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Running Title: Differentially expressed genes in amaranth leaves under Ca^{2+} stress

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Identification of calcium stress-induced genes in amaranth leaves through suppression subtractive hybridization

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Summary

Calcium (Ca^{2+}) is a critical ion for the growth and development of plants and plays an important role in signal transduction pathways in response to biotic and abiotic stresses. We investigated the Ca^{2+} stress responsive-genes in amaranth leaves by using the suppression subtractive hybridization (SSH) technique. Screening of the SSH libraries generated 420 up-regulated transcripts and 199 down-regulated transcripts. The differentially expressed transcripts were associated with stress response, transcription factors, gene regulation, signal transduction, and unknown function. Selected genes were used to study their differential regulation by RT-PCR. Among the up-regulated transcripts, a fragment containing the motif of C3HC4-type RING-Zinc family was further characterized. The phylogenetic tree showed that the ORF of amaranth Zinc Finger protein (*Ah*Znf) has a closer relationship with its ortholog from *Ricinus communis* and is distantly related to the *Arabidopsis thaliana* C3HC4-type ortholog. We have identified a novel putative zinc finger protein among other novel proteins such as the wall associated kinase (WAK), Slingshot phosphatase (SSH), Rhomboid protease, and vacuolar cation/proton exchange (CAX1) involved in response to Ca^{2+} stress. Further characterization of the unknown genes in amaranth could provide new insights on the plant Ca^{2+} signal pathways.

Keywords:
Amaranthus hypochondriacus L.; Calcium stress; Subtractive cDNA libraries; Zinc finger proteins

Abbreviations:

*Ah*Znf, *Amaranthus hypochondriacus* Zinc Finger protein
cDNA, Complementary DNA
EST, Expressed Sequence Tag
G.h.fbr-sw, *Gossypium hirsutum* fibre-secondary wall
ORF, Open Reading Frame
PCR, Polymerase Chain Reaction
RACE, Rapid Amplification of cDNA Ends
ROS, Reactive Oxygen Species
RT-PCR, Reverse Transcription-Polymerase Chain Reaction
SSH, Suppression Subtractive Hybridization
USP, Universal Stress Protein
WAK, Wall Associated Kinase

Introduction

Plants are exposed to several environmental stresses such as drought, salinity and extreme temperatures. The survival and reproduction of plants under these adverse environments relies on stress perception and signal transduction to switch on adaptive responses (Chinnusamy et al., 2004). However these stress sensors are not well known and most of the signaling intermediates have not been identified to date. In addition, there is little information regarding cross-talk between different stress signal transduction pathways in plants, some of which are specific, but others may cross-talk at various steps (Agarwal and Zhu, 2005; Chinnusamy et al., 2004). Stress signals result in cytosolic Ca^{2+} perturbations, which are unique and precisely decoded by Ca^{2+}-sensing proteins to relay the signaling cascade (Mahajan et al., 2008; Tuteja and Sopory 2008). Ca^{2+} serves as a secondary messenger, and has been described as a major point of signaling cross-talk because it can be elicited by numerous abiotic and biotic stress cues (Agarwal and Zhu, 2005). Molecular, genetic and biochemical studies have demonstrated that the Salt-Overly-Sensitive (SOS) is a novel signal transduction pathway involved in the perception and transduction of salt stress signals in plants. This pathway also emphasizes the significance of calcium (Ca^{2+}) signal in reinstating cellular ion homeostasis (Chinnusamy et al., 2004). The mechanisms giving rise to the changes in cytosolic Ca^{2+} levels, and the Ca^{2+}-responsive genes/proteins are just beginning to be unraveled.

Several studies related to biotic and abiotic stresses have been reported using model plants such as *Arabidopsis thaliana*, rice, maize, and wheat (Chen et al., 2002; Li et al., 2008; Zheng et al., 2004). Stress-tolerant species may have specific

response mechanisms, that could be the key to the natural stress adaptation phenomena; hence some studies have focused on *Thellungiella halophila* (http://thellungiella.org/), among others halophyte (Sahu and Shaw, 2009; Wang et al., 2010). Although these halophytes exhibit tolerance to several stresses, they do not represent crops used as food and/or feed resources (Umezawa et al., 2006).

Amaranth is a dicotyledonous plant with C_4 metabolism that produces seeds with high nutritive and nutraceutical properties (Barba de la Rosa et al., 2009). Amaranth leaves contain, on dry weight, high levels of protein (27.8 to 48.6%), unsaturated oil (45% linoleic acid), fiber (11 to 23%), vitamins A and C, and minerals such as iron, magnesium, potassium and calcium. Calcium in amaranth leaves was reported to be as high as 210 mg/100 g (NAS, 1984). In addition, amaranth grows in semi-arid environments and soils containing high salt concentrations of around 60 to 250 mM NaCl (Huerta-Ocampo et al., 2009; Omami et al., 2006). Until date, identification of genes related to stress-responsive in amaranth is lacking. The aim of this work was directed towards a better understanding of the effect of Ca^{2+} on the growth and calcium-responsive genes in *Amaranthus hypochondriacus*. The differential expression of transcripts in amaranth leaves was analyzed using SSH libraries and the Ca^{2+} responsive-genes were identified by their putative functions. Here we report some of the amaranth decoders of the Ca^{2+} signals in amaranth. In addition to this, we have cloned and characterized the full length cDNA of a transcript containing the RING Zinc-Finger motif.

Materials and methods

Plant materials, growth conditions and stress treatment

Amaranth seeds (*Amaranthus hypochondriacus* L.) *cv* Nutrisol were germinated on sterile soil Special Blend (SunGro Horticulture, Bellevue, WA). Seedlings with three to four true leaves were transplanted into plastic pots containing sterile soil. Experiments were carried out in a growth chamber illuminated by fluorescent lamps (300 $\mu mol\ m^{-2}\ s^{-1}$), and operated by periods of 12h/12h (light/darkness) at 25 °C (Omami et al., 2006). Two-weeks-old seedlings were watering every third day with 100 mL of 0 to 100 mM $CaCl_2$ solution at pH 6. On day 45, the leaves were collected, snap frozen in liquid nitrogen and stored at -80°C. Three independent experiments of each treatment were analyzed.

RNA extraction and generation of subtractive cDNA libraries

Leaf samples from individual plants were pooled and homogenized with a mortar and pestle in the presence of liquid nitrogen. Ground leaves tissue was transferred into Eppendorf tubes and total RNA was extracted using RNeasy MiniKit system (Qiagen, GMBH, Hilden Germany) according to manufacturer´s instructions. cDNA was synthesized from total RNA (1.5 µg) using the SMART cDNA Synthesis Kit (Clontech, Palo Alto, CA), and purified through Chroma-Spin-1000 columns (Clontech). SSH-libraries were constructed using the PCR-Select cDNA subtractive kit (Clontech). Forward subtraction involved the isolation of gene fragments which showed increased expression level following the treatment. Leaves from plants stressed with 50 mM $CaCl_2$ were the “tester”, while the control samples (20 mM $CaCl_2$) were the “driver”. Reverse subtraction involved the isolation of gene

1 fragments that showed a decrease in expression following the treatment. This was
2 carried out with the control sample as 'tester' and the treated sample as the 'driver'.
3 The subtracted cDNAs were cloned into the pGEM®-T Easy Vector (Promega
4 BioSciences, San Luis Obispo, CA) and *Escherichia coli* TOP 10F´ strain
5 chemically competent cells were transformed to generate the forward and reverse
6 subtractive cDNA libraries. About 600 colonies were picked and grown in LB
7 medium with ampicillin (100 μg mL^{-1}) and the presence of the insert was confirmed
8 by restriction analysis.

9

10 **DNA sequencing and analysis**

11 A total of 250 differentially expressed clones were selected for sequencing
12 (MCLab, Molecular Cloning Laboratory, San Francisco, CA, USA). The DNA
13 sequences were edited to remove the vector sequences and then searched
14 against the GenBank database at NCBI
15 (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), the TIGR *Arabidopsis thaliana*
16 (http://www.tigr.org/tdb/e2k1/ath1/), and TIGR Rice Genome Annotation Project-
17 Web (http://tigrblast.tigr.org/euk-blast/index.cgi?project=osa1). Sequence
18 comparisons were made using the BLAST algorithm.

19

20 **Confirmation of expression profile by semi-quantitative RT-PCR analysis**

21 The expression patterns of selected clones were further confirmed by semi-
22 quantitative RT-PCR using a gene-specific primer pair based on the nucleotide
23 sequence of each clone (Table 1). Total RNA (1.5 μg) was reverse transcribed
24 using the Super Script™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and

1 the SMART^{TM} PCR cDNA synthesis kit (Clontech). The conditions of the PCR
2 reaction were: denaturing at 94°C for 4 min; 27-30 cycles of 1 min at 94°C, 45 s at
3 54-62°C, 1 min at 72°C, and a final extension of 7 min at 72°C. The RT-PCR
4 products were separated on a 1.2% agarose gel and stained with ethidium
5 bromide. The expression level of genes in each sample was analyzed and
6 calculated based on the intensity of the band by Quantity One^{TM} v4.5.0 (Bio-Rad,
7 Hercules, CA). β-a*ctin* gen was used as an internal standard. Each reaction was
8 performed in triplicates.
9
10 **Isolation of the full-length cDNA of the putative amaranth Zinc-Finger**
11 **transcripts**
12 The 5´and 3´ rapid amplification of the cDNA ends (RACE) were conducted to
13 obtain the full-length cDNA of the amaranth zinc finger (*AhZnf*) transcripts. Total
14 RNA from amaranth leaves was obtained as described before. The 5´-RACE
15 fragment was synthesized using 10 pmol of SMARTII A (SMART PCR cDNA
16 synthesis kit, Clontech) and 10 pmol of ZF-R primer. The 3´-RACE fragment was
17 synthesized using 10 pmol of ZF-F primer and 3´SMART CDS primer IIA (SMART
18 SMART™ RACE cDNA Amplification Kit, Clontech). The ZF-F and ZF-R specific
19 primers (Table 1) were designed based on the *AhZnf* transcript sequence obtained
20 from our SSH library. The PCR conditions were: 1 min at 94°C, 30 cycles of 94ºC/1
21 min, 60ºC/1 min, 72ºC/2 min, and final extension step of 10 min at 72°C. The
22 amplified fragments were cloned into pCR4-TOPO (Invitrogen) and transformed
23 into *E. coli* TOP 10F´ competent cells. The 5´ and 3´ RACE cDNA fragments were
24 sequenced, and the full-length ORF of *AhZnf* cDNA was analyzed.

Results

Morphological response of amaranth to Ca^{2+} stress

We determined that the $CaCl_2$ concentration for normal growth of amaranth ranged from 10 to 20 mM. Below 10 mM and above 20 mM, plant growth was stunted and resulted in the development of fewer and small leaves (data not shown). At 50 mM the leaves presented damage symptoms (dark spots). Based on these observations, plants watered with 20 mM $CaCl_2$ served as the control plants, and the ones watered with 50 mM $CaCl_2$ as Ca^{2+} stressed plants.

Identification of differentially expressed transcripts by Suppression Subtractive Hybridization (SSH)

The SSH libraries generated 420 up-regulated transcripts and 199 down-regulated transcripts. The EST sequences of up-regulated genes were grouped into 6 functional categories including stress response, signal transduction, transcription factors, gene regulation, and genes with unknown or hypothetical function (Fig. 1A). With respect to the down-regulated genes, the ESTs were grouped into 10 functional categories including response to stress, signal transduction, transcription factors, metal-binding, metabolism, protein folding, photosynthesis, defense against pathogen, and unknown function or hypothetical (Fig. 1B). From those, 48 unigenes were up-regulated and 46 unigenes were down-regulated when comparing control plants (20 mM $CaCl_2$) against 50 mM $CaCl_2$-stressed plants (Table 2 and 3). Around 30% of up and down-regulated genes corresponded to genes with unknown or without homology in the databases. This group of genes

could be a source of novel proteins important in amaranth Ca^{2+} signaling, further work is in progress for their characterization.

Some of the up-regulated transcripts found in amaranth were green ripe-like 1(*grl*1), LRR, and G.h.fbr-sw. GRL1 has been identified as a member of the family of reversion to ethylene sensitivity1 (*rte*1), both genes have the capacity to alter ethylene signaling (Resnick et al., 2006). Leucine-rich repeat (LRR) is a part of a gene family that takes part in developmental signaling and gene regulation (Forsthoefel et al., 2010). The G.h.fbr-sw sequences that were expressed in cotton fibre, some had matches in the GenBank to proteins that may be represented by rare transcripts and/or those that have pivotal roles in modulating development, including transcription factors, protein kinases, hormone-responsive proteins and glysosyltransferases. Other group of G.h.fbr-sw genes did not have any matches, representing proteins with novel functions in the highly specialized secondary wall phase of fibre development (Haigler et al., 2005). Another up-regulated transcript in amaranth was the RNA-directed DNA polymerase; this gene has been reported to be a key regulator during *Medicago truncatula* regeneration (Imin et al., 2008).

Gene expression in response to Ca^{2+} stress

Selected genes up-regulated by Ca^{2+}-stress in amaranth leaves were examined by RT-PCR (Fig. 2). Those genes were: S-adenosyl-methionine synthase (SAMS), transcription factor (Znf), calmodulin (CaM), metallothionein (MT2A), and wall associated kinase (WAK). Other transcripts such as USP, Green ripe-like 1 (GRL1), CaM, the vacuolar cation/proton exchanger (CaX), and MT2A, were analyzed by Northern blot (data not shown). The RT-PCR and Northern Blot results

correlated with the up or down-regulation observed in the transcript analysis performed using the SSH approach.

Characterization of the full-length sequence of the putative amaranth Znf

The nucleotide and the protein sequence of the putative *Ah*Znf (Acc. No. HM77322) is shown in Fig. 3. The full-length cDNA of *Ah*Znf contained an ORF of 518 bp and was predicted to encode a polypeptide of 173 amino acids. Using the SMART tool and the TMHMM2 algorithm (Letunic et al., 2008) a transmembrane domain was predicted from residues 25 to 47. The *Ah*Znf domain (residues 101 to 142, underlined) was classified as a RING finger C3HC4 type. The RING fingers play a key role in the ubiquitination pathway and are involved in mediating protein-protein interactions. The full *Ah*Znf ORF was compared with other zinc finger proteins reported in the NCBI databases and multiple sequence alignment was generated using the ClustalW (http://www.ebi.ac.uk/Tools/clustalw/). AhZNF showed the classical C-X2-C-X(9-39)-X-X(1-3)-H-X(2-3)-C-X2-C-X(-448)-C-X2-C conserved motif of RING Znf proteins (Fig. 4), however low homology among different species was observed. A phylogenetic tree was constructed with the RING sequences and several other Znf proteins available in the database (Fig. 5). No similarity was observed between *Ah*Znf and its orthologs from *Arabidopsis thaliana*, *Capsicum annuum* or *Zea mays.* The closest similarity found was with a one Ring-H2 finger protein from *Ricinus communis*, a plant that is naturally resistant to water stress (Zeng et al., 2009).

Discussion

Calcium is absorbed passively via the fine roots, and is transported and stored in vacuoles. Amaranth showed normal growth and development when treated with 10 to 20 mM of $CaCl_2$. This is a higher concentration than that reported (4 mM) for maize normal growth (Guerra-Peraza et al., 2009).

General salt stress-responsive genes

We have found that one metallothionein-2A (*Ah*MT2A), corresponding to the MT type expressed in leaves (Guo et al., 2003), was up-regulated in response to Ca^{2+} stress. MTs are proteins involved in the metabolism of metals such as Cu, Cd, Zn, and play a key role in Zn homeostasis (Cobbet and Goldsbrough, 2002). Likewise, the S-Adenosyl-L-Methionine Synthase (SAMS) was also found up-regulated, but only in 50 mM $CaCl_2$ treated plants. SAMS provides the methylene group used in the biogenesis of cyclopropane and as a source of alkyl and amino groups used for the biogenesis of polyamines and biotin. Polyamines are considered to be essential for life; they are involved in regulation of gene expression, translation, cell proliferation, membrane stabilization, among other processes (Kusano et al., 2008). They also have direct effects on several ion channels and receptors, resulting in the regulation of Ca^{2+}, Na^+, and K^+ homeostasis. Polyamines interact with voltage-activated Ca^{2+} channels and cyclic nucleotide-gated channels. Therefore, changes in intracellular or extracellular levels of polyamines could alter K^+, Na^+ or Ca^{2+} trafficking (Chan et al. 2003; Kusano et al., 2008).

We found one homolog of *At*UspA to be up-regulated in response to Ca^{2+} stress. The universal protein A (USP), a conserved group of protein superfamily, was

originally identified in *Escherichia coli*; however, the biological and biochemical functions of these proteins are unknown. Genetic evidence has shown that *UspA* mediates the survival of cells starved for a wide variety of nutrients, toxic chemicals, osmotic stress, light damage, and heat stress (Nystrom and Neidhardt, 1992). In tomatoes, the *UspA* domains form a part of the well studied signaling pathway that mediates resistance to bacterial speck disease (Sessa and Martin, 2000).

At least three different SLT1 genes (Na^+ and Li^+ Tolerant 1) were found, of which one was up-regulated and two were down-regulated. The functions of SLT genes are not well understood, but it has been suggested that they may play a role in signaling regulatory molecules that mediate salt tolerance by modulating Na^+ homeostasis (Matsumoto et al., 2001).

Among the transcription factors that were up-regulated by Ca^{2+} stress, we found the CID9, and 23S pseudouridine synthase (Table 2). CID9 plays an important role in the regulation of translation and the control of mRNA stability in eukaryotes. It has also been found to be over-expressed in response to drought and salt stress (Ma et al., 2006). The 23S pseudouridine synthase, is a gene regulator for the RNA pseudouridylation. Uridine has the effect of enhancing local RNA stacking in both single-stranded and duplex regions, resulting in increased conformational stability (Liang et al., 2009). In contrast, the glycine-rich RNA-Binding protein (GRP) was found to be down-regulated by Ca^{2+} stress (Table 3). The knowledge regarding the functional roles of GRPs is limited; studies indicated that they function as RNA chaperones during the cold adaptation process in monocotyledonous, as well as in dicotyledonous plants (Kim et al., 2010).

Ca^{2+} signaling

We have found in amaranth leaves the up-regulation of one CaM and one CAX (vacuolar H^{+}/Ca^{2+} antiporter) transcripts in response to Ca^{2+} stress. Intracellular Ca^{2+} signals are sensed by calmodulins (CaMs), and calcineurin B-like protein (CBL-4) known as the SOS3. CAX1 has been identified as an additional target of SOS2 activity, reinstating cytosolic Ca^{2+} homeostasis under salt stress (Mahajan et al. 2008). The biological roles of CAX transporters in cell growth and in response to environmental stresses are just emerging; these are proposed as potential genes for increasing abiotic stress tolerance in plants (Zhao et al., 2008).

When free Ca^{2+} is increased in the cytosol, complexes of Ca^{2+}-binding proteins trigger the release of phosphatidylinositol phosphates (Kato et al., 2010). In this work, we have found the up-regulation of phosphoinositide binding protein (SSH1). SSH1 belongs to the Slingshot family of protein phosphatases with no clear function (Wang et al., 2005). The loss of SSH function in *Drosophila* leads to disorganized epidermal cell morphogenesis, including malformation of bristles and wing hairs (Nishita et al., 2004). In mammals, SSH1 changes during the cell division cycle in cultured cells, insulin induced the accumulation of SSH1 and active downstream effectors of PI3K, together with phospatidylinositol onto membrane protrusion (Nishita et al., 2004; Niwa et al., 2002). Proteins anchored in the plasma membrane are bound with phosphatidylinositol phosphates, and liberation of phosphoinositides triggers an intricate network of enzymes and phospholipid messengers that are crucial regulators of most, if not all, cellular processes (Bunney and Katan, 2010).

Interactions between neighboring cells play a vital role in the control of cell expansion; while cells expansion is controlled by cell wall architecture, cytoskeleton, and wall membrane interactions. It has been reported that SOS5 helps in the maintenance of cell wall integrity and architecture, SOS5 shows similarity with cell to cell adhesion proteins (Mahajan et al., 2008; Shi et al., 2003). In this work we found up-regulation of one receptor-like kinase, a wall associated kinase (WAK). WAK have been associated with the communication between plant cell wall and cytoplasm. A novel WAK from rice (*Os*WAK) was up-regulated under biotic or mechanical stress (Li et al., 2009). In *A. thaliana*, WAK plays important roles in cell expansion, stress tolerance, and resistance to pathogenic bacteria (Hou et al., 2005). Also one Hyp-Rich glycoprotein (HRGP) was found to be up-regulated; SOS5 is predicted to contain at its N-terminal a signal peptide for its plasma membrane localization and signal sequence at C-terminal for the addition of GPI (glycosylphosphatidylinositol) lipid anchor. HRGPs represent a family of proteins that self-assemble and that are key protein constituents of the cell wall (Lee et al., 2007). They are involved in plant defense response to pathogen attack, and in wall strengthening by formation of intra- and inter-molecular cross-links (Deepak et al., 2007). Further work is ongoing to characterize this HRGP protein and its relation with SOS pathway.

Organelle-related transcripts

The Rhomboid and DnaJ genes were found to be amongst the down-regulated transcripts. The Rhomboid protein is an ancient conserved family of intramembrane serine proteases that catalyze the cleavage of transmembrane

segments within the lipid membrane to achieve a wide range of biological functions (Sherrat et al., 2009). Recent reports have indicated that a subfamily of rhomboids are gatekeepers of mitochondrial dynamics and apoptosis, thus introducing a new paradigm of how the mitochondria uses these unique type of proteases to direct the stress responses, to signal to the nucleus, and other key mitochondrial activities in health and disease (Hill and Pellegrini, 2010). In plants little is known about this type of proteins and further studies should be done.

At least 26 DnaJ Heat shock proteins (HSP) are predicted in *Arabidopsis*, most of which have a chloroplast targeting signal, but only a few of them have been characterized. Some data obtained with DNA microarray analysis demonstrated that the lack of one of the DnaJ proteins triggered a global stress response in plants and therefore confered more tolerance to oxidative stress induced by high light or methyl viologen treatments (Chen et al., 2010).

It was also found the that down-regulation of genes coding for proteins of photosynthetic system such as the photosynthetic oxygen-evolving complex (OEC), involved in the redox reactions leading to water oxidation, chlorophyll A-B binding protein/LHCI type I (CAB), oxygen-evolving enhancer protein (PsbP-1), an extrinsic subunit of photosystem II that plays important roles in the water splitting reaction, and photosystem I subunit XI precursor. It has been reported that a decreases the fixation of CO_2 and light, resulting in the attenuation of photosynthesis and therefore the plant growth is stunted (Gao et al., 2008).

Characterization of Znf transcription factor in response to Ca^{2+} stress

There are several reports demonstrating that the expression of Znf proteins from different sources enhance plant growth or improve drought and salt stress tolerance in Arabidopsis (Park et al., 2010; Saad et al., 2010; Tian et al., 2010). We have found the up-regulation of a putative RING-Znf protein in amaranth during Ca^{2+} stress. Up-regulation of Zinc-finger proteins under calcium stress has been reported in fungi (Shumacher et al., 2008), but this is the first report of the characterization of one Znf protein in plants in response to Ca^{2+} stress. Phylogenetic relationships have suggested an evolutionary history of the RING finger domains (Lim et al., 2010). The Znf domain is found in many organisms including Archaea, Bacteria, and Eukarya. From our phylogenetic tree, it was observed that amaranth Znf is closely to the predicted RING from *Ricinus communis* (castor bean). The ortholog from *A. thaliana* is a distantly related to *A. hypochondriacus*. This clearly indicates that although the stress-responsive genes in amaranth seem to be similar to the responses in other plants, the structure of functional proteins could be an important factor in the adaptation to tolerance/resistance to environmental factors.

Several abiotic stresses such as salinity induce the amount of $[Ca^{2+}]$cyt, and in turn Ca^{2+} triggers the cell responses. Then, it is important to understand the Ca^{2+} signaling processes involved in perception and transduction of stress stimuli, especially in naturally tolerant plants. Our results open future studies in the characterization of novel and unknown transcripts, and their roles in the Ca^{2+} signaling pathways and the abiotic stress cross-talk.

Acknowledgments- This work was supported by the European Commission 6th Framework Programme, AMARANTH:FUTURE-FOOD, Contract No. 032263. Hugo Sergio Aguilar gives thanks to IPICyT for the Ms.Sc. fellowship. We thank the support of Fundación Produce San Luis Potosi. We also thank CONACYT-Mexico for Grants: 56787 (Laboratory for Nanoscience and Nanotechnology Research-LINAN). We thank to Dr. Sheetal Rao from Texas A&M University for manuscript review.

References

Agarwal M, Zhu J-K. Integration of abiotic stress signaling pathways. In:Matthew A. Jenks and Paul M. Hasegawa, editors. Plant abiotic stress. Blackwell Publishing Ltd, 2005. p 214-247.

Barba de la Rosa AP, Fomsgaard IS, Laursen B, Mortensen AG, Olvera-Martínez JL, Silva-Sánchez C, Mendoza-Herrera A, De León-Rodríguez A, González-Castañeda J. Amaranth (*Amaranthus hypochondriacus*) as an alternative crop for sustainable food production: Phenolic acids and flavonoids with potential impact on its nutraceutical quality. J Cereal Sci 2009;49:117-121.

Bunney TD, Katan M. PLC regulation: emerging pictures for molecular mechanisms. Trends Biochem Sci 20011;36:88-96.

Chan CWM, Schorrak LM, Smith RK, Bent A, Sussman MR. A cyclic nucleotide-gated ion channel, CNGC2, is crucial for plant development and adaptation to calcium stress. Plant Physiol 2003;132:728-731.

Chen KM, Homstrom M, Raksajit W, Suorsa M, Piippo M, Aro EM. Small chloroplast-targeted DnaJ proteins are involved in optimization of photosynthetic reactins in Arabidopsis thaliana. BMC Plant Biol 2010;10:43-58.

Chinnusamy V, Schumaker K, Zhu JK. Molecular genetic perspectives on cross-talk and specificity in abiotic stress signaling in plants. J Exp Bot 2004;55:225-236.

Cobbett C, Goldsbrough P. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Ann Rev Plant Biol 2002;53:159-182.

Deepak S, Shailasree S, Kini RK, Hause B, Shetty SH, Mithofer A. Role of hydroxyproline-rich glycoproteins in resistance of pearl millet against downy mildew pathogen *Sclerospora graminicola*. Planta 2007;26:323-333.

Forsthoefel NR, Davo TP, Vernon DM. PIRL1 and PIRL9, encoding members of a novel plant-specific family of leucine-rich repeat proteins, are essential for differentiation of microscopores into pollen. Planta 2010;232:1101-1114.

Gao C, Wang Y, Liu G, Yang C, Jiang J, Li H. Expression profiling of salinity-alkali stress responses by large-scale expressed sequence tag analysis in *Tamarix hispida*. Plant Mol Biol 2008;66:245-258.

Guerra-Peraza O, Nguyen HT, Stamp P, Leipner J. *ZmCOI6.1*, a novel, alternatively spliced maize gene, whose transcript level changes under abiotic stress. Plant Sci 2009;176:783-791.

Haigler CH, Zhang D, Wilkerson CG. Biotechnological improvement of cotton fibre maturity. Physiol Plantarum 2005;124:285-294.

Hill RB, Pellegrini L. The PARL family of mitochondrial rhomboid proteases. Semin Cell Dev Biol 2010;21:582-592.

Huerta-Ocampo JA, Briones-Cerecero EP, Mendoza-Hernández G, De León-Rodríguez A, Barba de la Rosa AP. Proteomic analysis of amaranth (*Amaranthus hypochondriacus* L.) leaves under drought stress. Int J Plant Sci 2009;170:990-998.

Imin N, GoffardN, Nizamidin M, Rolfe BG. Genome-wide transcriptional analysis of super-embryogenic *Medicago truncatula* explants cultures. BMC Plant Biol 2008;8:110.

Kato M, Nagasaki-Takeuchi N, Ide Y, Tomioka R, Maeshima M. PCaPs, possible regulators of PtdInsP signals on plasma membrane. Plant Signal Behav 2010;5:848-850.

Kim JY, Kim WY, Kwak KJ, Oh SH, Han YS, Kang H. Glycine-rich RNA-binding proteins are functionally conserved in *Arabidopsis thaliana* and *Oryza sativa* during cold adaptation process. J Exp Bot 2010;61:2317-2325.

1 Kusano T, Berberich T, Tateda C, Takahashi Y. Polyamines: essential factors for
2 growth and survival. Planta 2008;228:367-381.
3 Lee JH, Waffenschmidt S, Small L, Goodenough U. Between-species analysis of
4 short-repeat modules in cell wall and sex-related hydroxyproline-rich
5 glycoproteins of *Chlamydomonas*. Plant Physiol 2007;144:1813-1826.
6 Letunic I, Doerks I, Bork P. SMART 6: Recent updates and new developments.
7 Nucleic Acids Res 2008;37:D229-D232.
8 Li H, Zhou S-Y, Zhao W-S, Su S-Ch, Peng Y-L. A novel wall-associated receptor-
9 like protein kinase gene, *OsWAK1*, plays important roles in rice blast disease
10 resistance. Plant Mol Biol 2009;69:337-346.
11 Liang B, Zhou J, Kahen E, Terns RM, Terns MP, Hong L. Structure of a functional
12 ribonucleoprotein pseudouridine synthase bound to a substrate RNA. Nature
13 Struct Mol Biol 2009;16:740-746.
14 Lim SD, Yim WCh, Moon JCh, Kim DS, Lee BM, Jang Ch S. A gene family
15 encoding RING finger proteins in rice: their expansion, expression diversity, and
16 co-expressed genes. Plant Mol Biol 2010;72:369-380.
17 Ma S, Gong Q, Bohnert HJ. Dissecting salt stress pathway. J Exp Bot
18 2006;5:1097-1107.
19 Mahajan S, Pandey GK, Tuteja N. Calcium- and salt-stress signaling in plants:
20 Shedding light on SOS pathway. Arch Biochem Biophys 2008;15:146-158.
21 Matsumoto TK, Pardo JM, Takeda S, Bressan RA, Hasegawa PM. *Tobacco* and
22 *Arabidiopsis SLT1* mediate salt tolerance of yeast. Plant Mol Biol 2001;45:489-
23 500.
24 NAS. National Academy of Sciences, Amaranth: Modern Prospects for an Ancient
25 crop, National Academy Press, Washington, D.C. 1984.
26 Nishita M, Wang Y, Tomizawa C. Suzuki A, Niwa R, Uemura T, Mizuno K.
27 Phosphoinositide 3-kinase-mediated activation of cofilin phosphatase slingshot
28 and its role for insulin-induced membrane protrusion. J Biol Chem
29 2004;279:193-7198.

Niwa R, Negata-Ohashi K, Takeichi M, Mizuno K, Uemura T. Control of actin reorganization by slingshot, a family of phosphatases that dephosphorylate ADF/cofilin. Cell 2002;108:233-246.

Nystrom T, Neidhardt FC. Cloning, mapping and nucleotide sequencing of a gene encoding a universal stress protein in *Escherichia coli.* Mol Microbiol 1992;6:3187-3198.

Omami EN, Hammes PS, Robbertsc PJ. Differences in salinity tolerance for growth and water-use efficiency in some amaranth (*Amaranthus* spp.) genotypes. New Zeal J Crop Hort 2006;34:11-22.

Park SJ, Kwak KJ, Jung HJ, Lee HJ, Kang H. The C-terminal zinc finger domain of Arabidopsis cold shock domain proteins is important for RNA chaperone activity during cold adaptation. Phytochem 2010;71:543-547.

Resnick JS, Wen CK, Shockey JA, Chang C. Reversion to ethylene sensitivity1, a conserved gene that regulates ethylene receptor function in *Arabidopsis*. Proc Natl Acad Sci USA 2006;103:7917-7922.

Saad RB, Zouari N, Ramdhan WB, Azaza J, Meynard D, Guiderdoni E, Hassairi A. Improved drought and salt stress tolerance in transgenic tobacco overexpresssing a novel A20/AN1 zinc-finger "AlSAP" gene isolated from the halophyte grass *Aeluropus littoralis*. Plant Mol Biol 2010;72:171-190.

Sahu BB, Shaw BP. Isolation, identification and expression analysis of salt-induced genes in *Suaeda maritima*, a natural halophyte, using PCR-based suppression subtractive hybridization. BMC Plant Biol 2009;9:69-94.

Sessa G, Martin GB. Protein kinases in the plant defense response. Adv Bot Res 2000;32:379-404.

Sherrat AR, Braganza MV, Nguyen E, Ducat T, Goto NK. Insights into the effect of detergents on the full-length rhomboid protease from *Pseudomonas aeruginosa* and its cytosolic domain. Biochim Biophys Acta 2009;1788:2444-1453.

Shi H, Kim Y, Guo Y, Stevenson, B, Zhu JK. The Arabidopsis *SOS5* locus encodes a putative cell surface adhesion protein and is required for normal cell expansion. Plant Cell 2003;15:19-32.

1 Shumacher J, Larrinoa IF, Tudzynski B. Calcineurin-responsive zinc finger
2 transcription factor CRZ1 of Botrytis cinerea is required for growth, development,
3 and full virulence on beans plants. Eukaryot Cell 2008;7:584-601.
4 Tian Z-D, Zhang Y, Liu J, Xie C-H. Novel potato C2H2-type zinc finger protein
5 gene, stZFP1, which responds to biotic and abiotic stress, plays a role in salt
6 tolerance. Plant Biol 2010;12:689-697.
7 Tuteja N, Sopory SK. Chemical signaling under abiotic stress environment in
8 plants. Plant Signal Behav 2008;3:526-536.
9 Umezawa T, Fujita M, Fujita Y, Yamaguchi-Shinozaki K, Shinozaki K. Engineering
10 drought tolerance in plants: discovering and tailoring genes to unlock the future.
11 Curr Opin Biotechnol 2006;17:113-22.
12 Wang W, Wu Y, Li Y, Xie J, Zhang Z, Deng Z, Zhang Y, Yang C, Lai J, Zhang H,
13 Bao H, Tang S, Yang C, Gao P, Xia G, Guo H, Xie Q. A large insert
14 *Thellungiella halophila* BIBAC library for genomics and identification of stress
15 tolerance genes. Plant Mol Biol 2010;7291-7299.
16 Wang Y, Shibasaki F, Mizuno K. Calcium signal-induced cofilin dephosphorylation
17 is mediated by slingshot via calcineurin. J Biol Chem 2005;280:12683-12689.
18 Zeng C, Wang W, Zheng Y, chen X, Bo W, Song S, Zhang W, Peng M.
19 Conservation and divergence of micro RNAs and their functions in
20 *Euphorbiaceous* plants. Nucleic Acids Res 2009;38:981-985.
21 Zhao J, Barkla BJ, Marshall J, Pittman JK, Hirschi KD. The *Arabidopsis cax3*
22 mutants display altered salt tolerance, pH sensitivity and reduced plasma
23 membrane H^+-ATPase activity. Planta 2008;227:659-669.
24 Zheng J, Zhao J, Tao Y, Wang J, Liu Y, Fu J. Isolation and analysis of water stress
25 induced genes in maize seedlings by subtractive PCR and cDNA macroarray.
26 Plant Mol Biol 2004;55:807-23.
27

1 **Table 1.** Gene-specific primer pairs used for RT-PCR

Clone	Primer	Sequence (5´-3´)	Amplicon (bp)	Ta (°C)
Metallothionein	MT2A-F	GTCTTGCTGCTGGAGGAAAC	271	55
	MT2A-R	CTTGGCTCTGCGTCTTTC		
Zinc Finger	ZF-F	GCTCTACTGTGGCTTCTTTG	402	58
	ZF-R	CCTGAACTATCTAACCGTGCC		
WAK	KF-F	CGCCCGGCAGGTACTTTTTT	437	60
	KF-R	CCGATTGAAGACTTTGCG		
SAMS	SAM-F	ACCATCTTCCACCTCAACCC	352	54
	SAM-R	GCCTGAAATCAAAGTTCTCC		
Calmodulin	CAM-F	GATAAAGATGGTGATGGCTG	293	54
	CAM-R	GTGAGTATCTCTCCCAGATTTG		
STS	STS-F	GTGAGTTTCTCTCCCAGATTTG	390	62
	STS-R	GTGTGGGTATTGTCTTGTTGC		
5´RACE	ZF-R	CCTGAACTATCTAACCGTGCC		60
	SMARTII	AAGCAGTGGTATCAACGCAGA GTACGCGGGG		
3´RACE	3´SMART	AAGCAGTGGTATCAACGCAGA GTACTT(25)		60
	ZF-F	GCTCTACTGTGGCTTCTTTG		
ACT	ACT-F	TCACCGAGGCCCCCATCAACC	300	55
	ACT-R	CGACCGGAAGCGTACAGGGACA		

2 F=Forward; R=Reverse; Ta=Temperature of annealing

3

1 **Table 2.** Differentially up-regulated transcripts of *Amaranthus hypochondriacus* L. isolated from cDNA SSH library after

2 $CaCl_2$ treatment

Contig	Clone number	Accession No.	Annotation	Source	E value	Identities (%)	Amplicon Size (bp)
Stress Response							
17	16R	AF268027.1	Metallothioenin	*Amaranthus cruentus*	0.0	100	426
23	1R	AB221009.1	S-Adenosyl-L-Methionine Synthetase (SAMS)	*Beta vulgaris*	0.0	90	524
24	40R	CN782050.1	Universal Stress Protein (USP)	*Chenopodium quinoa*	1e-112	79	891
16	42	NP_565864.1	SLT1 (Sodium and Lithium Tolerant 1)	*Arabidopsis thaliana*	3e-58	86	435
Calcium signaling							
18	41R	CAA46150.1	Calmodulin	*Oryza sativa*	2^{e-76}	97	843
26	8R	Os02g21009	Vacuolar cation/proton Exchanger (CAX)	*Oryza sativa*	7.4^{e-04}	60	363
10	3b	1814452D	Hyp-rich glycoprotein	*Sorghum bicolor*	0.021	35	1144
Signal transduction							
1	2b,10,73,77	Os03g44050	Wall associated kinase (WAK)	*Oryza sativa*	8.7^{e-06}	58	380-619
13	17	AF024651.1	Polyphosphoinositide binding protein Ssh1p (SSH1)	*Glycine max*	6^{e-64}	71	752

3

Transcription factors							
14	10b	NM_112305.1	CID9, RNA binding	*Arabidopsis thaliana*	8^{e-65}	78	345
27	66R	NP_567926.	Zinc Finger (C3HC4-type RING finger)	*Arabidopsis thaliana*	6^{e-15}	45	753
Gene regulation							
4	9,15,33, 39	AM748496.1	23S rRNA pseudouridine synthase	*Vigna unguiculata*	5^{e-12}	95	162-689
Unknown function							
2	5b,6b,7b, 81	NP_566427.1	Unknown protein	*Arabidopsis thaliana*	2^{e-47}	62	289-679
3	34,74	CAN61451.1	Hypothetical protein	*Vitis vinifera*	4^{e-23}	72	49-540
5	1,49	EE743832.1	Leucine-rich repeat (LRR)	*Quercus suber*	$8e^{-04}$	94	435-494
9	6	DQ372900.1	Green Ripe-Like 1	*Solanum tuberosum*	4^{e-65}	76	571
11	14B	ABO83407.1	RNA-directed DNA polymerase	*Medicago truncatula*	8^{e-03}	35	885
15	5	CO491319.1	G.h.fbr-sw	*Gossypium hirsutum*	2^{e-08}	100	340
25	1,2,9,14,1 5,66,95,98	ABD34618.1	Green Ripe-Like 1	*Solanum tuberosum*	7.2^{e-28}	65	246-885

No similarity sequences		
6	2,4,83	447-773
7	44,47	237-253
8	3	445
12	13b	712
19	46	514
20	4b	1192
21	20	324
22	**84**	405

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1 **Table 3.** Differentially down-regulated transcripts of *Amaranthus hypochondriacus* L. isolated from cDNA SSH library after
2 $CaCl_2$ treatment

Contig	Clone number	Accession Nₒ	Annotation	Source	E value	Identities (%)	Amplicon size (bp)
Response to stress							
2	11,39,63	NM_201896.2	SLT1 (Sodium and Lithium Tolerant 1)	*Arabidopsis thaliana*	1^{e-64}	79	416-435
16	91	AB112476.1	Salt-induced hydrophilic protein (AnSIHP1)	*Atriplex nummularia*	3^{e-45}	74	375
20	62	NM_202565.1	SLT1 (Sodium and Lithium Tolerant 1)	*Arabidopsis thaliana*	2^{e-19}	67	608
Signal transduction							
11	67,70, 78	P11898	Glycine-rich protein HC1	*Chenopodium rubrum*	1^{e-32}	54	323-761
Transcription factors							
7	71	AAP23943.1	CCR protein	*Citrofortunella microcarpa*	5^{e-31}	51	596
Metal-binding							
15	92	CAA33971.1	Metal ion binding protein	*Oryza sativa*	1.8^{e-05}	63	845
Metabolism							
13	88	Os02g22100	Rhomboid family protein	*Oryza sativa*	1.1^{e-03}	59	298
Protein folding							
3	19,30,65	NP_179378.1	DNAJ Heat Shock Protein putative (HSP)	*Arabidopsis thaliana*	1^{e-16}	38	461-984

Photosynthesis								
8	66	At3g54890.2	Chlorophyll A-B binding protein / LHCI type I (CAB)	*Arabidopsis thaliana*	9.4^{e-09}	62	334
9	44	X05511.1	Photosynthetic Oxygen-Evolving Complex (OEC)	*Spinacia oleracea*	0.0	81	292
14	34	NP_172153.1	PSBP-1 (Oxygen Evolving Enhancer Protein 2); calcium ion binding	*Arabidopsis thaliana*	4^{e-102}	75	875
17	86	CAB53034.1	Photosystem I subunit XI precursor	*Arabidopsis thaliana*	3^{e-32}	85	240
Defense against pathogen							
1	2,3,7,12, 17,36,38,40, 43,51,57,58, 59,61,64,68,77	P82010	AX2 (Antifungal Cysteine-Rich Protein Peptide)	*Beta vulgaris*	8^{e-07}	44	275-315
Unknown function							
4	79,80,96	Os04g14220	RPM1 putative expressed disease resistance protein	*Oryza sativa*	9.1^{e-04}	64	318-421
6	94	NM_101907.2	Tubulin family protein	*Arabidopsis thaliana*	3^{e-29}	71	404
18	87	EF122398.1	Putative auxin-repressed/ dormancy associated protein	*Citrus hybrid*	4^{e-24}	69	339
No similar sequences							
10	**83**						308

Figure legends

Fig. 1. Distribution of the differentially expressed genes in (A) up-regulated transcripts (forward) and (B) down-regulated (reverse). The classifications are based on their putative gene function.

Fig. 2. Expression pattern analysis of the selected differentially expressed SSH amaranth genes by RT-PCR; SAMS (S-Adenosyl Methionine Synthase), Znf (C3HC4-type RING type), CaM (Calmodulin), MT2A (Metallothionein), WAK (receptor like kinase), *Actin* gene was used to normalize the amount of loaded samples. Plants were watered with $CaCl_2$: Lane 1 = 20 mM, and lane 2 = 50 mM of $CaCl_2$. Gene function and corresponding expression patterns extracted by SSH are listed in Table 3 and 4.

Fig. 3. Nucleotide and deduced amino acid sequence of cDNA encoding for amaranth *Ah*Znf (Acc. Number HM77322). The transmembrane region is marked in squares. The RING domain C3HC4 of Znf domain is underlined.

Fig. 4. Multiple sequence alignment of the deduced amaranth Znf amino acid sequences and eight previously reported as novel Znf proteins. The alignment was performed using the ClustalW. Boxes indicates the RING domain sequence, the arrows indicates the cysteins involved in Zn interactions. Accession numbers of published sequences in the GenBank are as follows: *Arabidopsis thaliana* C2H2 type (AC013427.3); *Arabidopsis thaliana* C3HC4 type (NM111096.3); *Capsicum*

1 *annuum* Cys3-His type (DQ862464.1); *Zea mays* ZMCOI6.1 (DQ060243.1); *Oryza*
2 *sativa* (AP003249.3); *Sorghum bicolor* Znf (XM_002452671).
3
4 **Fig. 5**. Phylogenetic tree describing the relationship among Znf proteins from
5 different plants species constructed with ClustalW. The amino acid sequences
6 corresponded to: *Aeluropus littoralis* (DQ885218.1), *Capsicum annuum*
7 (AF539746.1), *Sorghum bicolor* (XM_002452671), *Capsicum annuum*
8 (DQ862464.1), *Capsicum annuum* (AY196704.1), *Oryza sativa* (AP003249.3),
9 *Arabidopsis thaliana* (AC013427.3), *Ricinus communis* (XM_002532246), *Zea*
10 *mays* (DQ060243.1), *Arabidopsis thaliana* (At1g04360), *Arabidopsis thaliana*
11 (NM_111096.3), *Populus trichocarpa* (CM_000342), *Vitis vinifera*
12 (XM_002269852), *Arabidopsis thaliana* (At5g17600), *Arabidopsis thaliana*
13 (At3g02290), *Medicago sativa* (AFO28841.1), *Oryza sativa* (NM_001061989).
14