arrived. At the time of the Spanish conquest, amaranth was one of the most important crops in Mesoamerica and expanded further to the north of the continent (Espitia-Rangel et al., 2010).

Amaranth was one of the most important foods of pre-Columbian cultures in the Americas. Among the Aztecs and their neighbors, it was not only used as food but also had great importance in their religious ceremonies. It is known that large amounts of amaranth seed were sent to Mexico-Tenochtitlan as part of the tribute paid by peoples subdued to the Aztec Empire.

Manifestations of the presence of amaranth as a rooted crop in pre-Columbian cultures of Mesoamerica include descriptions of the plant and its many uses such as those made by Francisco Hernandez in his "Natural History of New Spain" and by Fray Bernardino de Sahagun in the "Florentine Codex" (Figure 1.1).

It is believed that the cultivation of amaranth was banned by the conquerors to consider it a symbol of paganism. After the conquest the amaranth crop remained underutilized (Sandoval, 1989).

Currently, the commercial production of amaranth is mainly concentrated in the states of Morelos, Tlaxcala, Puebla and Mexico City and to a lesser extent in the states of Oaxaca, Mexico, Guanajuato, Guerrero, Durango, Queretaro and recently in Chihuahua and San Luis Potosi (Espita-Rangel et al., 2010).

1.1.3 Botanical characteristics of the genus *Amaranthus*

The genus *Amaranthus* belongs to the family *Amaranthaceae* (Table 1) comprising about 70 genera and 800 species of herbs of tropical origin. The plants belonging to this family have evolved in warm regions, dry and saline soils, which

have resulted in special anatomical and physiological adaptations to cope with these harsh environments (Espitia-Rangel et al., 2010).

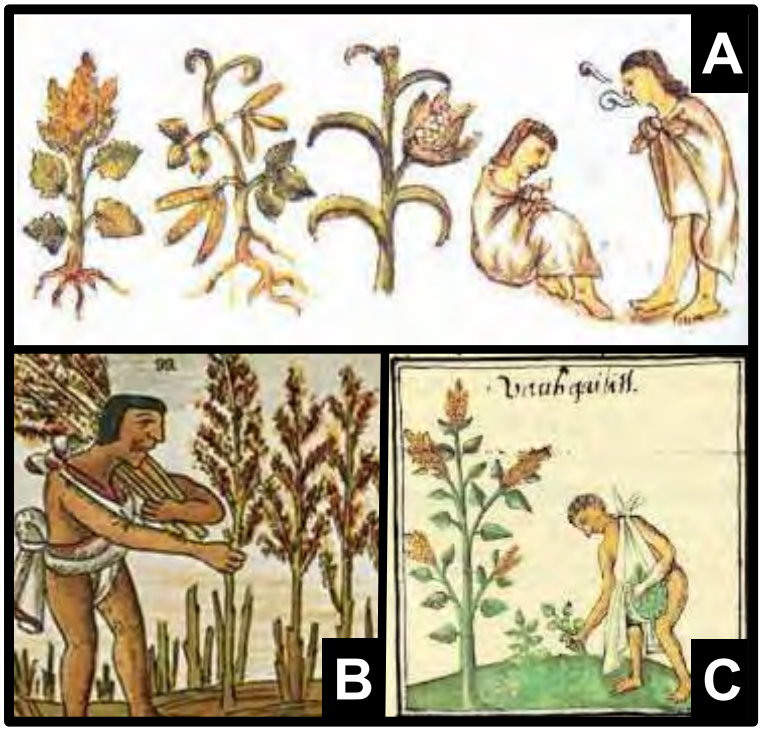

Figure 1.1. Images taken from the Florentine Codex by Fray Bernardino de Sahagun showing (A) amaranth as one of the staple crops for pre-Columbian cultures along with corn and beans (B), harvest of grain amaranth and (C) harvest of amaranth as a vegetable.

Table 1. Botanical classification of amaranth*

Kingdom	*Plantae*
Division	*Magnoliophyta*
Class	*Eudicotyledonae*
Order	*Caryophyllales*
Family	*Amaranthaceae*
Genus	*Amaranthus*
Species**	*hypochondriacus, cruentus and caudatus*

* Source: Tapia ME (1997). ** For grain production.

Amaranths are annual species, herbaceous or shrubby of various colors ranging from green to purple with different colors in between. The root has abundant branching and multiple thin rootlets which spread rapidly after the stem begins to branch, facilitating the absorption of water and nutrients. The main root serves as support to the plant (Garcia-Pereyra et al., 2010).

Primary roots can become woody; allowing the plant to be firmly anchored to the ground and sometimes it reaches considerable dimensions, especially when the plants grow all separated from one another. The stem is cylindrical, angular with thick longitudinal ridges that give a corrugated appearance, whose thickness decreases from base to apex. It has different colors that generally match the color of the leaves, but sometimes has streaks of different colors, sometimes may have ramifications in many cases start from the base or mid-height and which arise from leaf axils (Figure 1.2 A). The number of branches is dependent on the density at which it has grown (Garcia-Pereyra et al., 2010).

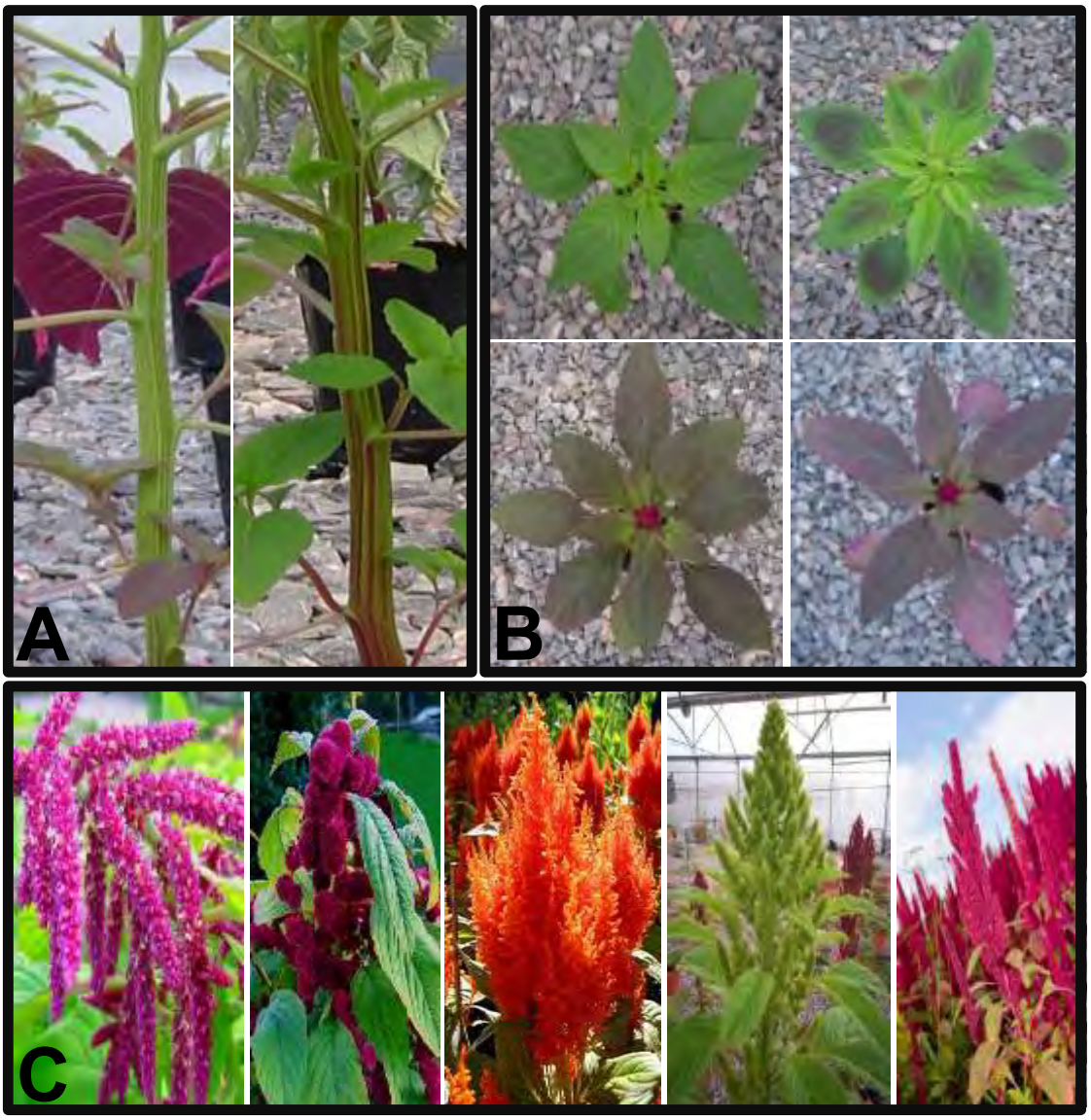

Figure 1.2. Various aspects of amaranths morphology. (A) Stem, (B) leaves and (C) inflorescence.

The leaves are petioled, oval to elliptical, opposite or alternate with prominent veins on the underside, smooth or slightly hairy green or purple whose size decreases from base to apex. Leaves have entire margins and are resizable (Figure 1.2 B). The inflorescences are very showy glomerular panicles, they can be terminal or axillary, which vary from fully erect to decumbent, with colors ranging from yellow, orange, brown, red, pink, to purple, the size can vary from 50 to 90 cm (Tapia, 1997) (Figure 1.2 C).

The fruit is a small capsule, it opens transversely at maturity dropping the top called operculum to expose the lower part called urn, containing the seed (Figure 1.3 A). The seed is small, smooth and shiny, measuring 1.5 mm in diameter, slightly flattened and the color can be white, yellow, pink, gold, red, purple and black (Figure 1.3 B). The number of seeds per gram varies from 1000 to 3000 per gram (Nieto, 1990).

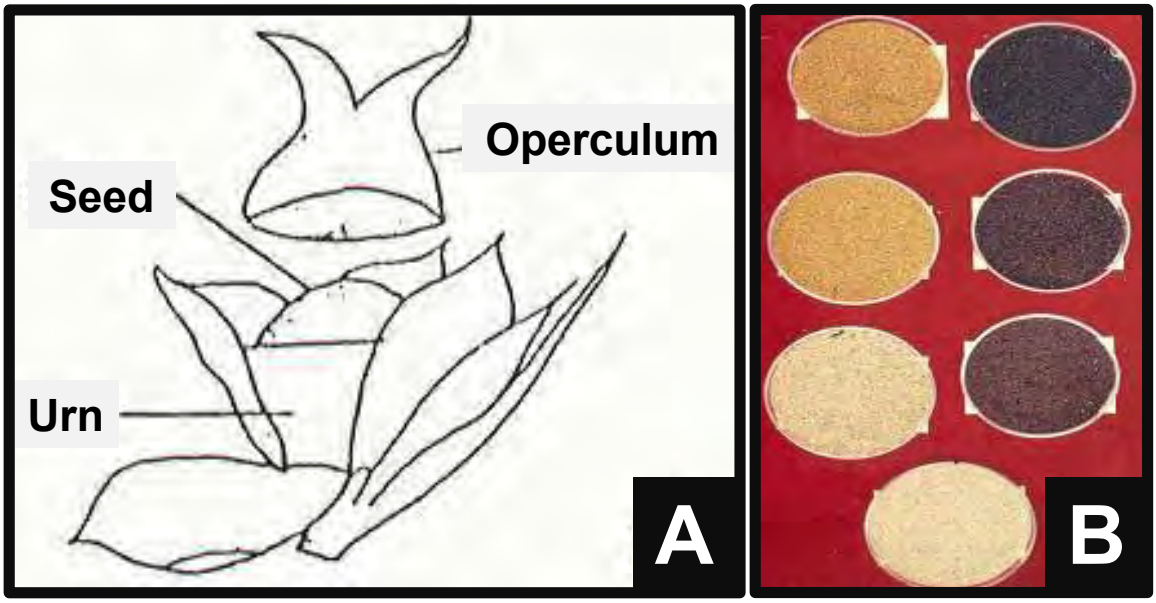

Figure 1.3. (A) Amaranth capsule containing the seed, (B) the wide color range of amaranth seeds.

The species for grain production are easily distinguished from other species of amaranth because of their high seed production, increased plant size and larger inflorescence. Uncultivated plants produce dark seeds and the production period is longer (Espitia-Rangel et al., 2010).

1.1.4 Amaranth species and races

There are three species of amaranth used for grain production. *Amaranthus hypochondriacus*, a native of northern Mexico and the southwestern United States,

Amaranthus cruentus, native to southern Mexico and Central America, and *Amaranthus caudatus*, a native of the Andean region of South America. Another important species is *Amaranthus hybridus*, this species is a weed widely distributed from the United States to northern South America and is not used for grain production. Its importance lies in its use in breeding programs that seek to exploit their desirable characteristics such as plant height and earliness (Espitia-Rangel et al., 2010). Because of the variability within each of the species used for grain production; Espitia-Rangel (1994) suggested the designation of race. Each race has a well-defined geographical distribution and has been developed under different growing conditions (Table 2).

Table 2. The races of amaranth, distribution and outstanding characteristics

Race	Species	Origin	Outstanding characteristics
Azteca	*hypochondriacus*	Mexico (temperate locations)	Great size and higher performance, late cycle and less lateral branching.
Mercado	*hypochondriacus*	Mexico (subtropical regions)	Good yield potential, sensitive to photoperiod and develops numerous side branches.
Mixteca	*hypochondriacus*	México (Oaxaca and Michoacán)	Long maturation period, small to medium seed size, very sensitive to photoperiod.
Nepal	*hypochondriacus*	India and Nepal	Good yield, mild to moderate seed fall, sensitive to photoperiod, early maturing has been observed in some collections.
Picos	*hypochondriacus*	India and Nepal	Low height, with a tendency to lodging, moderate seed fall.
Mexicana	*Cruentus*	México (warm and temperate zones)	High potential for high altitudes, medium to large seed size, slightly sensitive to photoperiod.
Guatemalteca	*Cruentus*	Guatemala and southern Mexico	Small Inflorescence, moderate to severe seed fall, early to half maturing, little sensitive to photoperiod.
Africana	*Cruentus*	Africa	Used as a vegetable, black seed in most of the native populations. Early and short, not very sensitive to photoperiod.
Sudamericana	*Caudatus*	Andean region of South America	Erect or decumbent Inflorescence, moderate seed fall, seed size medium to large, sensitive to photoperiod.
Edulis	*Caudatus*	Northwest Argentina	Determinate growth, seeds of medium to large, highly susceptible to root diseases.
Prima	*Hybridus*	North and Central America	Short, the inflorescence born in the leaf axils, small to medium black seeds

Source: Espitia-Rangel et al., 2010.

Currently there are 5 varieties improved for the production of amaranth grain in Mexico (Table 3).

Table 3. Improved amaranth varieties used for grain production in Mexico

Variety	Especie	Race	Outstanding characteristics
Nutrisol	*Hypochondriacus*	Azteca	High performance variety for high valleys
Rojita	*Hypochondriacus*	Nepal	Early variety for high valleys, suitable for mechanical harvesting
Revancha	*Hypochondriacus*	Mercado	Improved for mechanical harvesting
Amaranteca	*Cruentus*	Mexicana	Early variety for semi-tropical zones
Dorada	*Cruentus*	Mexicana	Variety for semi-tropical zones

Source: Espitia-Rangel et al., 2010.

1.1.5 Nutritional value

Amaranth seeds contain 16 to 17% protein; this percentage is higher than wheat (12-14%), rice (7-10%) and maize (9-10%) (Espitia-Rangel et al., 2010). In addition the amino acid composition of amaranth seeds is close to the optimal balance required for human diet. Amaranth seed proteins are rich in essential amino acids such as lysine (Barba de la Rosa et al., 1992) and amaranth leaves have a high content of minerals such as calcium, iron, magnesium and phosphorus, plus vitamins A and C (Barba de la Rosa et al., 2006).

Due to the high protein quality of amaranth seeds, crops such as corn, wheat and potato have been transformed with amaranth seed storage proteins in order to increase its nutritional value and content of essential amino acids (Chakraborty et al., 2010; Rascón-Cruz et al., 2004; Tamás et al., 2009).

1.1.6 Nutraceutical value

In addition to its recognized nutritional value, amaranth has proven to be a non-allergenic food with remarkable nutraceutical properties. Amaranth seed storage proteins demonstrated the presence of encrypted bioactive peptides with anti-hypertensive activity (Barba de la Rosa et al., 2010). Peptides with anti-tumor activity have also been reported in amaranth seed (Maldonado-Cervantes et al., 2010).

Amaranth seed consumption has shown to improve plasma lipid profiles in laboratory animals (Czerwiński et al., 2004). Anti-oxidative and anti-diabetic effects of amaranth grain and its oil have also been proved in diabetic rats (Kim et al., 2006). Anti-helmintic activity of methanolic extracts of three species of amaranth (Kumar et al., 2010) and anti-diarrheic properties for aqueous extracts have also been reported (Sawangjaroen and Sawangjaroen, 2005). The presence of

secondary metabolites with antioxidant activity in amaranth has also been documented (Barba de la Rosa et al., 2009).

However, a better understanding of the mechanisms by which bioactive peptides exert such functions is needed and it is also necessary to identify the compounds responsible of the demonstrated effects in aqueous and alcoholic extracts obtained from different amaranth tissues (Huerta-Ocampo and Barba de la Rosa, 2011).

1.1.7 Uses of amaranth

Amaranth grain and flour can be used in a wide variety of products such as: soups, pancakes, breakfast cereal, muffins, toast, tortillas, crackers, chips, pastries, snacks and drinks (National Research Council, 1984). There are different products found in the local markets such as the traditional sweet called "alegría" (“joy”) as well as tamales, pulque, marzipan, milkshakes and atole. The tender stems and leaves are usually eaten fried or boiled (Espitia-Rangel et al., 2010).

Amaranth is also a source of natural pigments with great potential for the food industry such as amaranthine and betalain. Amaranth oil contains a relatively high content of squalene (7-8%) this is important for the cosmetics industry and is also used as a lubricant for machinery and as steroid precursor (Espitia-Rangel et al., 2010).

1.1.8 Economic importance of amaranth

According to the National Association of Amaranth, it can be postulated that amaranth cultivation has the potential for an agronomic and industrial development similar to soybeans, where the production chain and the economic benefit can be very important. Because it is a short-cycle and drought-tolerant crop with interesting nutritional properties and multiple uses and forms of exploitation, it is considered as an alternative crop for many places where there is low and erratic rainfall (Espitia-Rangel et al., 2010).

There is an increase in the number of products on the market based-on or including amaranth in some proportion. In Mexico, there are companies dedicated to the cultivation and processing of amaranth *i.e.* Amaranthumn y Agrotecnia Nacional and San Miguel de Proyectos Agropecuarios). Famous food companies like Kellogg's and Bimbo now offer food products made with amaranth and a similar phenomenon occurs in the U.S., Japan and Europe.

1.2 Abiotic stress

1.2.1 Abiotic stress responses in plants

Higher plants are sessile and therefore cannot escape from abiotic stress. They are continuously exposed to different abiotic stress without any protection. This enabled them to develop unique molecular mechanisms to cope with different stresses. However, variations do exist in tolerance mechanisms among plants. Certain morphological features of some plants make them to withstand stress factors, but it may not be the case for all plants. The only option such plants is to alter their physiologies, metabolic mechanisms, gene expressions and developmental activities to cope with the stress effects. Therefore plants possess unique and sophisticated mechanisms to tolerate abiotic stresses. Those plants with better resistance, protection and acclimation mechanisms can survive while others cannot. Gene products play a key role in the molecular mechanisms of stress tolerance in plants (Madhava-Rao et al., 2006).

Abiotic stresses are commonly caused by drought, salinity, high or low temperatures, light, deficient or excess nutrients, heavy metals, pollutants etc., either individually or in combinations (Figure 1.4). The stress caused by abiotic factors alter plant metabolism leading to negative effects on growth, development and productivity of plants (Figure 1.5). If the stress become harsh and/or continues for longer period it may lead to unbearable metabolic burden on cells leading to reduced growth and in extreme cases results in plant death. However, plant stress may vary from zero to severe through mild and moderate levels. In nature, plants may not be totally free from stresses. Plants are expected to experience some degree of stress of any factor or factors. To combat these stresses, plants exhibit several mechanisms which make them withstand the stress with the formation of new molecules and molecular mechanisms of stress tolerance (Madhava-Rao et al., 2006).

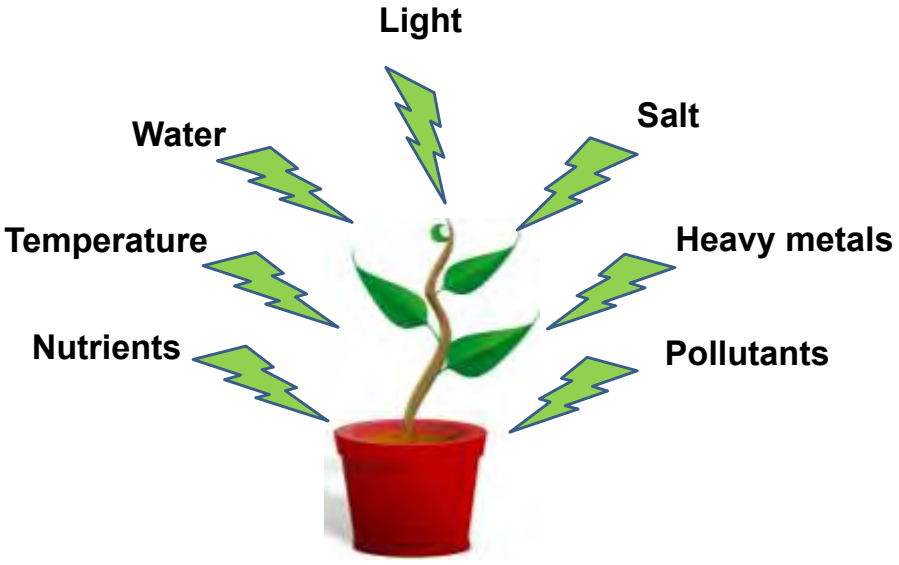

Figure 1.4 Some common abiotic stress factors that affect plants.

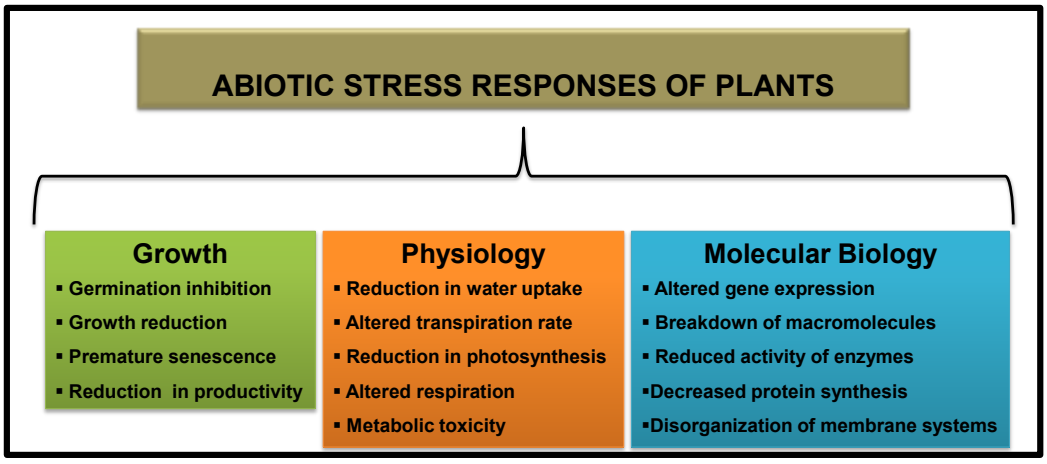

Figure1.5 Some of the common plant abiotic stress responses.

The complex plant response to abiotic stress, which involves many genes and biochemical-molecular mechanisms, is schematically represented in Figure 1.6.

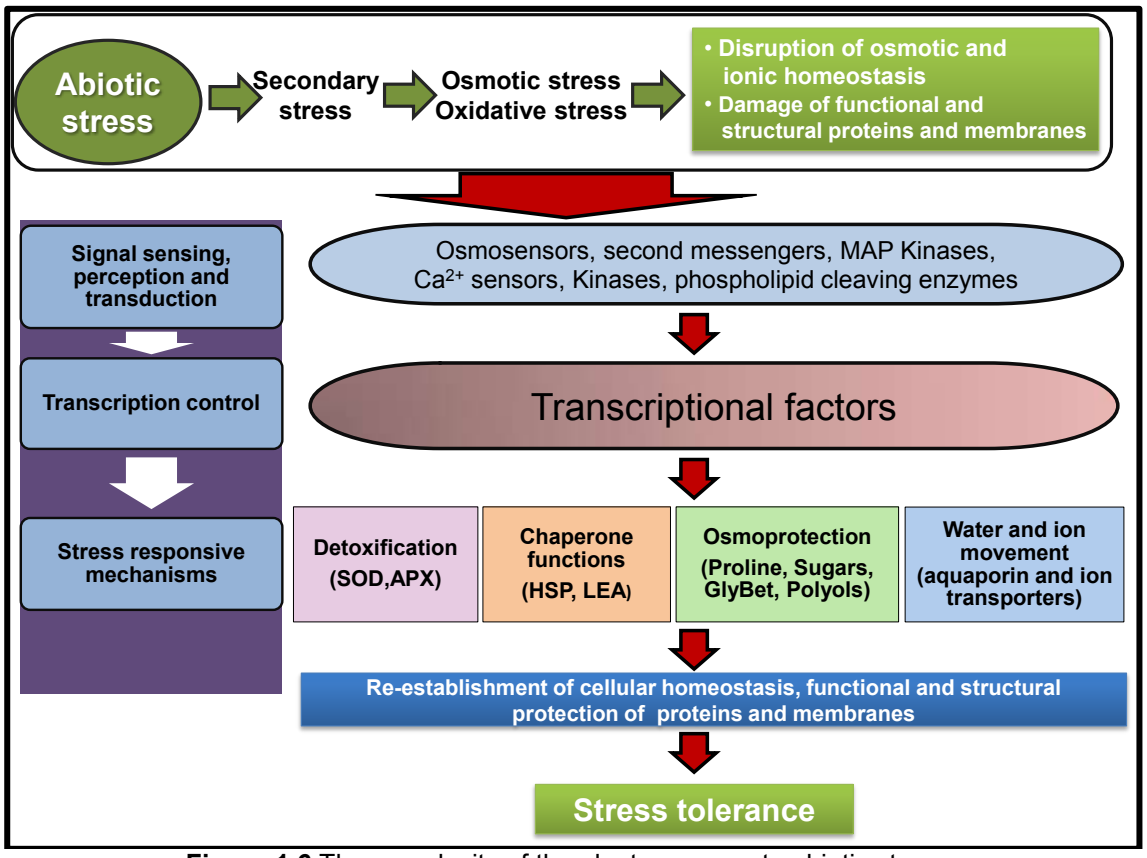

Figure 1.6 The complexity of the plant response to abiotic stress.

Primary stresses, such as drought, salinity, cold, heat and chemical pollution are often interconnected and cause cellular damage and secondary stresses, such as osmotic and oxidative stress. The initial stress signals (e.g. osmotic and ionic

effects, or temperature, membrane fluidity changes) trigger the downstream signaling process and transcription controls which activate stress-responsive mechanisms to re-establish homeostasis and protect and repair damaged proteins and membranes. Inadequate response at one or several steps in the signaling and gene activation may ultimately result in irreversible changes of cellular homeostasis and in the destruction of functional and structural proteins and membranes, leading to cell death (Wang et al., 2003).

Plants exhibit great variations in their tolerance mechanisms, within species, between species and among the plants of different groups. These variations are highly significant in developing stress tolerance in plants (Madhava-Rao et al., 2006).

1.2.2 Metabolic engineering for abiotic stress tolerance

As immobile organisms, many plants have evolved remarkable adaptations for various environmental stress factors. Stress triggers a response in plants, which brings change in its metabolite profile like formation of compatible solutes, antioxidants, phytoalexins, protein protectants and cryoprotectants often due to up-regulation of metabolic pathways (Bohnert and Shen, 1999). Stress may also be responsible for recession or complete shutting down of various cell functions and maintenances, such as cell division (Lerner, 1999). Plant breeders and geneticists have utilized natural variability for stress tolerance within germplasm collections for selecting cultivars with improved stress tolerance (Bolaños et al., 1993; Ceccarelli et al., 1991). However, this approach is limited by the available variability within the germplasm and crossability of the species.

Metabolic engineering can be defined as the manipulation of cellular, enzymatic, regulatory and transport processes using recombinant-DNA technology for the purpose of enhancing specific product yield (Bailey, 1991). Development of methods to transfer genes across species has led to research aiming to produce stress-tolerant transgenic crops using metabolic engineering. Today, scientific community is dedicated to identify potential target genes for metabolic engineering of crops for enhanced biotic and abiotic stress tolerance. Advancement in revealing the human, yeast, bacterial, *Arabidopsis* and other model crops genomes and quickly growing bioinformatics databases are playing a major role in this goal (Rathinasabapathi and Kaur, 2006).

One of the major limitations has been finding out which one of a multistep pathway can be manipulated to achieve a desired stress tolerance phenotype. In many cases, the connections between a pathway (or its end product or a specific gene) and stress tolerance have not been well established. Therefore, most research in model species aims to provide that connection between a gene and a stress tolerance phenotype. For the application of metabolic engineering in major crops, thorough field-level assessments of stress tolerance are required. Such assessments have not been realized in many of the studies. Despite the

complexity and lack of field studies, the past two decades have seen some impressive progress especially on understanding how plants cope with and adapt to various environmental stress factors (Rathinasabapathi and Kaur, 2006).

Current engineering approaches depend upon the transfer of one or several genes encoding either biochemical pathways or endpoints of signaling pathways. These gene products guard plants against abiotic stresses both in direct and indirect ways. Major components or targets for engineering stress tolerance in plants are listed in Table 4.

Table 4. Major metabolic and transport components for engineering abiotic stress tolerance in plants

Components	Mode of action
Growth regulators	Modification in hormone homeostasis
Heat shock proteins	Prevention or reversal of protein unfolding
Ion/proton transporters	Elimination and sequestration of toxic ions from the cytoplasm and organelles; ion uptake and transport
Membrane fatty acid composition	Increase in membrane fluidity and chilling tolerance
Osmoprotectants	Osmotic adjustment, protection of proteins and membranes, scavenging of reactive oxygen
Reactive oxygen scavenger	Detoxification of reactive oxygen species
Signaling pathway	Ca2+-sensors/phosphorylation intervened signal transduction
Transcription factors	Transcriptional activation or up-regulation of specific structural genes
Water status	Stomatal behavior; regulation of aquaporin in tonoplast and plasma membrane

Perception of the stress, signal transduction, activation of transcription factors and expression of structural genes are the main steps in stress response (Krauss, 2001). Since the availability of whole genome sequence of *Arabidopsis thaliana* and rice, identification of genes for use in metabolic engineering for stress tolerance has enormously improved.

Many high throughput techniques such as microarray analyses, proteomics and metabolomics are available to quickly identify a large number of transcripts, proteins or metabolites altered in response to stress. These techniques provide valuable data to make testable hypotheses. Additional validation can come from mutants affected for their stress tolerance or correlative data from different genotypes. Availability of T-DNA insertion mutants in most of the genes in Arabidopsis, makes it possible to test the function of genes whose functions are currently unknown (Rathinasabapathi and Kaur, 2006).

The discovery of novel genes, the determination of their expression patterns in response to abiotic stress and an improved understanding of their roles in stress adaptation obtained by the use of “omics“ technologies will provide the basis for new metabolic engineering strategies leading to novel crop cultivars. Table 5 provides selected examples of metabolic engineering where a stress tolerant phenotype has been achieved.

Table 5. Selected examples of stress tolerant phenotypes obtained by metabolic engineering

Gene (plant/product)	**Phenotype**
Alfin1 (alfalfa / transcription factor)	Salt tolerance
JERF3 (tomato / jasmonate and ethylene-responsive factor 3)	Salt tolerance
OSISAP1 (rice / A20-AN1-type zinc-finger protein)	Drought, cold and salt tolerance
DREB1A (*Arabidopsis* / DRE-binding protein 1)	Drought and salt tolerance
SNAC1 (rice / stress-responsive NAC1)	Drought and salt tolerance
HVA1 (barley / late embryogenesis abundant protein)	Tolerance to water and salt stress
TPS1 (*Arabidopsis* / trehalose 6-phosphate synthase)	Drought and salinity stress tolerance
BADH (spinach / betaine aldehyde dehydrogenase)	Tolerance to salt stress
PINO1 (*Porteresia* / L-myo-inositol-1-phosphate synthase)	Salt tolerance
AhCMO (*Atriplex hortensis* / choline mono-oxygenase)	Salt tolerance
AgNHX1 (*Atriplex gmelini* / Na+/H+ antiporter)	Salt tolerance
GhNHX1 (cotton / putative vacuolar Na+/H+ antiporter)	Salt tolerance
SsNHX1 (*Suaeda salsa* / Na+/H+ antiporter)	Salt tolerance
GlyI/GlyII (*Brassica juncea* / glyoxalase I/II)	Salt tolerance
GmTP55 (soybean / antiquitin-like protein)	Salt, dehydration and oxidative stress

1.2.3 Functional genomics for the analysis of abiotic stress tolerance

Functional genomics allows large-scale gene function analysis with high throughput technology and incorporates interaction of gene products at cellular and organism level. The information coming from sequencing programs provide enormous input about genes to be analyzed. The availability of *Arabidopsis* and rice genome sequence among other plants has paved the way for studying the function of genes on a genome-wide scale. The non-availability of information about genomes from other plants will also be compensated in part by the availability of large collection of ESTs and cDNA sequences. The basic interest behind these EST projects is to identify genes responsible for critical functions. In many cases bioinformatics tools will come in handy (Tyagi et al., 2006).

The gene function is defined by studying the transcripts, proteins and metabolites and also by altering the activity of a gene *per se*, e.g. by large-scale

mutagenesis. These different ingredients of functional genomics have developed in their own right and termed as transcriptomics, proteomics, metabolomics, and phenomics (Chory et al., 2000; Holtorf et al., 2002). ESTs, cDNA libraries and microarrays are used to analyze global gene expression profiles in a functional genomics program. Complementing large-scale expression studies are large mutant collections (Tyagi et al., 2006).

There are several reports where transgenic systems have been used to study gene expression involved in stress response. The function can be studied either by overexpression or suppression of gene expression using antisense or RNAi strategy (Tyagi et al., 2006).

1.2.4 Proteomics

The large-scale functional analysis of gene products, being expressed as proteins, is called proteomics (Pandey and Mann, 2000). Proteomics involves identification, quantification, activity and molecular interactions of all proteins from an organism. A proteome is a total protein complement of a genome (Wasinger et al., 1995) and the analysis of a functional proteome provides a powerful tool for linking gene expression to cell metabolism. Proteomics tells us what fraction of the genome is functional and at what levels. Proteome analysis provides better annotation of genome sequences by taking into account very small open reading frames (ORFs) which are functional. It can also assign an unidentified ORF to a particular protein product. Protein micro-characterization and their post-translational modifications can be determined by proteome methodologies (Park, 2004).

Proteomics concept is based upon high quality separation of proteins. This science of proteins dates back to late 1970"s with the advent of two-dimensional gel electrophoresis (2-DE) (O"Farrell, 1975). The proteins are separated by 2-DE, estimated using image analysis and then the mass spectrometer based identification of gel-separated proteins is carried out. Two-dimensional electrophoresis separates peptides according to their molecular mass and isoelectric point. Proteomics approach is dependent on high purity and reproducibility. Several computer softwares have been developed for the quantitative analysis of complex 2-DE patterns but the major advancement in proteomics has been a combination of 2-DE with mass spectrometry (MS) in 1990"s (Yates, 1998). MS technique has replaced the sensitive methods of Edman"s protein microsequencing, which has remained time and cost intensive.

Although the platform based on 2-DE is still the most commonly used, the use of gel-free and the second-generation of quantitative proteomic techniques has increased. Proteomic data are beginning to be validated using complementary "OMICS" or classical biochemical or cellular biology techniques. As a result, the coverage of the plant cell proteome and the plant biology knowledge is increasing.

However, plant biology research has yet to exploit fully the potential of proteomics, in particular its applications to PTMs and interactomics (Jorrín-Novo et al., 2009).

2. Justification

Food shortage is one of the serious problems that humanity has to face in this century. Cereals such as rice, wheat and corn provide half the calories consumed by the world's population. The world population is increasing at the rate of eighty million per year. It is estimated that we will have to produce 50 % more food grains by 2025 (Khush, 2001).

Many cultivated lands are not optimal in conditions for growth of conventional crops. A large proportion of the productive potential is lost due to unfavorable conditions and different types of biotic and abiotic stress factors.

Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50% (Boyer, 1982). Drought is one of the main abiotic stress factors limiting the agricultural productivity worldwide. Losses due to drought are superior than that caused by any other biotic or abiotic stress factor, while more than 800 million hectares of land throughout the world are salt affected (FAO, 2008). Increased salinization of arable land is expected to have devastating global effects, resulting in 30% land lost within the next 25 years, and up to 50% by the year 2050 (Wang et al., 2003).

Exposure of plants to abiotic stress factors activate various physiological and developmental changes ruled by the expression of different genes and proteins, they activate a diverse set of physiological, metabolic and defense systems to survive and to sustain growth. Understanding of the basic biochemical and molecular mechanisms for abiotic stress perception, transduction and tolerance is still a major challenge in biology (Valliyodan & Nguyen, 2006).

In crop plants, extensive efforts have been devoted to the generation of stress tolerant genotypes by conventional breeding, but more frequently conventional breeding has moved towards the exploitation of transgenics. The more frequent use of 'omics' technologies has allowed the identification of genes and proteins associated with abiotic stress tolerance (Witzel et al., 2009).

The analysis of gene expression at the transcriptional level provides important information regarding early stage transmission from the genome to cellular machinery. However, mRNA levels are not always consistent with the abundance of their respective proteins. Furthermore due to processes such as alternate splicing, protein proteolysis and post-translational modifications, one gene can produce many different protein species (Chen and Harmond, 2006). Post-transcriptional regulation plays a major role in stress-responsive gene expression and highlights the need for combined transcriptomic and proteomic analysis.

The study of stress responsive mechanisms in plants by the proteomic approach is a tool that may help to identify genes and metabolic pathways that are crucial for tolerance to abiotic stress (Salekdeh and Komatsu, 2007).

Amaranth can adapt to different growing conditions such as nutrient-poor soils, low water conditions and high temperatures. It is considered drought and salt stress tolerant. Amaranth is a good model to study tolerance to abiotic stress conditions such as drought and salt stress [Omami et al., 2006; Huerta-Ocampo et al., 2009; Huerta-Ocampo et al., 2011).

The objective of the project Amaranth Future Food (www.amaranth-future-food.net) was to provide the tools for an extensive and sustainable exploitation of amaranth (Fomsgaard et al., 2010).

3. Objective

The general goal of the present work was the identification of differentially accumulated proteins in amaranth plants under abiotic stress conditions such as drought and salt stress by a proteomic approach.

3.1 Specific Aims

- To identify differentially accumulated proteins in response to drought stress in amaranth leaves and roots.
- To identify differentially accumulated proteins in response to salt stress in amaranth roots.
- To quantify free proline and soluble sugars in response to drought stress in amaranth leaves and roots.
- To quantify free proline, soluble sugars and Na^+ and K^+ contents in amaranth roots subjected to salt stress.

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5. Results

5.1 Article “Proteomic analysis of amaranth (*Amaranthus hypochondriacus* L.) leaves under drought stress". *International Journal of Plant Sciences* 2009, Vol. 170, No. 8: pp. 990-998.

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PROTEOMIC ANALYSIS OF AMARANTH (*AMARANTHUS HYPOCHONDRIACUS* L.) LEAVES UNDER DROUGHT STRESS

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Amaranth (*Amaranthus hypochondriacus* L.) is a plant that produces seed with high protein content, is rich on nutraceutical compounds, and can grow under environmental conditions where most of the basic crops are not able to develop. But little is know about the amaranth stress-responsive genes/proteins. The aim of this work was to apply the comparative proteomics approach to study the differential expression of amaranth leaf proteins under drought stress. However, the protein extraction from amaranth tissues is difficult as a result of high endogenous concentrations of interfering compounds; we have made some modifications of the classical trichloroacetic acid–acetone precipitation method to improve the quantity and quality of extracted proteins. Satisfactory and reproducible two-dimensional electrophoresis protein profiles were obtained; the method was also tested for*Agave tequilana* and *Opuntia* spp., two more examples of plants that are tolerant to drought stress. Drought-responsive proteins in amaranth leaves were identified by liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS). The upregulated proteins identified included chloroplast chaperonins involved in refolding and protein complexes protection. Downregulated proteins include Rubisco large subunit, cytochrome *b6f*, oxygen evolving complexes, and the ascorbate peroxidase mitochondrial. The results have shown that chloroplasts and mitochondria may play a central role in amaranth adaptation to abiotic stress, and further studies should be done at the subcellular level.

Keywords: abiotic stress, agave, LC/ESI-MS/MS, nonmodel plants, *Opuntia* spp., recalcitrant plants.

Introduction

Drought and salinity are the most common problems in agriculture worldwide. Plant survival and hence crop productivity depend greatly on the ability to adapt to, respond to, and tolerate variable environmental conditions (Rossignol et al. 2006). When plants are subjected to stress, genes are turned on or off, with the resulting changes of the transcripts levels and their translated proteins responsible for the plant adjustments in the adaptation to the environmental conditions. Amaranth (*Amaranthus hypochondriacus* L.) produces seeds with high-quality protein, and according to recent reports, the seeds contain nutraceutical compounds and peptides with anticarcinogenic and antihypertensive activities (Silva-Sánchez et al. 2008; Barba de la Rosa et al. 2009). In areas of subtropical and tropical Asia, Africa, and Central America, *Amaranthus* ssp. are grown as green vegetables. The leaves are especially high in calcium and, on a fresh-weight basis, contain more fiber, niacin, and vitamin C than spinach (Abbot and Campbell 1982). The plant can grow at high temperatures with small quantities of water, and its cultivation has been extended worldwide (Sellers et al. 2003; Gimplinger et al. 2008).

Proteomics approach has a great potential for research using nonmodel plant species, where no genomic sequencing data are available and the identification of candidate genes can be difficult because of the divergence between orthologous genes. However, protein sequences are better conserved and make possible the identification of proteins in nonsequenced organisms (Carpentier et al. 2008). Not only is the recent progress in crop proteomics giving clues about nutritional value, but also major emphasis is taking on crop responses to abiotic stresses (Salekdeh and Komatsu 2007). Protein sample preparation is the most critical step for any proteomic study based on two-dimensional electrophoresis (2-DE) analyses (Görg et al. 2004), and researchers have turned special attention to the development of optimal protocols for 2-DE and proteomic analysis of different recalcitrant plant tissues (Giavalisco et al. 2003; Saravanan and Rose 2004; Carpentier et al. 2005; Song et al. 2006; Xie et al. 2007). Some of these protocols are known as standard or universal protocols (Wang et al. 2006), but they are not as useful for plants that have not been tested previously, where the best choice is to develop a specific optimized protocol rather than applying a given universal protocol (Wang et al. 2007; Gómez-Vidal et al. 2008; Sheoran et al. 2009).

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The aim of this work was to identify proteins in response to drought stress in amaranth leaves. We have optimized a protocol for amaranth leaf protein extraction; the protocol was also useful for *Agave tequilana* Weber var. azul and *Opuntia* spp., two examples of plants tolerant to abiotic stress with high biotechnological interest but which also contain different compounds that interfere with protein extraction. Amaranth, agave, and nopal could play an important role in the search of novel proteins/genes related to abiotic stress.

Material and Methods

Plants

Amaranth (*Amaranthus hypochondriacus* L.) cv. Nutrisol leaves were harvested from 45-d-old plants. Leaves were frozen and powdered under liquid nitrogen using a mortar and pestle. Six-month-old agave (*Agave tequilana* Weber var. azul) plants were kindly donated by the Herradura Company, and the leaves were frozen and milled. Two wild types of nopal (*Opuntia* spp.), locally named tapon morado and tempranillo, were collected from the Mexican High Plateau. The young cladodes were cleaned of spines, cut into small pieces, and freeze-dried. The dried plant was pulverized and sieved through a 100-mesh screen.

Amaranth Plants Drought Stress Treatment

Amaranth (*Amaranthus hypochondriacus* L.) cv. Nutrisol seeds were germinated on soil for horticulture (Sunshine Premix 3, Sun Gro Horticulture, Bellevue, WA). After germination, seedlings were transferred into 2000-mL pots with the same soil and grown in a greenhouse at 30°/27°C day/night temperatures with a 12L : 12D photoperiod for 29 d. Soil water content was monitored in the afternoon (4:00 p.m.) daily and maintained in humidity levels ~30% (Hydrosense, Campbell Scientific). Drought stress was initiated on day 22 by cessation of watering. On day 29, leaves were harvested from control and drought-stressed plants in parallel; leaves were flash frozen in liquid nitrogen and stored at −80°C until protein extraction. Leaf relative water content (RWC) was measured according to Barrs and Weatherley (1962) and calculated as follows:

$$\mathrm{RWC} = \frac{\text{fresh weight} - \text{dry weight}}{\text{turgid weight} - \text{dry weight}} \times 100.$$

Protein Extraction Procedures

The classic trichloroacetic acid–acetone (TCA-C) extraction protocol described by Damerval et al. (1986) was tested. Three grams of the powdered plant tissue were mixed with 10 mL of cold acetone containing 10% TCA and 0.07% 2-mercaptoethanol (2-ME). The suspension was sonicated (GE-505, Ultrasonic Processor) for 15 min at 4°C and centrifuged for 2 min at 3,000 *g* (Super T21, Sorvall), and the supernatant was incubated at −20°C for 2 h and then centrifuged at 10,000 *g* for 20 min. The protein pellet was resuspended and rinsed with ~1 mL of cold acetone containing 0.07% 2-ME to remove pigments and lipids. Washes were repeated until the pellet was colorless.

The TCA-C method was improved with the addition of acetone-ethanol washes (TCA-E protocol). Three grams of plant tissue were mixed with cold acetone containing 10% TCA and 0.07% 2-ME (1 mL for 0.3 g of tissue). The mixture was sonicated, centrifuged, and incubated at −20°C as for the TCA-C protocol. The pellet was washed with 1 mL of cold acetone containing 0.07% 2-ME, and one final wash was done with cold absolute ethanol.

In the third protocol, named TCA-A, 3 g of plant tissue were mixed with 4 volumes of ice-cold acetone containing 10% TCA, 1% PVPP, and 2% 2-ME (Saravanan and Rose 2004; Yao et al. 2006). The mixture was sonicated for 15 min and centrifuged as described above. The supernatant was incubated at −20°C for 2 h, and protein was pelleted by centrifugation at 13,000 *g* for 20 min at 4°C. The pellet was washed two times with 1 mL of cold acetone containing 0.07% 2-ME, once with cold absolute methanol, and once with cold acetone. Protein concentration was determined using Protein Assay (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard, according to the manufacturer's instructions.

Two-Dimensional Polyacrylamide Gel Electrophoresis

The protein samples obtained from the different protocols described above were resuspended in rehydration buffer: 8 M urea, 2% CHAPS (3-[3-(chloroamidopropyl)dimethylammonio]-1-propanesulfonate), 0.002% bromophenol blue, 0.28% DTT (dithiothreitol), and 0.5% w/v IPG buffer. Protein samples (350 μg for amaranth and agave leaves) were loaded onto an IPG strip holder with 13-cm linear gradient IPG strips, pH 3–10 or pH 4–7 (GE Healthcare). *Opuntia* spp. cladodes protein samples (25 μg) were loaded onto 7-cm pH 3–10 linear gradient IPG strips. The strips were rehydrated for 14–16 h at room temperature. Focusing was carried out in an Ettan IPGphor system (GE Healthcare) at a constant 50 mA per strip at 20°C, with the following program for 7-cm strips: (1) 500 V gradient until 0.01 kVh, (2) 4000 V gradient until 5.6 kVh, and (3) hold at 5000 V until 2.5 kVh. For 13-cm strips, the program was (1) 500 V gradient until 0.01 kVh, (2) 4000 V gradient until 5.6 kVh, and (3) hold at 8000 V until 15.2 kVh. After isoelectrofocusing (IEF), the IPG strips were immediately equilibrated for 15 min with equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, and 0.002% bromophenol blue) containing 1% w/v DTT. The strips were then transferred to 13% SDS-PAGE gels for two-dimensional electrophoresis with a Hoefer SE 600 Ruby Cell (GE Healthcare) for 13-cm strips or to a Bio-Rad mini-protean III apparatus (Bio-Rad) for 7-cm strips, using SDS electrophoresis buffer (25 mM Tris pH 8.3, 192 mM glycine, and 0.1% SDS) and resolved at 20 mA/gel until the dye (bromophenol blue) reached the bottom of the gels. Two-dimensional electrophoresis gels were stained with Sypro Ruby (Molecular Probes) or Coomassie brilliant blue R-250.

The stained gels were scanned with the Gel Doc (Bio-Rad), 16-bit TIF images were analyzed, and spot detection was carried out with PDQuest (ver. 7.2.0; Bio-Rad). All gels were

produced in triplicate. Gel image analysis of control and drought-stressed amaranth plants included spot detection, spot measurement, background subtraction, and spot matching. To correct the variability and to reflect quantitative variation, the spot volumes were normalized as the percentage of the total volume in all of the spots in the gel. The molecular masses of proteins on gels were determined by coelectrophoresis of molecular mass standards, and the isoelectric point of proteins was determined by migration of protein spots on 13-cm IPG linear gradient strips (pH 4–7). Data analysis of differential expressed proteins was performed by one-way ANOVA (Statgraphics Plus, ver. 5.0); the effect of the treatment was significant when $P < 0.05$.

Liquid Chromatography Tandem Mass Spectrometry

The protein spots were carefully excised from the Coomassie stained gels, distained, washed, digested with modified porcine trypsin (Promega, Madison, WI), and extracted as described previously (Xolalpa et al. 2007). The volume of the extracts was reduced by evaporation in a vacuum centrifuge at ambient temperature and then adjusted to 20 mL with 1% formic acid.

Mass spectrometric analysis was carried out on a 3200 Q TRAP hybrid tandem mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Ontario), equipped with a nanoelectrospray ion source (NanoSpray II) and a MicroIonSpray II head. The instrument was coupled online to a nanoAcquity Ultra Performance LC system supplied by Waters México (Waters, Milford, MA). Mass calibration of the hybrid triple quadrupole linear ion trap spectrometer was done with polypropylene glycol standard solutions. The instrument was then tuned and tested using [Glu1]-fibrinopeptide B (Sigma). Samples were desalted by injection onto a Symmetry C_{18} UPLC trapping column (5 μm, 180 μm $\times$ 20 mm; Waters) and washed with 0.1% formic acid in Milli Q water at a flow rate of 15 μL/min. After 3 min, the trap column was switched in line with the analytical column. Peptides were separated on a BEH C_{18} UPLC column (1.7 μm, 75 μm $\times$ 100 mm; Waters) equilibrated with 2% acetonitrile/0.1% formic acid using a linear gradient to 0.1% formic acid/70% acetonitrile over a 60-min period at a flow rate of 0.25 μL/min. Spectra were acquired in automated mode using information-dependent acquisition. Precursor ions were selected in Q1 using the enhanced MS mode (EMS) as a survey scan. The scan range for EMS was set at m/z 300–1500 and 4000 amu/s, with an ion spray voltage of +2.2 kV applied to a Picotip emitter FS150-20-10-N (New Objective, Woburn, MA). The interface heater for desolvation was held at 150°C. The survey scan was followed by an enhanced resolution scan (ER) of the three most intense ions at the low speed of 250 amu/s over a narrow (30 amu) mass range to determine the ion charge states. Two enhanced product ion scans (EPI) of the three most intensive peptide signals were performed at 4000 amu/s. The precursor ions were fragmented by collisionally activated dissociation (CAD) in the Q2 collision cell. Collision voltages were automatically adjusted on the basis of the ion charge state and mass using rolling collision energy. The fragment ions generated were captured and mass analyzed in the Q3 linear ion trap.

Data interpretation and protein identification were performed from the MS/MS spectra data sets using the MASCOT search algorithm (ver. 1.6b9; Matrix Science, London; available at http://www.matrixscience.com). Searches were conducted using the *Viridiplantae* subset of the National Center for Biotechnology Information nonredundant database (http://www.ncbi.nih.gov). Trypsin was used as the specific protease, and one missed cleavage was allowed with tolerances of 0.5 Da for the precursor and 0.3 Da for the fragment ion masses.

Results and Discussion

Total Protein Extraction and 2-DE Patterns

Yield of total protein extracted from amaranth leaves varied according to the method used. The TCA-A method gave the highest protein yields (0.99 ± 0.03 mg protein/g fresh tissue). TCA-C and TCA-E gave similar values (0.11 ± 0.03 mg protein/g fresh tissue. Equal amounts of the protein extracted from each of the three methods were separated by 2-DE (fig. 1). The 2-DE profiles of amaranth leaf proteins using the classic method (TCA-C) showed poor quality of electrophoretic patterns (fig. 1*a*). Vertical streaking was observed, and the average number of protein spots observed was 319 spots, with few spots with a molecular mass greater than 50 kDa. With the TCA-E method, the protein profile was somewhat improved, but there was still some horizontal streaking. The protein spots increased to 384 and were distributed all along the pH gradient, but only a few spots with a molecular mass greater than 50 kDa were observed (fig. 1*b*). The TCA-A protocol, which included polyvinylpolypyrrolidone (PVPP), greatly improved the quality of gels, with an increasing protein spot count to 453 and with improved resolution of spots above 50 kDa (fig. 1*c*). Basically, with the three methods tested, the 2-DE amaranth leaf profiles are the same, with the only changes being the resolution and number of spots as a result of the increased gel resolution. Sheoran et al. (2009) described differences on protein spots depending of the tested method, but Natarajan et al. (2005) reported only few differences from soybean extracts using different methods, and Saravan and Rose (2004) reported large differences in 2-DE spot patterns in tomato samples. The contrary results could be mainly due to the nature of the sample: it is well known that glycosylations and other posttraductional modifications affect the protein solubility, size, and charge under different extraction conditions (Saravan and Rose 2004). In amaranth samples, PVPP seems to help to extract proteins of high molecular mass.

Proteomic analysis is becoming an important tool for protein characterization of nonmodel plants (Carpentier et al. 2008). We tested the TCA-A protocol for total protein extraction from *Agave tequilana* Weber var. azul leaves and *Opuntia* spp. cladodes. Agave and nopal are two examples of plants that contain several interfering compounds and plants of high biotechnological potential as amaranth. The protein extraction from agave leaves using the TCA-A protocol resulted in 2-DE gels of good quality, and no background was observed. Using 13-cm strips at pH 3–10, there was a count of 269 spots

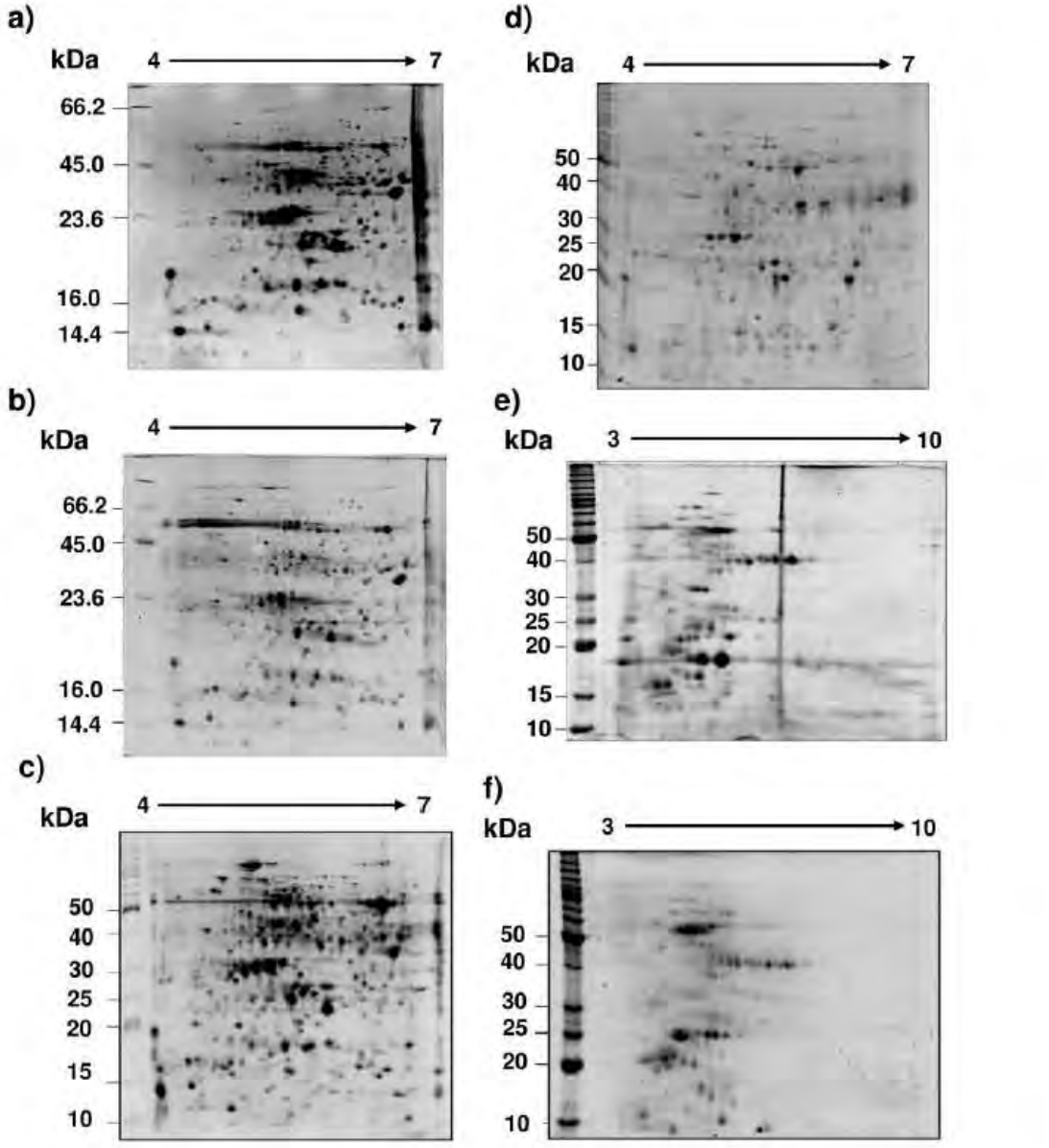

Fig. 1 Two-dimensional electrophoresis (2-DE) of total proteins from amaranth leaves extracted with different protocols: TCA-C, traditional TCA method (*a*); TCA-E, based on TCA-C with ethanol washes (*b*); TCA-A, based on PVPP extraction with methanol washes and cold acetone (*c*). The TCA-A method was tested in *Agave tequilana* leaves (*d*), *Opuntia* spp. tapon morado cladodes (*e*), and *Opuntia* spp. tempranillo cladodes (*f*).

(figure not shown), where the majority of spots were located in the pH range of 4–7. The 2-DE patterns of agave in the strips in the range of pH 4–7 gave a count of 369 protein spots (fig. 1*d*). This is mainly due to the fact that in a narrow pH range, more proteins of close p*I* could be separated. The 2-DE gel pattern for the *Opuntia* tapon morado sample was also of good quality, with 118 protein spots counted (fig. 1*e*), while in the *Opuntia* tempranillo sample 85 protein spots were counted (fig. 1*f*). The smaller number of spots resolved in *Opuntia* spp. samples is mainly due to the smaller amount of protein present in cladodes. Comparing the 2-DE profiles from the two *Opuntia* spp. samples, clear differences were found, and these patterns could be used as biomarkers for sample identification.

Comparative Proteomics of Amaranth Leaf Proteins under Drought Stress

The drought stress in amaranth was done by stopping the watering; soil water content decreased from levels of 16% ± 2% to 6% ± 1%. In this stage, the leaves of stressed plants started to roll. On day 29, the soil water content decreased to 4% ± 1%. The RWC declined from 95% in control plants to 45% in drought-stressed plants. When the plants were rewatered, the RWC in leaves returned to 90% in 5 h. The rapid recovery observed on amaranth after severe drought conditions is a distinctive characteristic of drought-tolerant plants. This is important to analyze the protein changes under abiotic stress, and comparative proteomics is the tool used for the analysis of the changes of plants under different environmental conditions (Salekdeh et al. 2002; Hajheidari et al. 2005; Salekdeh and Komatsu 2007; Xu and Huang 2008). We applied the comparative proteomics approach for the study of protein changes in amaranth leaves when the plant is grown under severe drought stress.

Leaf samples from control plants and stressed plants were taken, and total protein was extracted using the TCA-A method; protein profiles were observed by 2-DE gels. Digital image analysis of 2-DE gels profiles (p*I* range 4–7) showed that 453 spots were reproducibly detected. Figure 2 shows the amaranth leaves 2-DE pattern, and zoomed images compare the protein spots between the control (C) and the drought (D) treatment. The histograms in figure 3 show the changes in the amount of protein from the 22 differentially expressed proteins. Those spots were excised and tryptic digested, and peptides were characterized by liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS). From these, 17 proteins spots (77%) were successfully identified (table 1), with a significant score greater than 44 indicating the identity (homology at $P < 0.05$), while in five cases (23%) the identification was not possible (spots 5, 8, 9, 17, and 22 in fig. 2).

Identification of Drought-Responsive Proteins in Amaranth Leaves

Three spots that increased in leaves in response to drought were identified as chloroplast chaperone subunits. The chloroplast chaperonins consist of two distinct polypeptides, Cpn 60α and Cpn 60β, which share only ~50% amino acid identity. Spot 6 was identified as the α subunit of the chaperonin of 60 kDa (Cpn 60α), while spots 3 and 7 corresponded to the β subunit of this protein (Cpn 60β). Functional characterization of plant chaperonins is limited, but it is generally agreed that they are important in assisting plastid proteins as Rubisco (Boston et al. 1996). Spot 13 increased in response to drought stress and was identified as a heat shock protein (Hsp) of 70 kDa. Hsp 70 chaperones, together with their cochaperones, have essential functions in preventing aggregation and assisting refolding of nonnative proteins under normal and stress conditions (Hartl 1996). Some members of the Hsp 70 family are constitutively expressed and are involved in assisting the folding of the de novo synthesized polypeptides and the import/translocation of precursor proteins. Therefore, they are more involved in the refolding and proteolytic degradation of nonnative proteins under stress conditions (Wang et al. 2004). Spot 14, which appeared only after the drought treatment, was identified as an Hsp 70. Research into the cross-talk between Hsp/chaperones and other stress-responsive mechanisms in plants will provide a better understanding of acquired stress tolerance (Wang et al. 2004).

The majority of cytosolic protein degradation in eukaryotes occurs through the ubiquitin proteasome system. In this process, cellular proteins targeted for degradation are tagged by multimers of ubiquitin and are degraded by the 26S proteasome, a giant cytosolic protease. Proteins may display a hydrophobic patch as a result of denaturation that is recognized and routed for degradation. Spot 4 was identified as a member of the ubiquitin-conjugating enzymes family. These kinds of enzymes catalyze the second step in the ubiquitination reactions that label proteins for proteasome degradation (Nandi et al. 2006).

Spots 1, 2, and 21 decreased their concentration in response to drought stress and were identified as Rubisco large subunits. Rubisco quantity in leaves is controlled by its synthesis and degradation rates. Even in early drought stages, this protein still is relatively stable as a holoenzyme and has a half-life of some days (Webber et al. 1994). However, under drought stress, a rapid decrease in its transcription rate has been found in tomato, *Arabidopsis*, and rice (Bartholomew et al. 1991; Williams et al. 1994; Vu et al. 1999). Using the proteomic approach, the increased abundance of Rubisco fragmentation under drought stress has been reported in rice (Salekdeh et al. 2002), sugar beet (Hajheidari et al. 2005), and *Elymus elongatum* (Gazanchian et al. 2007). It is possible that fragmentation occurs during protein solubilization in vitro. In this work, no Rubisco fragmentation spots were identified between the analyzed proteins.

In oxygenic photosynthesis carried out by cyanobacteria, algae, and higher plants, the multisubunit cytochrome *b6f* integral membrane protein complex mediates electron transfer between the photosystem II, in which H_2O is the electron donor, and the photosystem I reaction centers. The electron transfer through the *b6f* complex is coupled to proton translocation across the membrane, which establishes an electrochemical potential gradient (Cramer et al. 2004). This complex is the most likely point of regulation in the electron transport chain (Golding and Johnson 2003). Cytochrome *b6f* complex iron sulfur subunit 2 (spot 11) was decreased in response to drought. Downregulation of this protein during drought may help to protect photosystem II by downregulating the electron transport to match the demands of carbon fixation by the Calvin cycle. Electron transport to oxygen and thus the production of reactive oxygen species will be minimized (Golding and Johnson 2003).

Spots 10 and 20, which decreased under drought, were identified as the oxygen evolving complex (OEC) 23-kDa subunit. These two forms are probably due to posttranslational modifications. The photosynthetic OEC is the enzyme in green plants that participates in the use of oxidants produced in the light to oxidize water, obtaining molecular oxygen as a by-product. This multimeric protein consists of at least three subunits named for their molecular masses (OE 33, OE 23, and OE 17) that are attached at the inner surface of the thylakoid membrane to the photosystem II reaction center (Hashimoto et al.

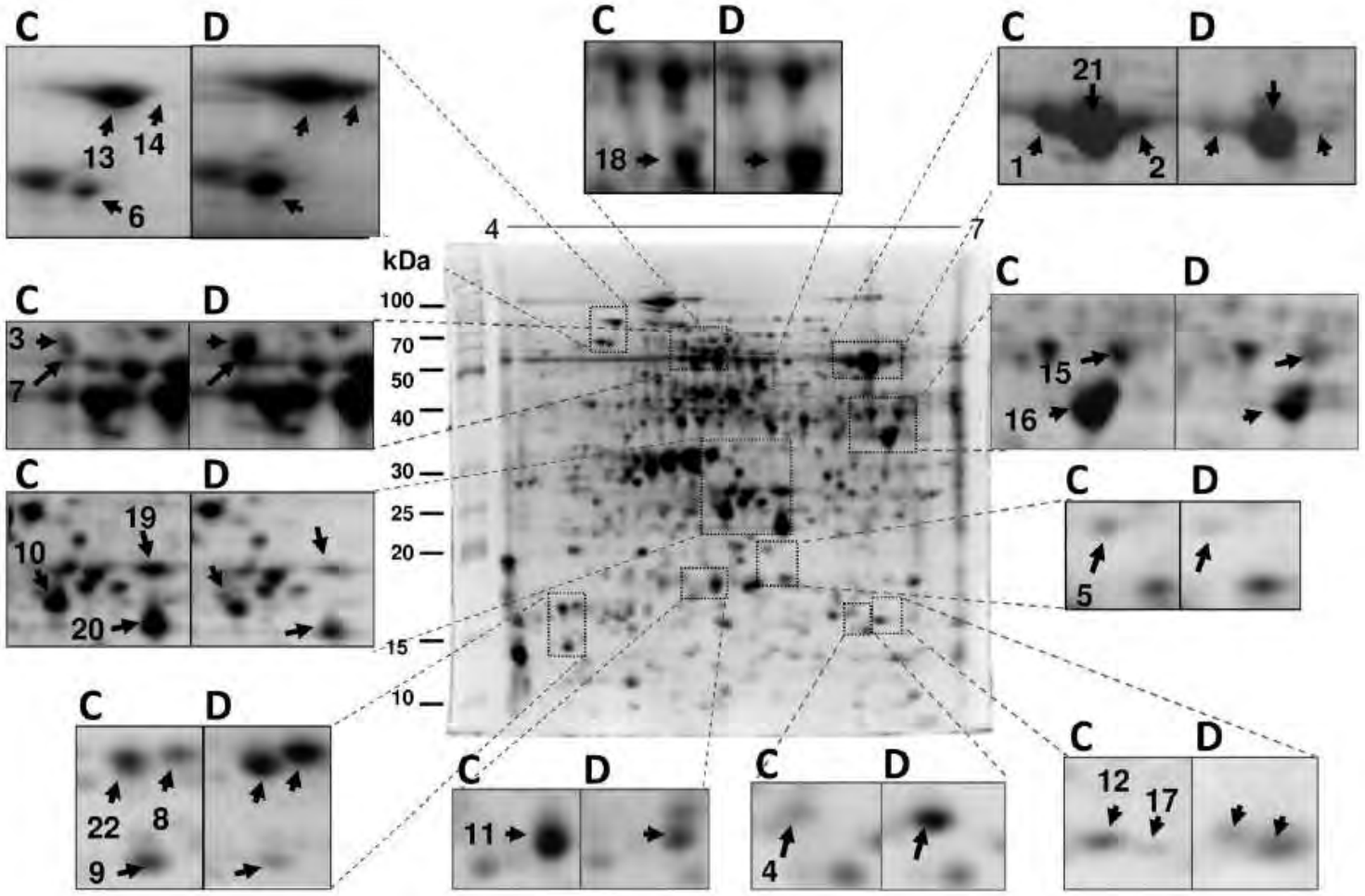

Fig. 2 Changes in protein abundance in amaranth leaves between control (*C*) and drought (*D*) conditions. Zoomed images show the differential protein spots characterized by LC/ESI-MS/MS.

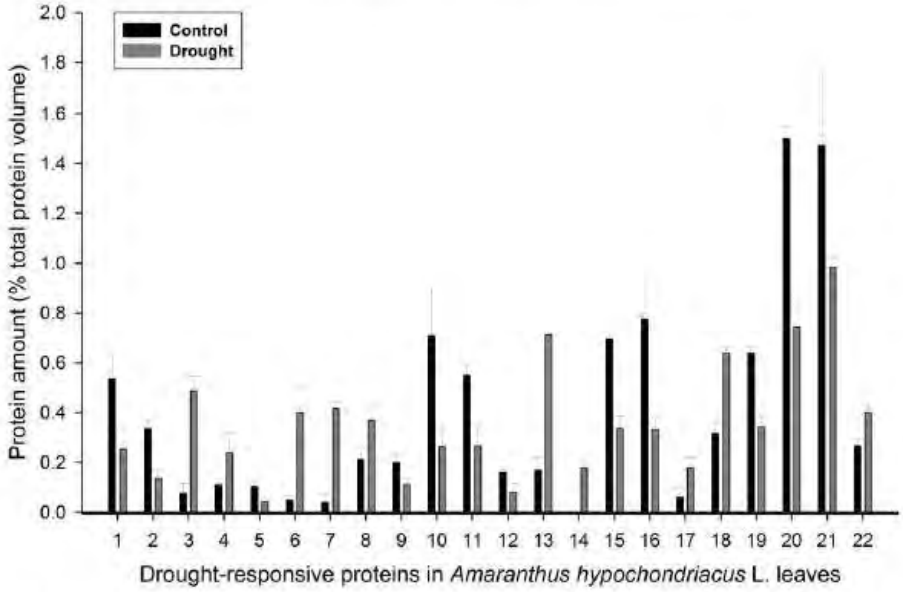

Fig. 3 Histogram showing changes in the amounts of differentially displayed proteins analyzed by LC/ESI-MS/MS.

1997). Under desiccation conditions, inhibition of the OEC occurs (Skotnica et al. 2000).

S-adenosylmethionine sintetase (SAMS, spot 18) increased in abundance following drought stress in amaranth leaves, and it catalyzes the production of S-adenosyl-L-methionine (SAM) from L-methionine and ATP. SAM serves as a methyl group donor in numerous transmethylation reactions. SAMS also play a role in the stress resistance and are encoded by small gene families that contain members that are differentially transcribed in response to various stress conditions (Sánchez-Aguado et al. 2004).

Spot 12, which decreased under drought stress, was identified as a hypothetical nucleoside diphosphate kinase (NDPK). This type of protein plays a significant role in hormone responses and MAPK-mediated H_2O_2 signaling, development, and growth. Yang et al. (2006) suggests that there might be a general stress response mechanism in relation to NDPK. The primary function of ascorbate peroxidase (APX) involves the reduction of hydrogen peroxide (H_2O_2), with the consequent oxidation of ascorbate to dehydroascorbate. H_2O_2 can act as a signaling molecule for the programmed cell death pathway and expression of defense-related genes that help to protect the cell from reactive oxygen species. Hossain et al. (2006) found a significant decrease in APX activity at the beginning of senescence, with a good correlation to an increase in the H_2O_2 content. This downregulation of APX seems to be a prerequisite for inducing senescence. In this work, spot 19, identified as APX, was decreased after severe drought stress in amaranth leaves. The increased expression of defense-related proteins cannot overcome the negative effects on the plant, and we saw that the leaves rolled and the plant started to wilt, but after rewatering, the plant recover its normal appearance.

Spot 16, found in decreased concentration, was identified as the mitochondrial malate dehydrogenase (mMDH). This enzyme catalyzes the reversible reduction of oxaloacetate to malate and is important in multiple metabolic pathways. Reduction of the activity of this enzyme showed an enhanced photosynthetic activity and aerial growth in transgenic tomatoes (Nunes-Nesi et al. 2005). Mitochondria are a key site of oxidative stress and play important roles in cell survival. It has been described that although plant cell bioenergetics is strongly affected by abiotic stresses, mitochondrial metabolism under stress is still largely unknown but may play a central role in cell adaptation to abiotic stresses that are known to induce oxidative stress at a cellular level (Pastore et al. 2001).

Spot 15 (fig. 2) was identified as the fructose-bisphosphate aldolase. The fructose-1,6 bisphosphate aldolase is an enzyme catalyzing a key reaction of glycolysis and energy production, converting D-fructose-1,6-bisphosphate into dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate. It is documented that both genes of fructose-bisphosphatase are downregulated by salt stress. The downregulation of proteins involved in respiration as fructose-bisphosphate aldolase may contribute to tissue adaptation to stress by lowering respiratory energy consumption (Chaves et al. 2009).

Conclusion

The method used in this work (TCA-A) was efficient for protein extraction from amaranth, agave, and nopal samples. The TCA-A method was efficient for comparative proteomic analysis of amaranth leaves when the plant was grown in normal conditions compared with leaves from plants grown

Table 1

Identification of Drought-Responsive Proteins in *Amaranthus hypochondriacus* L. Leaves by LC/ESI-MS/MS Analysis

Spot no.[a]	Proteins	Peptides matched/ sequence coverage	Mascot score[b]	Protein name	Accession no.[c]	Experimental mass (kDa)/p*I*	Theoretical mass (kDa)/p*I*	Species
1*	D	8/15%	339	Rubisco large subunit	gil1346961	52/6.27	50.4/6.51	*Amaranthus tricolor*
2*	D	7/15%	277	Rubisco large subunit	gil1346961	52/6.52	50.4/6.51	*Amaranthus tricolor*
3*	U	1/2%	54	Chaperonin-60 β subunit	gil1762130	62/5.20	63.3/5.72	*Solanum tuberosum*
4*	U	2/20%	74	Ubiquitin-conjugating enzyme-like protein	gil76160962	16.3/6.28	16.5/6.20	*Solanum tuberosum*
6*	U	3/5%	174	Chaperonin-60 α subunit	gil1710807	62/4.73	61.9/5.15	*Pisum sativum*
7*	U	1/2%	53	Chaperonin-60 β subunit	gil1762130	60/5.20	63/5.72	*Solanum tuberosum*
10*	D	5/26%	130	23-kDa oxygen evolving complex protein	gil148535011	24.4/5.47	21.5/5.94	*Salicornia veneta*
11*	D	4/41%	145	Cytochrome *b6f* complex iron sulfur subunit 2	gil146454654	18/5.40	16.6/6.04	*Sonneratia alba*
12*	D	5/21%	223	Hypothetical NDPK	gil125595441	15.8/6.45	21.5/8.38	*Oryza sativa*
13*	U	8/10%	335	Hsp 70 kDa	gil1143427	75/4.76	75.4/5.15	*Cucumis sativus*
14[d]		2/3%	58	Hypothetical protein (Hsp 70 kDa)	gil147805297	75/4.83	69.1/5.11	*Vitis vinifera*
15*	D	2/7%	107	Fructose-bisphosphate aldolase	gil113624	38.7/6.58	38.5/5.96	*Spinacia oleracea*
16*	D	4/14%	260	Malate dehydrogenase, mitochondrial precursor	gil126896	34.6/6.51	36.2/8.88	*Citrullus lanatus*
18*	U	7/23%	391	S-adenosylmethionine synthetase	gil48928010	46/5.69	43.2/5.52	*Solanum brevidens*
19*	D	6/34%	223	Ascorbate peroxidase	gil559005	27/5.83	27.4/5.43	*Nicotiana tabacum*
20*	D	6/26%	155	23-kDa oxygen evolving complex protein	gil148535011	23/5.83	21.5/5.94	*Salicornia veneta*
21	D	8/17%	351	Rubisco large subunit	gil7240502	52/6.38	51.7/6.12	*Achlys triphylla*

Note. U, upregulated proteins; D, downregulated proteins; Hsp, heat shock protein.

[a] Numbers correspond to spots in figure 2.

[b] Scores >44 indicate identity or extensive homology ($P < 0.05$).

[c] Accession numbers are from the National Center for Biotechnology Information Entrez database.

[d] Only under drought stress.

* Treatment effect is significant, $P < 0.05$.

under drought conditions. The results show that stress-responsive proteins in amaranth leaves are mainly chloroplast chaperonins. The decreased abundance of Rubisco large subunit, OEC, and cytochrome *b6f* complex, as well as the enzyme fructose-bisphosphate aldolase, show that the effect of drought on electron transport chain could be related to the reduction of carbon metabolism. The decreased abundance of the malate dehydrogenase mitochondrial could help to enhance the photosynthetic activity.

Although the genomes of the three plants used in this work are not sequenced, we have shown that proteomic analysis was successful for protein identification of differential protein spots from amaranth leaves. This work is expected to accelerate proteomic studies of *Amaranthus hypochondriacus* L., *Agave tequilana* Weber var. azul, and *Opuntia* spp., three nonmodel plants that are essential as food and feed resources in several places worldwide that grow under stress environments.

Acknowledgments

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5.2 Article: Water stress induces the up-regulation of DOF1 and MIF1 transcription factors and down-regulation of proteins involved in secondary metabolism in amaranth roots (*Amaranthus hypochondriacus* L.). *Plant Biology* 2011, Vol. 13, No. 3: pp. 472-482.

Supplemental material can be found in Annex 1.

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RESEARCH PAPER

Water stress induces up-regulation of DOF1 and MIF1 transcription factors and down-regulation of proteins involved in secondary metabolism in amaranth roots (*Amaranthus hypochondriacus* L.)

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Abiotic stress; drought; LC/ESI-MS/MS; proteomics; SSH libraries.

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ABSTRACT

Roots are the primary sites of water stress perception in plants. The aim of this work was to study differential expression of proteins and transcripts in amaranth roots (*Amaranthus hypochondriacus* L.) when the plants were grown under drought stress. Changes in protein abundance within the roots were examined using two-dimensional electrophoresis and LC/ESI-MS/MS, and the differential expression of transcripts was evaluated with suppression subtractive hybridisation (SSH). Induction of drought stress decreased relative water content in leaves and increased solutes such as proline and total soluble sugars in roots. Differentially expressed proteins such as SOD^{Cu-Zn}, heat shock proteins, signalling-related and glycine-rich proteins were identified. Up-regulated transcripts were those related to defence, stress, signalling (Ser, Tyr-kinases and phosphatases) and water transport (aquaporins and nodulins). More noteworthy was identification of the transcription factors DOF1, which has been related to several plant-specific biological processes, and MIF1, whose constitutive expression has been related to root growth reduction and dwarfism. The down-regulated genes/proteins identified were related to cell differentiation (WOX5A) and secondary metabolism (caffeic acid O-methyltransferase, isoflavone reductase-like protein and two different S-adenosylmethionine synthetases). Amaranth root response to drought stress appears to involve a coordinated response of osmolyte accumulation, up-regulation of proteins that control damage from reactive oxygen species, up-regulation of a family of heat shock proteins that stabilise other proteins and up-regulation of transcription factors related to plant growth control.

INTRODUCTION

Drought and salinity are two of the most important limiting environmental factors for worldwide agriculture. Plant survival, and therefore crop productivity, depends greatly on the ability to adapt, to respond and to tolerate such variable environmental conditions (Süle *et al.* 2004; Vij & Tyagi 2007; Carpentier *et al.* 2008a). In response to drought stress, plant gene induction leads to the synthesis and accumulation of osmoprotective products such as proline, glycine betaine, some sugars and other products that may act in maintaining cellular function through protecting cellular structures and osmotic adjustment (Yokota *et al.* 2006).

Plant roots are the primary sites of stress perception (Chang *et al.* 2000; Sauer *et al.* 2006). The morphological and functional qualities of roots are related to lignin content, which seems to be a key factor for plantlet survival (Pinker *et al.* 1995). Lignin is one of the most important biomolecules in vascular plants. Among other functions, lignin is uniquely involved in structural support and water transport. The emergence of lignin during evolution is believed to have been crucial for plant adaptation to survive on land (Xu *et al.* 2009). Lignin is a polymer of phenylpropanoid compounds formed through a complex biosynthetic route. Plants exposed to different stresses may change lignin content and composition (Silva-Moura *et al.* 2010).

Gene expression at the transcriptional level provides important information regarding early stage transmission from the genome to cellular machinery. However, mRNA levels are not always consistent with the abundance of cognate proteins. Furthermore, due to various processes, such as alternate splicing, mRNA processing, protein proteolysis and post-translational modifications (PTM), one gene can produce many different protein species (Chen & Harmon 2006). Previous reports suggest that post-transcriptional regulation plays a major role in stress-responsive gene expression (Jiang *et al.* 2007) and highlight the need for combined transcriptomic and proteomic analysis.

5.3 Book chapter "Amaranth: A pseudo-cereal with nutraceutical properties". *Current Nutrition & Food Science* 2011, Vol. 7, No.1: pp 1-9.

Amaranth: A Pseudo-Cereal with Nutraceutical Properties

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Abstract: Amaranth is a highly nutritious and non-allergenic crop with remarkable nutraceutical properties. Seed protein extracts following enzymatic digestion have been shown to inhibit Angiotensin Converting Enzyme (ACE). A possible mechanism of action of ACE inhibitor (ACEi) peptides involving the induction of Nitric Oxide (NO) production through endogenous Nitric Oxide Synthase (eNOS) activation has been proposed. The presence of lunasin, a peptide with proven antitumoral properties, has been confirmed in amaranth seeds and the gene encoding the amaranth lunasin peptide appears homologous to the bifunctional inhibitor/lipid transfer protein/seed storage 2S of the albumin family. Amaranth consumption has been shown to improve the plasma lipid profiles in animals. Methanolic extracts of amaranth have demonstrated anti-hyperlipidemic, anti-diabetic and anti-helmintic properties, while aqueous extracts have demonstrated anti-diarrheic, anti-fungal and anti-malarial properties. Specific polyphenols such as rutin, isoquercetin and nicotiflorin and some phenolic acids and amides with antioxidant effects have also been found in amaranth seeds. The aim of this mini-review is to provide an overview of the nutraceutical properties of amaranth.

Keywords: ACE-inhibitory peptides, amaranth, bioactive peptides, lunasin, nutraceuticals, polyphenols, rutin.

INTRODUCTION

The *Amaranthus* genus (family *Amaranthaceae*) consists of more than 50 species and includes plants with promising food crop potential due to their resistance to pests and abiotic stresses such as drought, heat and salinity [1-3]. Amaranth leaves and grains have high nutritional value [4, 5]. The cultivated commercial species of grain amaranth (*A. hypochondriacus*, *A. cruentus,* and *A. caudatus*) are native to America, but now are distributed throughout Asia and Africa. The weedy species *A. hybridus* grows all over the world. Some authors recognize the *A. mategazzianus* Pass or *A. edulis* Speg as a fourth cultivated species, but genetic studies do not seem to warrant recognition of this group [6].

Amaranth is an excellent food source owing to the high nutritional value of its leaves and seeds, the seeds contain high amounts of protein with a better balance of essential amino acids than that of cereals and legumes [4, 7]. The crop has gained importance in the US and is an emerging product in Europe, especially as a substitute for wheat in diets of patients with celiac disease. Amaranth seeds consumption positively affected plasma lipid profiles in cholesterol-fed rats, suggesting that these seeds would be a good substitute for hyper-cholesterolemic patients allergic to cereals.

The amaranth starch granule is one of the smallest found in nature (0.8 – 2.5 μm). Small granules have higher swelling power, lower solubility, greater water-binding capacity, and lower susceptibility to α-amylase digestion [8, 9]. The oil has high value due to its high squalene content [10]. Several attempts to develop technologies that increase the exploitation of amaranth seed constituents have been reported. The seeds flour has been incorporated in various food products, such as bakery goods (breads, pastries and cookies), extrusion-cooked products (cereal flakes, tortillas and pasta) [7, 11, 12] or by starch hydrolysis to obtain a carbohydrate-rich fraction and high protein content flour [13].

Because the high quality of amaranth proteins, maize has been transformed with the amaranth globulin 11S [14] and wheat with amaranth 35-kDa albumin [15]. The amaranth albumin AmA1, rich in all essential amino acids, has been used to increase the nutritive value of transgenic potatoes [16]. The addition of amaranth albumins to complement wheat flour not only improved the quality of crumb and loaf volume but also improved the balance of essential amino acids [17].

In this new millennium, a new era of amaranth research has started. *In silico* analysis of the potential active peptides present in amaranth proteins [18, 30] triggered renewed interest in this ancient crop. As a source of nutritive and nutraceutical compounds, it is hoped that amaranth will help reduce hunger and benefit human health. This mini-review discusses the current state of amaranth information; however further research is needed to confirm and exploit the full biotechnological potential of amaranth.

NUTRACEUTICAL PROPERTIES OF AMARANTH

Amaranth Seeds as a Source of Bioactive Peptides

Peptides liberated by enzymatic hydrolysis of food proteins have received much attention from food scientists. These bioactive peptides, containing 2 to 9 mostly hydrophobic amino acid residues in addition to proline, lysine or arginine, are inactive within the parent protein, but following enzymatic digestion or food processing can function as physiological modulators of metabolism [19]. *In silico*

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5.4 Salt stress-induced alterations in the root proteome of *Amaranthus cruentus* L.

5.4.1 Introduction

Salt stress is one of the mayor abiotic stress factors that adversely affect crop productivity. High concentrations of salts in soils account for large decreases in the yield of a wide variety of crops (Tester and Davenport, 2003). Increased salinization of arable land is expected to have devastating global effects, resulting in 30% land lost within the next 25 years, and up to 50% by the year 2050 (Wang et al., 2003). Exposure of plants to abiotic stress factors activate various physiological and developmental changes ruled by the expression of different genes and proteins, they activate a diverse set of physiological, metabolic and defense systems to survive and to sustain growth. Understanding of the basic biochemical and molecular mechanisms for abiotic stress perception, transduction and tolerance is still a major challenge in biology (Valliyodan and Nguyen, 2006).

In crop plants, extensive efforts have been devoted to the generation of stress tolerant genotypes by conventional breeding, but more frequently conventional breeding has moved towards the exploitation of transgenics. The more frequent use of 'omics' technologies has allowed the identification of genes and proteins associated with abiotic stress tolerance (Witzel et al., 2009). Recently, proteomic analysis has become one of the best strategies to reveal the dynamics of expression under salt stress (Guo et al., 2012).

Amaranth *(Amaranthus* spp.) is a promising vegetable species often grown under semiarid conditions prone to both drought and salinity (Omami and Hammes, 2006). Some of the amaranth species are recognized as weeds, while the grain amaranth species (*A. cruentus, A. caudatus and A. hypochondriacus*) are recognized by their high nutritional value. Due to the high quality of amaranth proteins, crops such as corn, wheat and potatoes have been transformed with amaranth seed storage proteins in order to increase the nutritional value and content of essential amino acids in these crops (Chakraborty et al., 2010; Rascón-Cruz et al., 2004; Tamás et al., 2009). In addition to its recognized stress tolerance and high nutritional values, amaranth has proven to be a non-allergenic food with remarkable nutraceutical properties (Huerta-Ocampo and Barba de la Rosa, 2011).

The increased ability to withstand drought stress and the high salt tolerance of grain amaranth has been associated with a high water use efficiency (Omami et al., 2006). Genetic information underlying the mechanisms that confer to amaranth its capacity to withstand drought and/or salt stress is limited although several abiotic stress related genes have been identified in amaranth and in phytogenetically related species such as spinach, cultivated and wild species of beet root, *Mesembryanthemum crystallinum* and the halophytes *Suaeda* spp., *Salicornia* spp., and *Atriplex* spp. (Delano-Frier et al., 2011).

The results of a combined proteomic/subtractive hybridization approach suggested that amaranth‟s root response to drought stress involves a coordinated response that includes osmolyte accumulation and the activation of stress-related genes needed for the scavenging of reactive oxygen species, protein stabilization and transcriptional regulation of plant growth (Huerta-Ocampo et al., 2011). Identification of calcium stress induced genes in amaranth leaves through suppression subtractive hybridization has also contributed to increase the relatively low number of grain amaranth expressed sequence tags (ESTs) responsive to abiotic stress conditions (Aguilar-Hernández et al., 2011).

The utilization of 454 pyrosequencing technology was employed as a tool to obtain genomic data from *Amaranthus tuberculatus*, a notorious weed of maize and soybean crops in the USA (Lee et al., 2009) and noteworthy, the publication of the first large scale transcriptomic analysis of grain amaranth where different sources of RNA (drought and acute salt stress among other conditions) were used to generate the cDNA libraries employed for pyrosequencing (Delano-Frier et al., 2011) open the door for a confident identification of more amaranth stress responsive proteins using the proteomic approach and surely will contribute to the elucidation of the salt tolerance mechanisms in amaranth. To date there are no reports on the analysis of proteins involved in the response to NaCl stress in amaranth roots. Because roots are the primary site of stress perception, the aim of this work was to identify differentially accumulated proteins in amaranth roots under salt stress.

5.4.2 Experimental Procedures

5.4.2.1 Plant materials, salt-stress treatment and plant harvest

Amaranth seeds (*Amaranthus cruentus* L. c.v. Amaranteca) were obtained from the National Institute for Investigation in Forestry, Agriculture and Animal Production (INIFAP, Mexico). Seeds were germinated on soil for horticulture (Peat Moss Tourbe, Premier Horticulture, Québec, Canada). After germination, clean seedlings were transferred to a greenhouse under natural temperature, light and photoperiod in plastic trays (60 x 45 x 15 cm) which were filled with ½ strength nutrient solution (Hydro-Environment, México) (EC 2.0-2.2 ds m^{-1}), and renewed every week. After pre-culture of amaranth seedlings for one month, 150 mM NaCl were added to the nutrient solution (EC 16.9-17.2 ds m^{-1}). Samples were collected after 1 h, 24h and 168 h after salt-stress imposition.

5.4.2.2 Total soluble sugar and proline contents

Free proline and total soluble sugar contents were determined in roots of plants subjected to progressive salinity stress. Free proline was determined using the ninhydrin reaction according to (Magné and Larher, 1992). Samples were milled and proline was extracted by boiling 0.5 g of powdered roots in 2 mL of

water. Then 0.5 mL of sodium citrate (0.2M pH 4.6) and 2 mL of 1% ninhydrin (acetic acid-water, 60:40) solution were added to 0.5 mL of the resulting extract. Mixture was incubated in boiling water for 1 h. The reaction mixture was extracted with 2 mL of toluene and proline concentration was calculated based on absorption of the resulting chromophore at 520 nm in a spectrophotometer (8453 UV-Visible Spectrophotometer, Agilent Technologies, Santa Clara, CA, USA) using toluene as blank and L-proline (Sigma-Aldrich, St Louis, MO, USA) as standard.

Total soluble sugars were determined using anthrone as previously reported (Aghaei et al., 2008). Roots (500 mg) were crushed in 5 mL of ethanol; the insoluble fraction was washed with 70% ethanol. Alcohol soluble fractions were centrifuged at 3500 x *g* for 10 min. The supernatants were collected and 3 mL of freshly prepared anthrone reagent (150 mg of anthrone in 100 mL of 72% H_2SO_4) was added to 0.1 mL of the alcoholic extract, the mixture was placed for 10 min in a boiling water-bath and reaction was stopped changing the tube to an ice-bath. After cooling, the absorbance was measured at 625 nm in a spectrophotometer using D-Glucose (Invitrogen, Carlsbad, CA, USA) as standard.

5.4.2.3 Ion content analysis

Prior to determination of ion content, the seedlings were uprooted and washed with deionized water. Root samples were dried in an 80 °C oven until constant weight. Dried materials were ashed in a furnace for 4 h at 500°C. Once at constant weight the ash was dissolved with 1.0N HCl solution and diluted in deionized water into a volumetric flask. Na^+ and K^+ contents were determined using an atomic absorption spectrometer (AAnalyst 400, PerkinElmer, Waltham MA, USA).

5.4.2.4 Protein sample preparation

Root samples were frozen in liquid nitrogen and milled in a coffee grinder (Braun, Naucalpan, Mexico) to a fine powder. The powder was suspended in extraction buffer (40 mm Tris pH 8.0, 1% PVPP and 2 mM PMSF) and mixture was sonicated (GE-505, Ultrasonic Processor, Sonics & Materials Inc., Newton, CT, USA) for 15 min at 4 °C and centrifuged for 10 min at 10,000 x *g* (Super T21; Sorvall, Kendro Laboratory Products, Newton, CT, USA). The supernatant was filtered with Miracloth (Calbiochem, Darmstadt, Germany) and treated with Nuclease Mix (GE Healthcare) according to manufacturer"s instructions. Mixed with three parts of cold acetone containing 10% TCA and 2% 2-ME, and incubated overnight at -20 °C. After 30 min of centrifugation at 13,000 x *g* the protein pellet was recovered and washed once in cold methanol and three times in cold acetone. The resulting pellet was dried and suspended in rehydration buffer [8 M urea, 2% CHAPS, 0.56% DTT, 0.002% bromophenol blue and 0.5% IPG buffer pH 4–7 (Bio-Rad, Hercules, CA, USA). Protein concentration was determined using the RC/DC

Protein Assay (Bio-Rad). BSA was used as standard. Root proteins were extracted from three independent replicates for each treatment.

5.4.2.5 2-DE and image analysis

Isoelectric focusing (IEF) was carried out onto 24 cm IPG linear gradient strips pH 4-7 (Bio-Rad). Strips were rehydrated with 500 µg and 1.5 mg protein for analytical and preparative gels respectively. Focusing was conducted at 20°C with an Ettan IPGphor system (GE Healthcare, Piscataway, NJ, USA) at constant 50 mA per strip under the following conditions: (I) 500 V gradient until 0.01 kVh, (II) 4000 V gradient until 5.6 kVh, and (III) holding at 8000 V until 60 kVh. After IEF, the IPG strips were stored at -20° C or immediately equilibrated twice for 15 min in equilibration buffer (6M urea, 30% glycerol, 2% SDS, 50 mM Tris-HCl buffer pH 8.8), the first time containing 1% DTT and the second time equilibration was performed in the same solution now containing 2.5% iodoacetamide instead of DTT. The strips were placed directly onto 13% polyacrylamide-SDS slab gels. Separation was conducted using the Ettan^{TM} Dalt*six* Electrophoresis unit (GE Healthcare). Preparative gels were stained with PhastGel^{TM}Blue R (GE Healthcare). Analytical gels were stained with Syrpo®Ruby (Molecular Probes) and documented with *Pharos FX* Plus Molecular Imager (BioRad). The resulting 16 bit pictures were exported to and normalized with PDQuest 2-D Analysis Software v8.0 (Bio-Rad). Proteins were considered as differentially accumulated when they displayed a fold change ≥2. Experimental molecular mass of each protein spot was estimated by comparison with molecular weight standards (BenchMark^{TM} Protein Ladder, Invitrogen). Experimental p*I* was determined by migration of protein spots on the IPG linear gradient strips.

5.4.2.6 *In-gel* digestion and Mass Spectrometry analysis (LC-MS/MS)

Differentially accumulated protein spots were excised from the preparative gels, reduced with 10 mM DTT in 25 mM ammonium bicarbonate followed by protein alkylation with 55 mM iodoacetamide. Protein digestion was carried out overnight at 37°C with sequencing grade trypsin (Promega, Madison, WI, USA). Nanoscale LC separation of tryptic peptides was performed with a nanoACQUITY UPLC System (Waters, Milford, MA, USA) equipped with a Symmetry C18 pre-column (5 µm, 20 mm x 180 µm, Waters) and a BEH130 C18 (1.7 µm, 100 mm x 100 µm, Waters) analytical column. The lock mass compound, [Glu^{1}]-Fibrinopeptide B (Sigma-Aldrich, St. Louis, MO, USA) was delivered by the auxiliary pump of the nanoACQUITY UPLC System at 200 nL/min at a concentration of 100 fmol/mL to the reference sprayer of the NanoLockSpray source of the mass spectrometer. Mass spectrometric analysis (LC-MS/MS) was carried out in a SYNAPT HDMS quadrupole/time-of-flight (Waters). The mass spectrometer was operated in V-mode and analyses were performed in positive mode ESI. The TOF analyzer was externally calibrated with [Glu1]-Fibrinopeptide B from *m/z* 50 to 2422. The data were post-acquisition lock mass corrected using

the doubly protonated monoisotopic ion of [Glu^1]-Fibrinopeptide B. The reference sprayer was sampled every 30 s. The RF applied to the quadrupole was adjusted such that ions from m/z 50-2000 were efficiently transmitted. Data independent accurate mass LC-MS/MS data were collected in an alternating, low energy, and elevated-energy mode of acquisition (MS^e).

5.4.2.7 Database search and protein identification

Data interpretation and protein identification were performed on the MS/MS spectra datasets using the MASCOT search algorithm (Matrix Science, London, UK available at http://www.matrixscience.com). Searches were conducted using the *Viridiplantae* subset of the National Center for Biotechnology Information non-redundant database and plants EST database (http://www.ncbi.nih.gov). Trypsin was used as the specific protease and one missed cleavage was allowed. The mass tolerance for precursor and fragment ions was set to 10 ppm and 0.1 Da respectively. Carbamidomethyl cysteine was set as fixed modification and oxidation of methionine was specified as variable modification. Identifications were considered successful when significant MASCOT scores (> 43 for nrNCBI and > 61 for Plants EST database) were obtained, indicating the identity or extensive homology at $P<0.01$.

5.4.2.8 Hierarchical clustering and protein classification

Hierarchical clustering of the expression profiles was performed on the log transformed induction factor values of differentially accumulated protein spots using the software Cluster v 3.0 (Available at http://bonsai.hgc.jp/~mdehoon/software/cluster/index.html). The program Tree-View (http://jtreeview.sourceforge.net/) was used to generate the graphical view of the results obtained from Cluster v 3.0. Identified proteins were classified into different categories of biological processes in which they are involved according to Gene Ontology (http://www.geneontology.org/).

5.4.3 Results and Discussion

5.4.3.1 Effect of salt-stress treatment on amaranth plants cultivated under hydroponic conditions

For the proteomic analysis of the root response to salt stress, it was necessary to prepare plants of uniform size and cultured under stable growth conditions. In this study, amaranth plants (*Amaranthus cruentus* L. cv. Amaranteca) were grown in ½ strength nutrient solution under hydroponic conditions (Figure 5.4.1 A). NaCl was added to 150 mM concentration in the nutrient solution. Therefore amaranth roots were directly in contact with salt. This

system allowed a reproducible sampling of the root tissue. After 1 h of NaCl addition it was clearly observed the first phase of salt stress, the resulting change in the osmotic pressure which starts immediately after the salt concentration around the roots increases and leads to a dramatic lost of turgor in amaranth plants (Figure 5.4.1 B). These plants were completely recovered after 24 h (Figure 5.4.1 C), indicating that the molecular mechanisms to cope with the osmotic phase of the stress were successful; and after 168 h (Figure 5.5.1 D) salt treated plants did exhibit slight phenotypic differences in comparison to controls, such as an slight curling of leaves and accelerated senescence of the oldest leaves indicating the presence of the ionic phase of salt stress.

Two phases occur during salt stress. A first, osmotic phase of rapid onset, when high concentrations of salts in the soil make it harder for roots to extract water and the resulting change in the osmotic pressure have an immediate effect on plant metabolism. The second, ion-specific phase, due to the accumulation of Na^+ in plant tissues (mainly leaves). In this phase salt accumulates to toxic concentrations in the old leaves which are no longer expanding and so no longer diluting the salt arriving in them as younger growing leaves do, and they die. If the rate at which they die is greater than the rate at which new leaves are produced, the photosynthetic capacity of the plant will no longer be able to supply the carbohydrate requirement of the young leaves, which further reduces their growth rate (Munns and Tester, 2008).

The hydroponic system employed in the present work allowed an easy sampling of amaranth roots during the different steps of salt stress, during the first osmotic phase, which starts after the salt concentration around the roots increases to a threshold level (after 1h); later when at the whole plant level amaranths are totally recovered of the osmotic phase of the stress (after 24h) and finally after 168 h of stress imposition during the ion-specific phase of plant response to salt stress. Gene and finally protein expression varies with the time after the salt shock is applied. After rice was suddenly exposed to 150 mM NaCl, the genes expressed in roots at 15 min were different from those expressed after 1 week (Kawasaki et al., 2001). It is likely that many genes and their translated proteins induced soon after salt is applied are related to the response to the osmotic component of salt stress and can be similar to that occurring during water stress rather than specifically to the ionic component of salt stress.

5.4.3.2 Changes in ion contents and osmolytes

As a result of the treatment Na^+ content increased very fast (two-fold) after only 1 h and after 24 h it reached about 120 mg/g DW. While after 168 h there was no significant difference in the root Na^+ content in comparison to that obtained after 24 h (Figure 5.4.1 E). K^+ accumulation was promoted during the first 24 h, K^+ content slightly increased after 1 h of salt stress imposition and after 24 h it doubled, however K^+ content dramatically declined after 168 h (Figure 5.4.1 F). As a consequence of the increase of Na^+ content and the decrease of K^+ with the time

progression the K^+/Na^+ ratios decreased gradually with the progression of salt stress imposition (Figure 5.4.1 G).

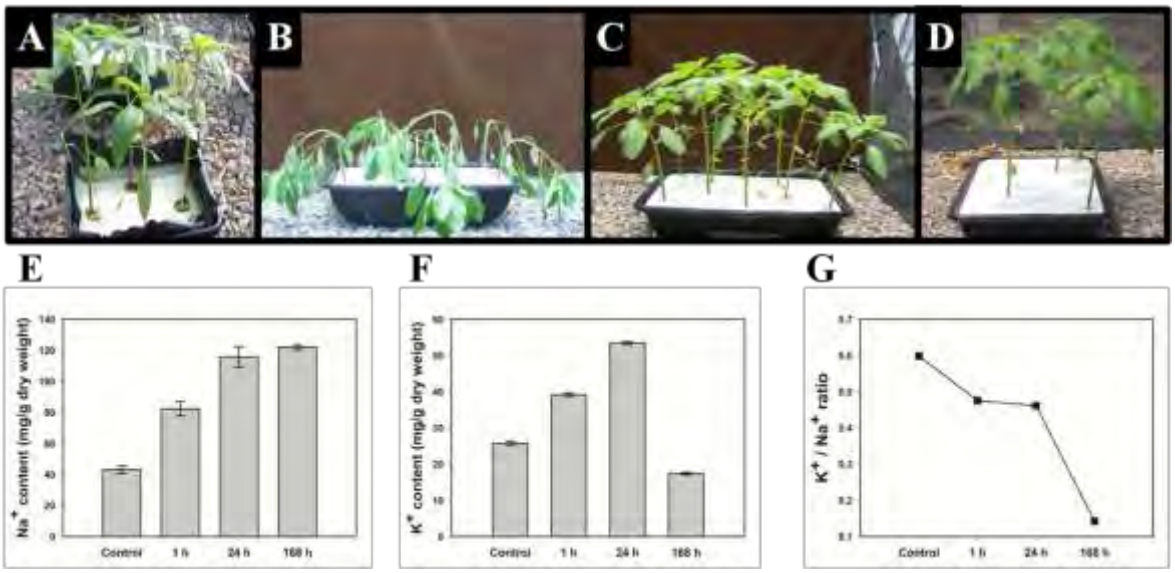

Figure 5.4.1. Salt-stress induced physiological changes of *Amaranthus cruentus* grown under hydroponic conditions. The plants were grown for 1 month in ½ strength nutrient solution (A) and then 150 mM NaCl were added to the nutrient solution. Root samples were taken after 1 h (B), 24 h (C) and 168 h (D) of salt stress imposition. The effect of salt stress on Na^+ content (E), K^+ content (F); and K^+/Na^+ ratio (G) are presented. The values are presented as means ±SE of three biological replicates.

Na^+ exclusion by leaf blades or by roots ensures that Na^+ does not accumulate to toxic concentrations within leaves. A failure in Na+ exclusion manifests its toxic effect after days or weeks, depending on the species, and causes premature death of older leaves. In plants without such mechanisms as *Amaranthus cruentus*, tolerance requires compartmentalization of Na^+ at the cellular and intracellular level to avoid toxic concentrations within the cytoplasm, especially in mesophyll cells in the leaf. Toxicity occurs with time, after leaf Na^+ increases to high concentrations in the older leaves. Restoration of water relations typically requires the uptake of osmoticum in the form of Cl^- and Na^+ ions. These are predominantly stored in vacuole with a concomitant loss of cellular K^+, the disproportionate presence of Na+ in cellular an extracellular compartments negatively impacts on the acquisition of essential nutrients such as Ca^{2+} and K^+(Maathuis, 2006). Synthesis of osmolytes is another important mechanism for tissue tolerance to salt stress.

In order to evaluate the effects of salt stress on the production of osmoprotective substances, root free proline and soluble sugars contents were determined in controls and in salt stressed plants. After addition of 150 mM NaCl to the hydroponic nutritive solution root free proline contents remain almost unchanged during the first 24 h, a slight decrease after 1 h of stress imposition was

observed. The maximum free proline contents were observed after 168 h of stress imposition and corresponded to a three-fold increase (Figure 5.4.2 A). Accumulation of free proline is a common response to abiotic stresses; several studies suggest that proline is involved in the osmotic adjustment between the cytoplasm and vacuole. The role of proline as a protective agent for enzymes, its function as a free-radical scavenger or as a storage compound for carbon and nitrogen have been also suggested (Aghaei et al., 2008).

The content of soluble sugars remained significantly unchanged after 24 h and 168 h after salt stress imposition. A slight decrease was observed after 1 h under salt stress (Figure 5.4.2 B). Soluble sugars contents observed in controls and under salt stress showed that the role of soluble sugars in amaranth subjected to salt stress are not so clear. Soluble sugars as osmoprotectants can counteract the ionic toxic effects in shoots of several species of plants under salt stress (Zhang et al., 2005).

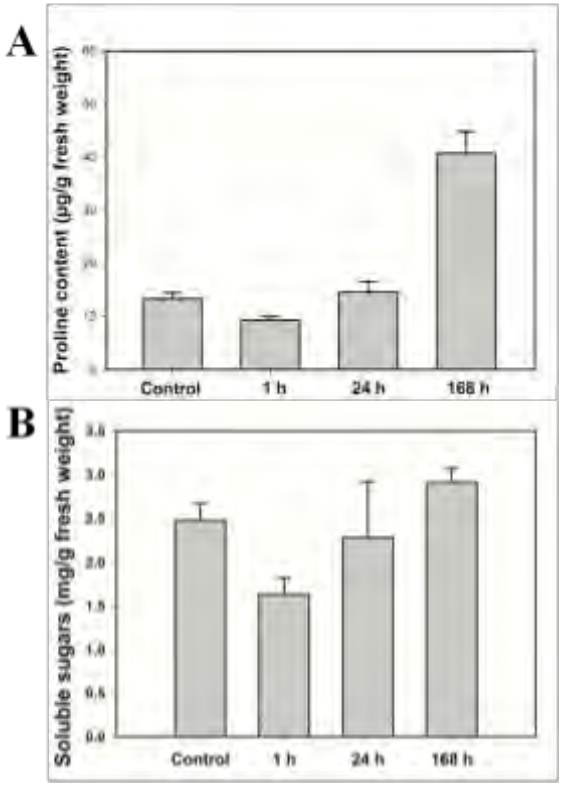

Figure 5.4.2. Effects of salt stress on the proline (A) and soluble sugars (B) contents in amaranth roots. The values are presented as means ±SE of three biological replicates.

5.4.3.3 Identification of salt-stress responsive proteins by 2-DE and LC-MS/MS

We applied a 2-DE gel coupled with LC-MS/MS approach in order to analyze the changes in protein accumulation in amaranth roots under salt stress in plants subjected to 150 mM NaCl. Roots were harvested and proteins were extracted and

separated by 2-DE. Representative 2-DE gels of the analyzed conditions are shown in Figure 5.4.3.

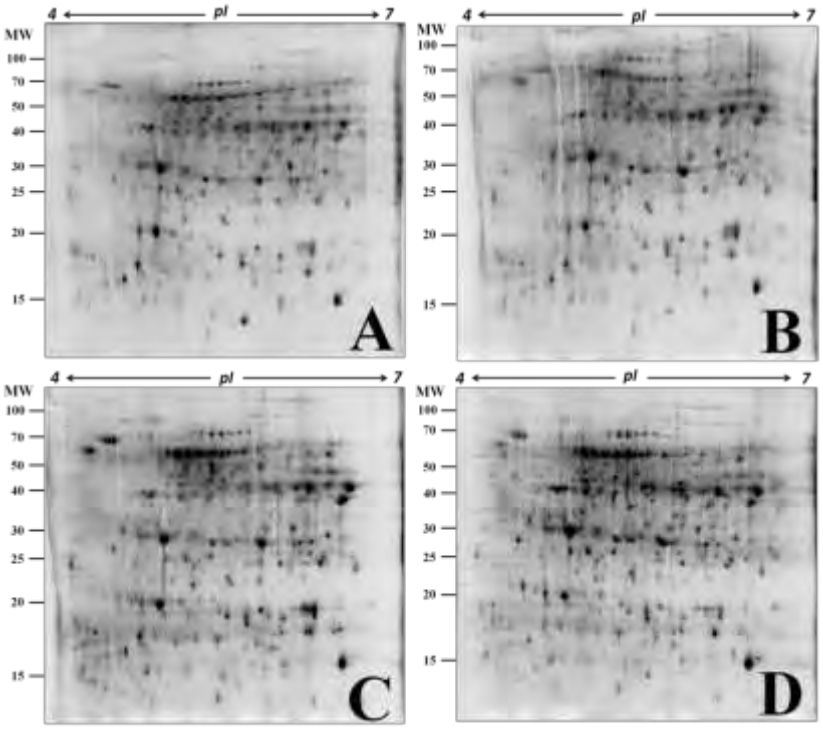

Figure 5.4.3. Representative 2-DE gels of amaranth root proteins subjected to salt stress. Three independent replicates for each condition were run. Control (A), after 1 h (B), 24 h (C) and 168 h (D) of salt stress imposition (150 mM NaCl) under hydroponic conditions.

Out of more than 600 reproducibly detected protein spots, 101 spots showed significant changes in abundance in salt stressed samples at least at one stage compared with control plants. Figure 5.4.4 shows the position of the 101 differentially accumulated protein spots on a 2-DE gel of root proteins from amaranth plants harvested after 168 h of salt stress imposition.

One hundred differentially accumulated protein spots are visible in the gel shown in Figure 5.4.4. Only one spot (spot 84) was not present under such stress conditions. A close-up and 3D-View of spot 84 and other four differentially accumulated protein spots is also shown in Figure 5.4.4.

Only 81 differentially accumulated protein spots were detected in all analyzed samples, 1 spot disappear after 168 h of salt stress imposition. Interestingly 19 spots absent in controls were accumulated in response to salt stress. Four spots appear after 1h, 7 after 24 h and 8 of them appear after 168 h of salt stress imposition. Close-up views and graphical expression patterns of 24 differentially accumulated protein spots are shown in Figure 5.4.5.

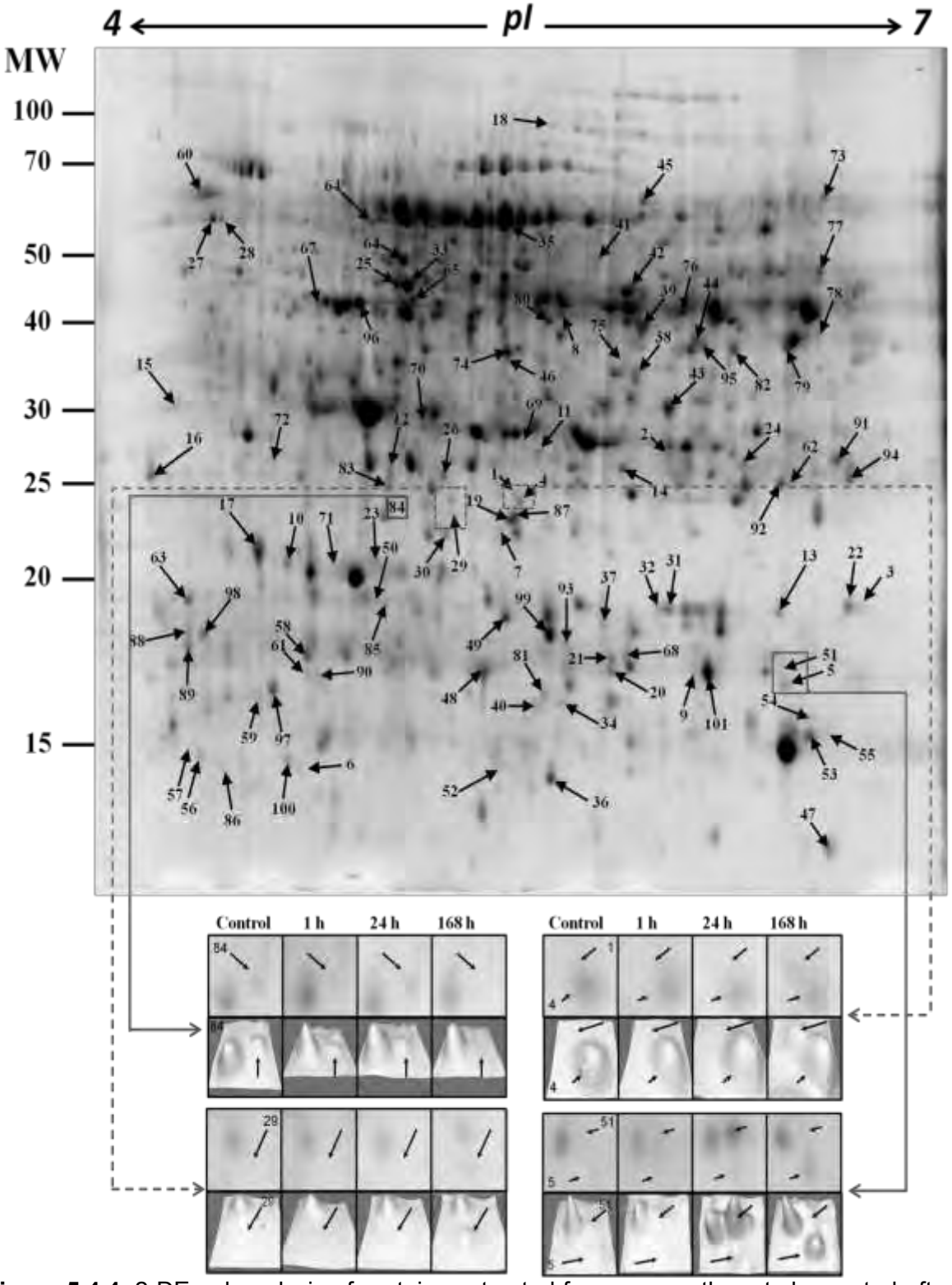

Figure 5.4.4. 2-DE gel analysis of proteins extracted from amaranth roots harvested after 168 h under salt stress. Arrows represent salt stress responsive spots analyzed by LC-MS/MS. Examples of changes in spot abundance including a 3D-View among the analyzed conditions are presented.

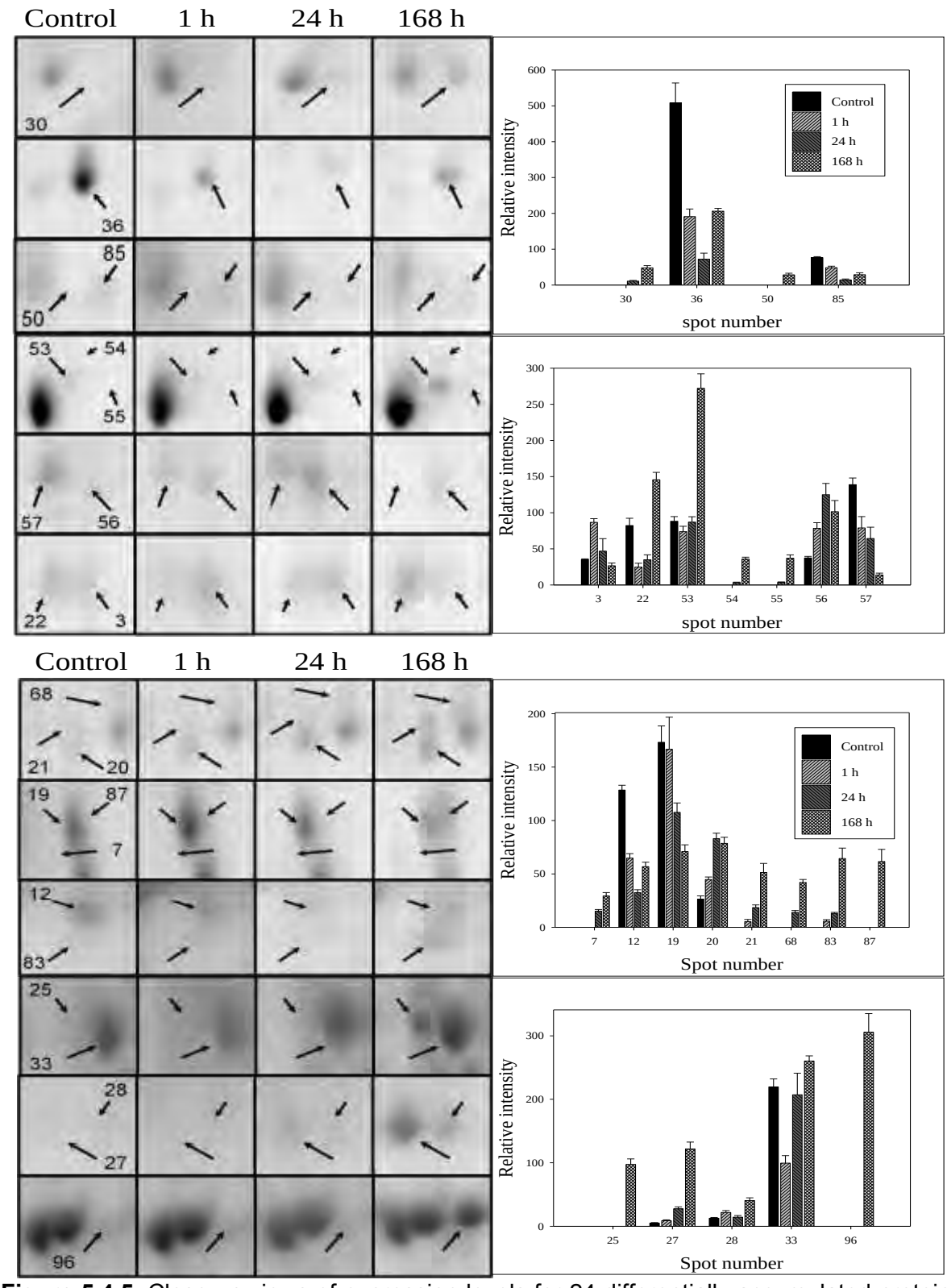

Figure 5.4.5. Close-up views of expression levels for 24 differentially accumulated protein spots on 2-DE gels of amaranth root proteins subjected to salt stress. Graphical expression levels are also shown for every spot. Numbers correspond to the numbers in Figure 5.4.4.

The number of significantly increased differentially accumulated protein spots in amaranth roots subjected to salt stress increased from 16 after 1 h to 33 after 24 h and up to 45 after 168 h of salt stress imposition. The number of significantly decreased differentially accumulated protein spots also increased from 17 after 1h to 27 after 24 h and up to 40 after 168 h of salt stress imposition (Figure 3.5.6).

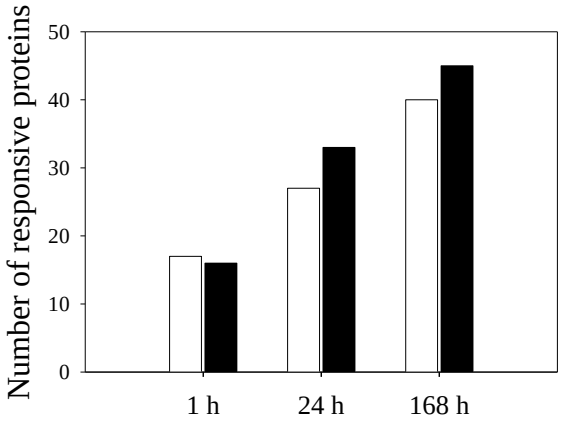

Figure 5.4.6 The number of protein spots differing significantly (by at least two-fold) in abundance in salt-stressed amaranth roots. Solid bars: Proteins significantly increased in stressed plants. Open bars: Proteins significantly decreased in stressed plants.

Visualization of the expression of the 101 differentially accumulated protein spots was simplified by hierarchical clustering analysis. As shown in Figure 5.4.7 hierarchical clustering offered a global view of the differential accumulation patterns of protein spots.

Protein spots were excised from preparative 2-DE gels and subsequently subjected to LC-MS/MS analysis; data were collected in an alternating, low energy, and elevated-energy mode of acquisition (MS^e). So far 51 spots have been successfully identified (P<0.01) by searching against nrNCBI and Plants EST databases. In 4 cases 2 protein per spot were identified. The resulting 55 proteins were grouped into different categories of biological processes in which they are involved according to Gene Ontology (Figure 5.4.8).

Among the differentially accumulated protein spots identified so far, proteins related to carbohydrate metabolism such as fructokinase among others were down regulated under salt stress. In some cases several spots identified as the same protein showed significant changes in opposite directions. Such is the case of 3 spots identified as cyclophilins. Noteworthy, this last protein is induced by salt stress among other abiotic stresses, and its absence turns plants hypersensitive to oxidative damage (Dominguez-Solis et al., 2008), but when over-expressed confer salt tolerance in rice (Ruan et al., 2011).

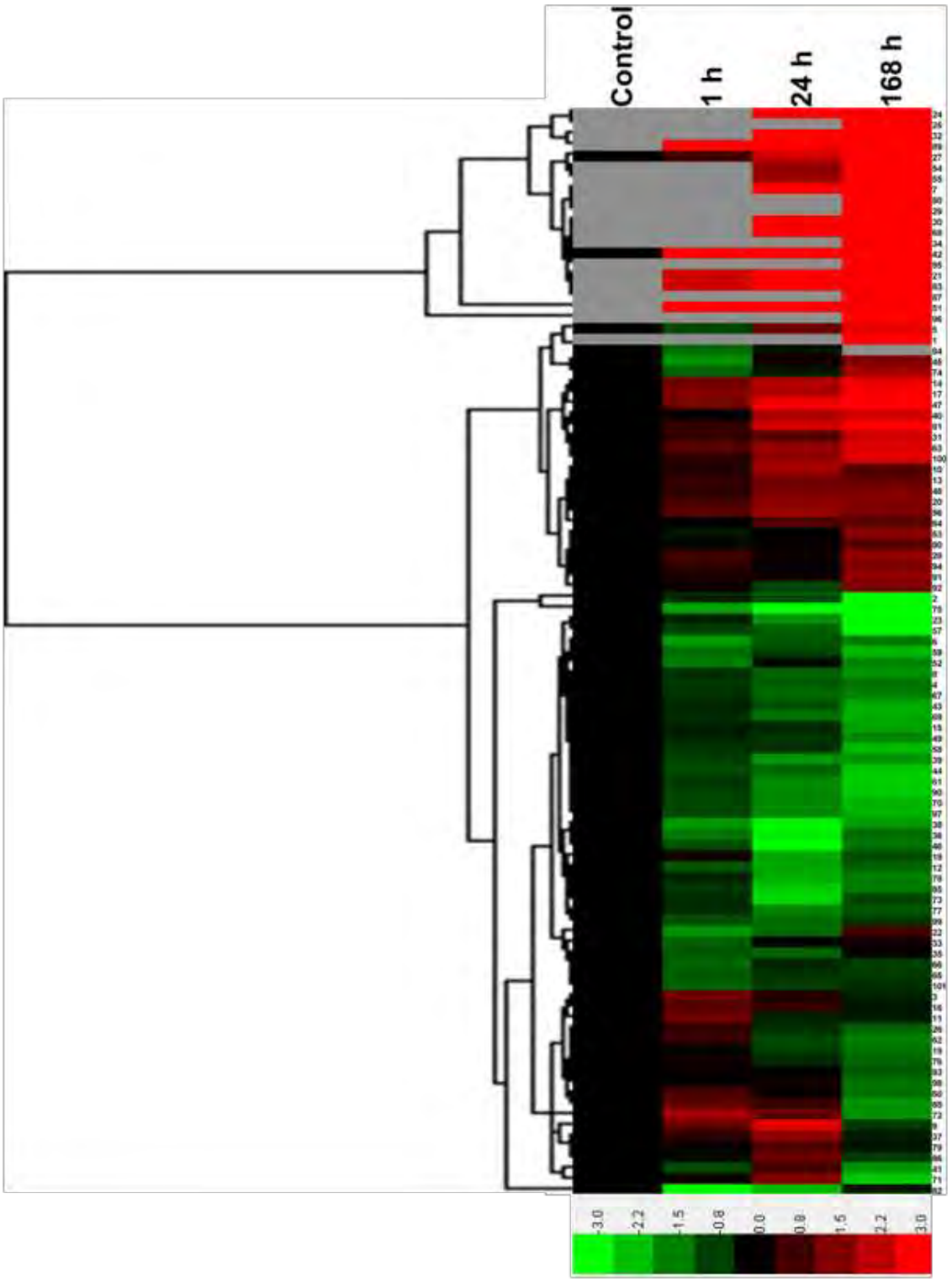

Figure 5.4.7. Hierarchical clustering of differentially accumulated amaranth root protein spots in response to salt stress. Columns represent the treatments and rows display the abundance of the protein spots as derived from induction factors obtained from normalized spot volumes. Increasing and decreasing protein spots are indicated in red and green respectively based on a relative scale (-3 to +3). Protein spots not detected at any of the employed conditions are indicated in gray.

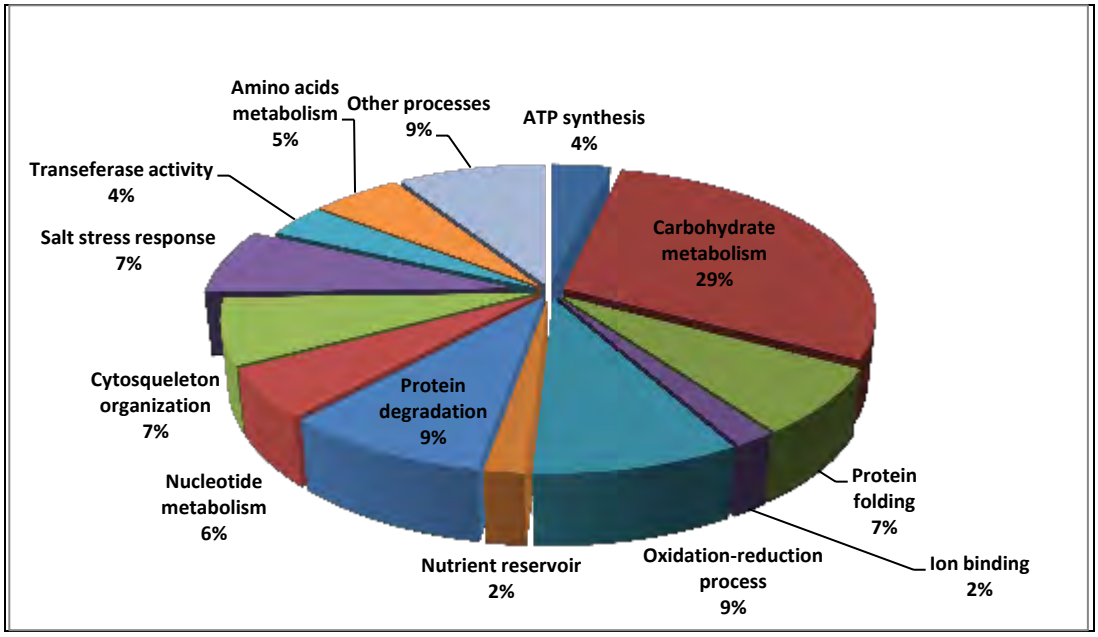

Figure 5.4.8. Classification of the identified proteins, the pie chart shows the distribution of the salt-stress responsive proteins into their biological process in percentage according to Gene Ontology (http://www.geneontology.org/).

5.4.3.4 Pending issues

Analysis with Mascot search engine of LC-MS/MS data sets of differentially accumulated protein spots identified in response to salt stress in amaranth roots against a private database generated from ESTs data obtained by our group by suppression subtractive hybridization approach in amaranths subjected to abiotic stress conditions plus the data obtained by the first large transcriptomic analysis of grain amaranth.

5.4.4 References

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5.5 Proteomic analysis of differentially accumulated proteins during ripening and in response to 1-MCP in papaya fruit.

Although this was not the focus of the thesis, in addition to the proteomic analysis of amaranth under abiotic stress an analysis of the ripening process in papaya fruits using the proteomic approach was also carried out.

The inhibitor of ethylene 1-MCP (1-methylcyclopropene), a non-toxic agent for humans and environment has been employed to delay ripening and minimize the sudden softening in papaya fruit. Physiological and biochemical modifications induced by 1-MCP are not well understood. In collaboration with a scientific group from the University of Colima, in this case proteomics was used as an approach to understand the molecular physiology of papaya fruit ripening and to analyze the physiological and biochemical modifications induced by 1-MCP.

As result 1 article and 1 book chapter were written. The article has been published and it can be found in Annex 3. The mentioned book chapter has been recently accepted for publication and can be found in Annex 4.

6. Conclusions

In the present thesis the 2-DE gel coupled with LC-MS/MS approach employed to analyze the amaranth response to abiotic stress conditions suggests that amaranth‟s response to drought stress involves a coordinated response that includes osmolyte accumulation and the activation of stress-related genes needed for the scavenging of reactive oxygen species, protein stabilization and transcriptional regulation of plant growth. In the case of salt stress the analysis of LC-MS/MS data sets against public databases allowed the identification of only 50% of the differentially accumulated protein spots, the analysis with Mascot search engine of such spots against a private database generated from ESTs data obtained by our group by suppression subtractive hybridization approach during the last years plus the data obtained by the first large transcriptomic analysis of grain amaranth will help us to increase the number of identified protein spots.

Differentially accumulated proteins in response to stress may be considered associated to abiotic stress tolerance. However, functional characterization of these proteins in order to demonstrate its importance in plant response to abiotic stress is needed. Functional complementation by heterologous expression in yeast, gene silencing, heterologous expression in insertional lines and evaluation of differential accumulation of these proteins in hybrids from cultivars with contrasting tolerance to abiotic stress that preserve the desired trait are among the alternatives to demonstrate the roles played by such proteins in plant responsive mechanisms to cope with abiotic stresses.

7. Annexes

Annex 1. Supplemental table from article titled "Water stress induces the up-regulation of DOF1 and MIF1 transcription factors and down-regulation of proteins involved in secondary metabolism in amaranth roots (*Amaranthus hypochondriacus* L.)". *Plant Biology*. 2011, Vol. 13, No. 3: pp. 472-482.

Supplemental Table Identification of drought-responsive proteins in *Amaranthus hypochondriacus* L. roots by LC/ESI-MS/MS analysis.

Spot no.[a]	Induction factor[b]	Peptides matched / Sequence coverage	Mascot Score [c]	Protein name	Accession no.[d]	Peptides	Exp.Mass (kDa) / pI	Theor.Mass (kDa) / pI	Source
1 *	1.35	2 / 11%	109	Chaperonin 21 precursor	gi|7331143	(K)YAGTEVEFDGSKHLILK(E) z (K)TAGGLLLTEAAK(E)	22.6 / 5.43	26.5 / 6.85	*L. esculentum*
2 *	0.39	8 / 35%	401	S-adenosylmethionine synthetase 2	gi|127046	(K)TNLVMVFGEITTK(A) Ox (M5) (K)VLVYIEQQSPDIAQGVHGHLTK(R) (K)RPEDIGAGDQGHMFGYATDETPELMPLSHVLATK(L) Ox (M13) (R)VHTVLISTQHDETVTNDEIAADLK(E) (K)EHVIKPVIPEK(Y) (R)FVIGGPHGDAGLTGR(K) (K)SIVASGLAR(R) (K)TAAYGHFGR(E)	43.4 / 5.66	43.2 /5.57	*Dianthus caryophyllus*

3 *	0.49	15 / 53%	506	S-adenosylmethionine synthetase	gi|71000465	(K)TNMVMVFGEITTK(A) Ox (M3,M5) (R)SIGFTSDDVGLDADK(C) (K)VLVNIEQQSPDIAQGVHGHLTK(R) (K)RPEEIGAGDQGHMFGYATDETPELMPLSHVLATK(L) Ox (M25) (K)EHVIKPVIPEK(Y) (K)TIFHLNPSGR(F) (R)FVIGGPHGDAGLTGR(K) (K)IIIDTYGGWGAHGGGAFSGK(D) (K)SIVANGLAR(R) (R)RAIVQVSYAIGVPEPLSVFVDTYGTGK(I) (L)SVFVDTYGTGK(I) (K)IPDKEILK(I) (R)FQKTAAYGHFGRDDPDFTWEVVKPLK(W) (K)TAAYGHFGR(D) (R)DDPDFTWEVVKPLK(W)	43.1 / 5.97	42.9/ 5.60	*Beta vulgaris*
4 *	4.68	1 / 10%	45	Glycine-rich RNA-binding protein 1	gi|6911142	(R)GSGGGGGGGGFRGPR(R)	15.4 / 6.54	14.1/8.71	*Catharanthus roseus*
5 *	2.10	1 / 9%	52	Glycine-rich RNA-binding protein 2	gi|6911146	(R)GSGGGGGGGGFRGPR(R)	18.8 / 6.33	16.2/7.82	*Catharanthus roseus*
6 *	2.79	3 / 21%	122	Cytosolic class I HSP 2B	gi|37704425	(K)VDLPGIK(K) (R)VLQISGER(S) (K)AAMENGVLTVTVPK(M)	16.8 / 5.65	15.3/5.82	*Nicotiana tabacum*
7 *	1.81	3 / 21%	93	17.5 kDa Class I HSP	gi|38639431	(R)VLQISGER(H) (R)LPENVKMDQVK(A) (K)ASMENGVLTVTVPK(A)	16.2 / 5.64	17.5/5.31	*Carica papaya*
8 *	2.40	2 / 9%	95	Low Molecular Weight HSP	gi|159138945	(K)ADLPGVK(K) (R)VLQISGER(H)	19.6 / 6.04	18.3/6.34	*Gossypium hirsutum*
10 *	1.31	4 / 24%	138	Small Heat Shock Protein	gi|16930753	(K)ETPEAHVFK(A) (R)VLQISGER(T) (R)FRLPENAK(V) (K)ASLENGVLTVTVPK(E)	17.5 / 5.47	17.9/5.82	*Retama raetam*
11 *	0.66	1 / 3%	48	Isoflavone reductase-like protein	gi|18410820	(K)ILVIGGTGYIGK(F)	32.5 / 5.46	33.7/5.66	*Arabidopsis thaliana*
12 *	0.73	4 / 16%	92	Adenosine kinase 1	gi|15232763	(K)LNNAILAEDK(H) (K)FNVEYIAGGATQN(S) (K)KPENWALVEK(A) (K)LVDTNGAGDAFVGGFLSQLVHGK(G)	38.5 / 5.11	37.8/5.29	*Arabidopsis thaliana*

13 *	0.60	3 / 9%	119	Caffeic acid 3-O-methyltransferase	gi|3176967	(K)NPDAATMLDR(M) Ox (M7) (R)TVFHVDAIMLAHNPGGK(E) (K)VMEFLK(Q)	36.8 / 6.14	38.6/ 5.70	*M. crystallinum*
16 *	2.0	2 / 16%	50	Heat Shock Protein 17.6 kDa	gi|15218934	(R)LPENVKMDQVK(A) (K)ASMENGVLTVTVPK(V)	16.8 / 6.11	17.7/6.85	*Arabidopsis thaliana*
19 *	1.33	1 / 8%	56	Superoxide dismutase [Cu-Zn]	gi|3334334	(R)AVVVHADPDDLGR(G)	15.6 / 5.41	15.2/5.46	*M. crystallinum*
20 *	1.45	6 / 38%	153	Nucleoside diphosphate kinase 1	gi|400404	(K)HYADLSAKPF(F)	12.6 / 6.55	16.3/6.42	*Spinacia oleracea*
						(K)GVVATGRKLIGATNPLASEPGTIR(G)			
						(R)KLIGATNPLASEPGTIR(G)			
						(K)LIGATNPLASEPGTIR(G)			
						(R)GDFAIDIGR(N)			
						(R)NVIHGSDAVDSATK(E)			

[a]The numbers corresponds to spots in the 2-DE gel showed in figure 2A. ,

[b]Percent volume of spot in stress condition / percent volume in control condition.

[c]Scores > 44 indicate identity or extensive homology (P <0.05)

[d]Accession numbers are from NCBI Entrez database

*The treatment effect is significant, P < 0.05. HSP, Heath Shock Protein.

Annex 2. Article "Proteomic analysis of differentially accumulated proteins during ripening and in response to 1-MCP in papaya fruit". *Journal of Proteomics*. 2012, Vol. 75, No. 7: pp. 2160-2169.

Supplemental material can be found in Annex 3.

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Proteomic analysis of differentially accumulated proteins during ripening and in response to 1-MCP in papaya fruit

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ABSTRACT

Papaya (*Carica papaya* L.) is a climacteric fruit susceptible to postharvest losses due to the ethylene-induced ripening. The inhibitor of ethylene action, 1-methylcyclopropene (1-MCP), has been used worldwide as a safe postharvest non-toxic agent, but the physiological and biochemical modifications induced by 1-MCP are not well understood. Using the 2-DE analysis, we report the changes in the protein profiles after 6 and 18 days of postharvest and the effect of the effect of 1-MCP treatment on fruits. Twenty seven protein spots showing differences in abundance during ripening were successfully identified by nano-LC-ESI/MS/MS. Some spots corresponded to the cell wall degrading enzymes related to fruit ripening; others were involved in oxidative damage protection, protein folding, and cell growth and survival that were induced by 1-MCP. This is the first proteomic report analyzing the effect of 1-MCP in papaya ripening. The present data will help to shed light on papaya fruit ripening process.

1. Introduction

Papaya (*Carica papaya* L.) is a tropical fruit widely cultivated and consumed. The fruit is rich in vitamins A and C, folate, niacin, thiamine, riboflavin, iron, calcium, and fiber. Papaya has reached the first place rank on nutritional scores among 38 common fruits [1]. The fruit, stems, leaves, seeds, and roots of papaya are used in a wide range of medical applications [2–4], as well as, for production of papain and chymopapain: valuable proteolytic enzymes used to tenderize meat [4].

Mexico has become the leading exporter of papaya with "Maradol" being an important Mexican cultivar grown throughout Central Mexico [5]. The fruit is green when unripe and turns yellow to orange with a few freckles when ripe; it has a shape like an elongated melon weighing between 800 and 2000 g. The edible mesocarp is sweet, soft and juicy with salmon pink or red flesh that has a slightly perfumed fruity flavor [6].

Papaya softening is a major factor that determines fruit quality and marketing [7,8]. Fruit ripening in papaya, as climacteric fruit, involves a complex series of physiological and

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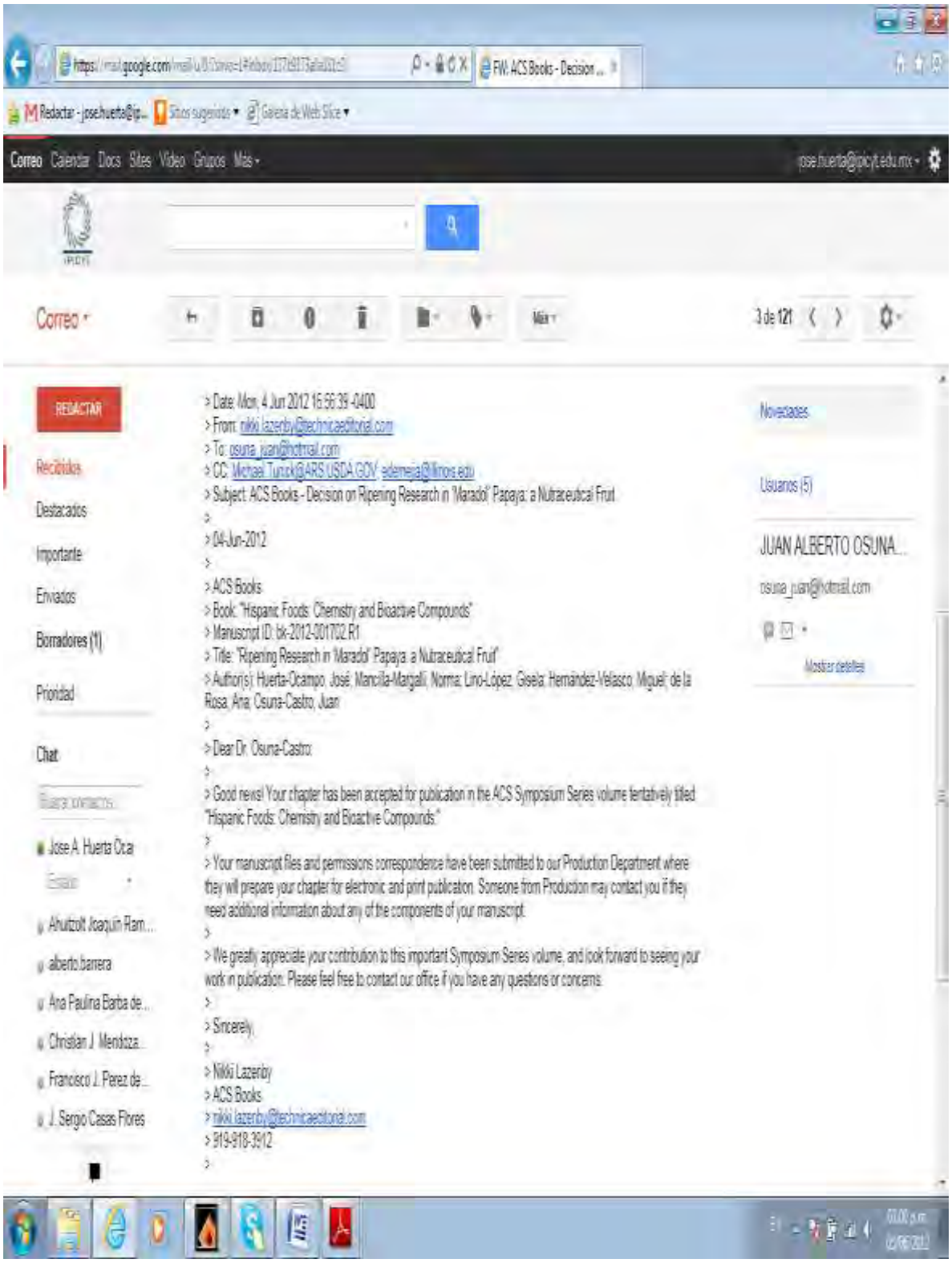
Annex 4. Book chapter "Ripening Research in „Maradol" Papaya: a Nutraceutical Fruit". In: "Hispanic Foods: Chemistry and Bioactive Compounds" ACS Books <u>Accepted/In Press</u>.



Ripening Research in 'Maradol' Papaya: a Nutraceutical Fruit

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Ripening Research in 'Maradol' Papaya: a Nutraceutical Fruit

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Papaya is among twenty top fleshy fruit produced worldwide and ranked first on nutritional scores among 38 common fruits. The fruit is mainly consumed fresh but it is also used in elaboration of drinks, jams, and as a dried and crystallized fruit candy. Several therapeutic uses have been claimed for papaya fruit. However, as climacteric fruit, it is linked to a dramatic increase in respiration and ethylene production and being susceptible to postharvest losses due to the ethylene-induced overripening and excessive softening. The inhibitor of ethylene action, 1-MCP, has been used worldwide as a safe chemical to control fruit posthaverst life