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# Comparative proteomic analysis of tomato (*Solanum lycopersicum*) leaves under salinity stress

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**Abstract**

Salinity is a major constraint to crop productivity. The mechanisms responsible for the effects of salt stress on tomato plants were examined by means of proteomic analysis. Two contrasting tomato genotypes (*cv.* Roma and *cv.* SuperMarmande) seedlings were cultivated using a hydroponic system in the controlled environment growth chamber. The salt stress (NaCl) was applied (0, 100 and 200 mM) and maintained for 14 days. Leaf osmotic potential significantly decreased with NaCl treatment in genotype SuperMarmande. However, genotype Roma maintained its leaf osmotic adjustment under salt treatment. Lipid peroxidation (estimated by MDA content) significantly increased under salt in both cultivars, but the rate of increment was higher in genotype SuperMarmande. Leaves of control and salt-stressed plants were also sampled for phenol protein extraction. Proteins were separated by two-dimensional gel electrophoresis (2-DE). 26 protein spots exhibited significant abundance variations between samples. Our results indicated that some proteins exhibited variation strictly related to salt stress whatever the genotype, while some other proteins also showed variation which could be related to the degrees of genotype tolerance. The up-regulation of Rubisco activases and RuBisCO large subunit under salt treatment, was correlated with an increase in abundance level of proteins that are involved in energy metabolism (pyruvate dehydrogenase, Glucose-6-phosphate dehydrogenase, Malate dehydrogenase), especially in salt-tolerant genotype (Roma). Accumulation of antioxidants enzymes (ascorbate peroxidase, glutathione peroxidase, peroxidase and mitochondrial peroxiredoxin) in the leaves of salt tolerant genotype (Roma) was well correlated with the level of lipid peroxidation of membranes. Up-regulation of Heat Shock Proteins and maintenance of water status as reflected by leaf osmotic potential are considered to be strongly correlated with to the degrees of tomato genotype tolerance in this study.

**Keywords**: Leaf; proteomic analysis; salt stress; tomato.
**Abbreviations:** MDA_Malondialdehyde; 2-DE_Two-Dimensional Electrophoresis; ROS_Reactive Oxygen Species; TCA_ Tricarboxylic Acid Cycle; LC-MS_Liquid Chromatography Mass Spectrometry; PSII_Photosystem II; HSP_Heat Shock Proteins; GPX_Glutathione Peroxidase, APX_Ascorbate Peroxidase; GRP_Glycine-Rich Proteins.

## Introduction

Salinity is one of the major abiotic stresses in plant agriculture worldwide. An excess amount of salt in the soil adversely affects plant growth and development. Nearly 20% of the world's cultivated area and about half of the world's irrigated lands are affected by salinity (Kaya et al., 2002). The detrimental effects of salt on plants are a consequence of both; a water deficit that results from the relatively high solute concentrations in the soil and a specific $Cl^-$ and $Na^+$ stress. Based on transcript profiling, it has been suggested that salinity may lead to a series of changes in basic photosynthesis, photorespiration, amino acid and carbohydrate synthesis (Sengupta and Majumdar, 2009; Chaves et al., 2009; Ahuja et al., 2010). To cope with salt stress, plants have evolved complex salt-responsive signaling and metabolic processes at the cellular, organ and whole-plant levels. However, our understanding of these mechanisms is incomplete because of the complexity of salt-induced stress, which has both an ionic component and an osmotic component (Munns and Tester, 2008). Proteomics offers a new platform for studying complex biological functions involving large numbers and networks of proteins and can serve as a key tool for revealing the molecular mechanisms that are involved in interactions between salinity and plant species (Zhang et al., 2012). Variation of the plant proteome under salt stress has already been studied in several plants, among others in soybean (Aghaei et al., 2008; Sobhanian et al., 2010a), rice (Kim et al., 2005; Parker et al., 2006), wheat (Wang et al., 2008; Peng et al., 2009), Arabidopsis (Jiang et al., 2007) and Poaceae (Sobhanian et al., 2010b). Previous physiological and proteomic studies (Manaa et al., 2011) established several adaptation strategies to salt stress in tomato root, including support for glycolysis, TCA cycle, amino-acid metabolism, ATP biosynthesis, photosynthesis, defence-related protein and detoxification. Ahsan et al. (2007) examined proteome variation in tomato leaves exposed to waterlogging stress and found that the expression of proteins associated with stress/defense mechanisms and energy/metabolism were increased, while photosynthesis and protein biosynthesis-related proteins were decreased. Among the identified photosynthesis-related proteins, the expression of RuBisCO was decreased in the total soluble proteins in response to water logging stress.

RuBisCO has a dual function: it acts as a carboxylase mediating photosynthetic $CO_2$ assimilation and as an oxygenase catalyzing the first step of the photorespiratory pathway. However there have only been a few reports on proteomic analyses about how salt stress affects the expression of proteins in tomato leaves.

In higher plants, leaves represent highly specialized organ that is primarily engaged in photosynthesis. It has been shown in several reports that rates and activities of photosynthesis are highly dependent on the development and age of the leaf, and this is also correlated with the accumulation of proteins such as ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and other photosynthesis-related proteins (Maayan et al., 2008; Urban et al., 2008). Leaves also play major role in transporting essential elements and water from the roots to aerial parts. Moreover, photosynthesis and cell growth are among the primary processes to be affected by salt stress (Munns and Tester, 2008). Hence, for better understanding of how plants respond and adapt to salt stress, it is important to focus on leaf system. On the other hand, tomato (*Solanum lycopersicum*) is one of the world's most important and widespread crops and it plays an important role in the human diet. Tomato has long served as a model system for plant genetics, development and physiology. The aim of the present research was to identify changes in leaf protein profile, due to exposure to salt stress, in tomato plants (*Solanum lycopersicum*) by comparative proteomics analysis between NaCl treated and non-treated leaves.

**Results and discussion**

***Leaf osmotic potential and lipid peroxidation***

The changes in leaf osmotic potential under different salinity levels are shown in Fig. 1A. SuperMarmande, showed the highest leaf osmotic potential under control treatment (-0.78 MPa) as compared to genotype Roma (-1.42 MPa). Leaf osmotic potential significantly decreased with salt treatment and was found to be directly proportional to the concentration of the salt added, especially in SuperMarmande genotype. However, Roma maintained its osmotic adjustment in leaves under salt stress, whatever the salinity levels. Maintenance of water status as reflected by leaf osmotic potential is considered to be one of the important adaptations to salt stress (Ashraf and Harris, 2004). In the present study, leaf osmotic potential significantly decreased with salt stress in genotype SuperMarmande, which can be linked to salt-induced water loss as has been earlier reported in tomato (Chen and Plant, 1999), pea (Kav et al., 2004) and *Brassica rapa* (Noreen et al., 2010). Lipid peroxidation is an indicator of membrane damage and leakage under salt stress conditions (Katsuhara et al., 2005). Salt stress affects both cultivars by means of lipid peroxidation. MDA content increased significantly under NaCl treatments (Fig. 1B). However the rate of increment was higher in genotype SuperMarmande. Lipid peroxidation was higher in both genotypes at 200 mM NaCl as compared to control treatment. Maintain of osmotic adjustment under salt stress in genotype Roma is in good correlation with low MDA content. Parallel to our results, low MDA content is important in terms of salt tolerance as represented in different studies. Salt tolerant barley cultivar (Liang et al., 2003) and salt resistant tobacco plant (Ruiz et al., 2005) also had lower levels of lipid peroxidation which is an important sign of higher oxidative damage limiting capacity under salinity.

On the other hand, salt stress treatment decreased plant growth for each genotype (see Supplementary data 1). Plant growth reduction was found to be directly proportional to the concentration of the salinity levels. Roma presented the least decrease in plant growth compared with SuperMarmande. Collectively, genotype SuperMarmande could be classified as the salt-sensitive and genotype Roma the salt-tolerant because of lower growth reduction, maintenance of osmotic adjustment in leaves and lower lipid peroxidation of membranes.

***Salt-responsive proteins***

To investigate salt responsive proteins of tomato leaves, seedlings were exposed to salt stress (0, 100, 200 mM NaCl) for 14 days. Proteins were extracted from leaf of untreated (control) and NaCl-treated plants of tow tomato genotypes (*cv*. Roma and *cv*. SuperMarmande). Leaf protein content (Fig. 1C) showed differentials variations that depend on genotype and salinity levels. Salt stress led to a decrease in leaf protein content for both genotypes. Protein content was gradually decreased with increasing of salinity levels. Salt-sensitive genotype (SuperMarmande) exhibited the highest reduction under NaCl stress, ranged by 43% and 55%, respectively for 100 and 200 mM as compared to control treatment. In contrast, protein content was unmodified by NaCl (100 mM) in tolerant genotype (Roma) and decreased by 25% with 200 mM NaCl. Similar to our findings, Porgali and Yurekli (2005) reported that, protein amount in salt sensitive *L. esculentum* plants decreased with the salt application as compared to control plants. In salt tolerant *L. pennellii* plants, total protein amount was more higher than control plants. Demiral and Turkan (2006) detected that total soluble protein content in salt tolerant rice cultivar (*O. sativa* cv. Pokkali) increased with salinity, while salt sensitive (*O. sativa* cv. IR-28) showed a decrease under salt stress. Ours data confirm that proteins synthesis putatively, linked to the degrees of genotype tolerance. As a consequence more information on salt-responsive proteins and the molecular mechanisms of salt tolerance in tomato plants is needed.

***Leaf proteomic analysis of salt stressed tomato***

A representative 2-DE Coomassie brilliant blue-stained gel of Roma genotype (control) is presented in Fig. 2 and considered as reference gel. To detect the proteins variably expressed in the salt-stress treatment, 2-DE patterns from control and the corresponding stress treatment were compared. In Fig. 3, a representative image from a 2-DE gel of control and salt treated plant of both genotypes is shown. The concentration of NaCl chosen was 100 mM as the salt concentrations similar to those that can be found in salty soils. The leaf protein spots showed a broad distribution in the *pI* range from 4.0 to 7.0 and the mass range from 10–120 kDa. Among about 600 detected spots by digital image analysis, 26 protein spots exhibited significant spot abundance variation ($P$ <0.01) under NaCl stress and responded to salinity by up- or down-regulation. For identification, the protein spots were excised and digested with trypsin and could be successfully identified using LC-MS/MS analysis. In total, it was possible to obtain good quality protein identification results (Table 1) with only one unidentified spot (26), it was not possible to determine the protein spot identity because of a lack of matching unigene sequences. Identified proteins are known to be involved in a wide range of physiological processes, i.e. energy and carbon metabolism (4 spots), photosynthesis-related proteins (5 spots), ROS ( reactive oxygen species) scavenging and

**Table 1.** Identification of salt responsive proteins in *Solanum lycopercicum* leaf varying among genotypes and salt treatment identified by LC-MS/MS.

| *a* Spot ID | Protein identification | Organisme | Function | *b* Accession | c NP | *d* C% | *e* Pi/Pm Experim | *f* Pi/Pm Theori | *g* SP | *h* Roma | *h* SM |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Glutathione peroxidase (GPX) | *Arabidopsis thaliana* | Oxidative stress | SGN-U322657 | 6 | 41 | 5.30/21.10 | 5.11/19.07 | cyt | 7.6/+5.3 | 3.4/+1.2 |
| 2 | Peroxidase (PER) | *Populus albaxPopulustremula* | Oxidative stress | SGN-U315420 | 22 | 59 | 5.40/37.40 | 5.17/35.16 | SP | 11.3/+4.2 | 9.1/+2.2 |
| 3 | Peroxidase (PER) | *Populus albaxPopulustremula* | Oxidative stress | SGN-U315420 | 18 | 60 | 6.10/34.20 | 5.18/35.17 | SP | 14.4/+5.1 | 10.8/+1.9 |
| 4 | Ascorbate peroxidase (APX) | *Solanum lycopersicum* | Oxidative stress | Y16773 | 12 | 47 | 6.20/30.00 | 5.73/27.46 | SP | 8.2/+2.4 | 6.3/+0.9 |
| 5 | Lactoylglutathione lyase | *Citrus paradisi* | Oxidative stress | Z97064 | 18 | 63 | 6.30/32.00 | 6.24/37.21 | SP | 33.7/-10.8 | 30.4/-7.1 |
| 6 | Heat shock hsp70 protein | *Ricinus communis* | Stress defence and heat shock | SGN-U314389 | 8 | 32 | 5.50/99.20 | 5.22/93.59 | cyt | 9.2/+3.2 | 8.1/+0.2 |
| 7 | Pathogenesis-related protein 10/PR10 | *Arabidopsis thaliana* | Stress defence and heat shock | AY088545 | 12 | 62 | 6.10/33.00 | 4.88/27.32 | SP | 18.6/+3.3 | 16.8/+2.1 |
| 8 | Class II small heat shock HSP 17.6 | *Solanum lycopersicum* | Stress defence and heat shock | AF090115 | 29,0 | 48 | 6.60/18.00 | 6.75/17.33 | PS | 21.4/+4.4 | 22.3/+0.8 |
| 9 | Class I small heat shock HSP 26.5 | *Arabidopsis thaliana* | Stress defence and heat shock | AC008016 | 12 | 53 | 5.80/24.00 | 6.86/26.54 | mTP | 15.1/+5.5 | 13.9/+4.2 |
| 10 | Malate dehydrogenase, cytosolic | *Solanum chilense* | Energy and carbon metabolism | SGN-U312385 | 15,0 | 51 | 6.40/42.20 | 6.10/35.74 | pTP | 37.5/+9.4 | 35.8/+3.8 |
| 11 | Heat shock hsp70 protein | *Cucumis sativus* | Stress defence and heat shock | CAA52149 | 14 | 27 | 5.70/72.00 | 5.15/75.42 | SP | 9.3/+2.7 | 10.5/+0.6 |
| 12 | Ascorbate peroxidase | *Solanum lycopersicum* | Oxidative stress | Q3I5C4 | 7 | 37 | 6.10/31.00 | 5.61/27.40 | SP | 24.5/+6.6 | 23.9/+2.1 |
| 13 | GRP-like protein 2 | *Gossypium hirsutum* | Stress defence and heat shock | Q0PW29 | 17 | 64 | 4.90/48.20 | 6.01/40.84 | SP | 20.7/+3.9 | 21.5/+2.6 |
| 14 | Mitochondrial peroxiredoxin | *Pisum sativum* | Stress defence and heat shock | Q6KBB1 | 19 | 28 | 6.50/26.50 | 8.41/21.47 | mTP | 15.1/+3.1 | 13.7/+2.0 |
| 15 | S-adenosylmethionine synthase | *Nicotiana tabacum* | Amino acid metabolism | SGN-U312580 | 14 | 52 | 6.50/47.10 | 5.96/42.60 | cyt | 35.6/+5.5 | 33.8/+5.8 |
| 16 | Putative ATP synthase beta chain | *Sorghum bicolor* | Energy and carbon metabolism | Q09EM2 | 11 | 15 | 6.40/67.00 | 5.71/55.68 | SP | 12.4/-3.9 | 9.2/-4.6 |
| 17 | Universal stress protein family | *Sorghum bicolor* | Stress defence and heat shock | C5YPL0 | 8 | 51 | 7.10/21.00 | 8.11/29.89 | mTP | 22.5/+5.0 | 20.4/+1.9 |
| 18 | Chlorophyll a/b-binding protein | *Solanum tuberosum* | Photosynthesis-related proteins | Q7M1K9 | 12 | 38 | 6.30/26.10 | 6.43/27.82 | cTP | 14.6/+2.8 | 15.5/+1.7 |
| 19 | Putative ferredoxin-NADP reductase | *Arabidopsis thaliana* | Electron transport | Q8W3L1 | 19 | 32 | 6.50/48.20 | 8.69/53.07 | SP | 13.3/+4.9 | 11.4/+2.5 |
| 20 | Glucose-6-phosphate dehydrogenase | *Solanum tuberosum* | Energy and carbon metabolism | P37830 | 12 | 41 | 5.70/60.10 | 5.97/58.47 | cyt | 35.3/+8.5 | 36.7/+11.0 |
| 21 | Rubisco large subunit | *Solanum lycopersicum* | Photosynthesis-related proteins | ABC56308 | 4 | 12 | 6.10/39.00 | 6.55/52.95 | cTP | 16.6/+4.1 | 15.7/+5.2 |
| 22 | Rubisco large subunit | *Solanum lycopersicum* | Photosynthesis-related proteins | ABC56308 | 5 | 11 | 5.90/38.10 | 6.56/52.96 | cTP | 17.0/+3.8 | 17.7/+1.1 |
| 23 | Rubisco activase | *Lycopersicon pennellii* | Photosynthesis-related proteins | O49074 | 12 | 55 | 6.80/50.20 | 8.61/50.70 | cTP | 10.1/+4.6 | 8.9/+1.7 |
| 24 | Rubisco small subunit | *Solanum lycopersicum* | Photosynthesis-related proteins | CAA29400 | 4 | 35 | 6.70/28.00 | 7.58/20.30 | cTP | 13.2/-3.7 | 14.5/-2.6 |
| 25 | Pyruvate dehydrogenase | *Solanum lycopersicum* | Energy and carbon metabolism | SGN-U315305 | 18 | 47 | 6.80/40.10 | 6.87/43.37 | pTP | 38.3/+9.1 | 29.5/-0.8 |
| 26 | Unidentified spot | - | - | - | - | - | 6.30/38.20 | - | - | 29.5/-6.7 | 24.3/-7.3 |

*(a)* Spot ID corresponds to position of the spot in the gel as illustrated in Fig. 2. (*b)* Database unigene/EST accession numbers. (*c)* Number of peptides matching to the unigene sequence. (*d)* Sequence coverage percentage. (*e)* Experimental pI and mass (kDa) of identified proteins. Experimental values were computed by Progenesis Software. (*f*) Theoretical pI and mass (kDa) of identified proteins. (*g)* Peptide signal according to 'TargetP' software: mTP, mitochondrial transit peptide; cTP, chloroplast transit peptide; pTP, plast transit peptide; cyt, cytoplasm; SP, secreted protein. (*h)* Normalized volume of identified proteins: control/increased or decreased in average volume after salt treatment.

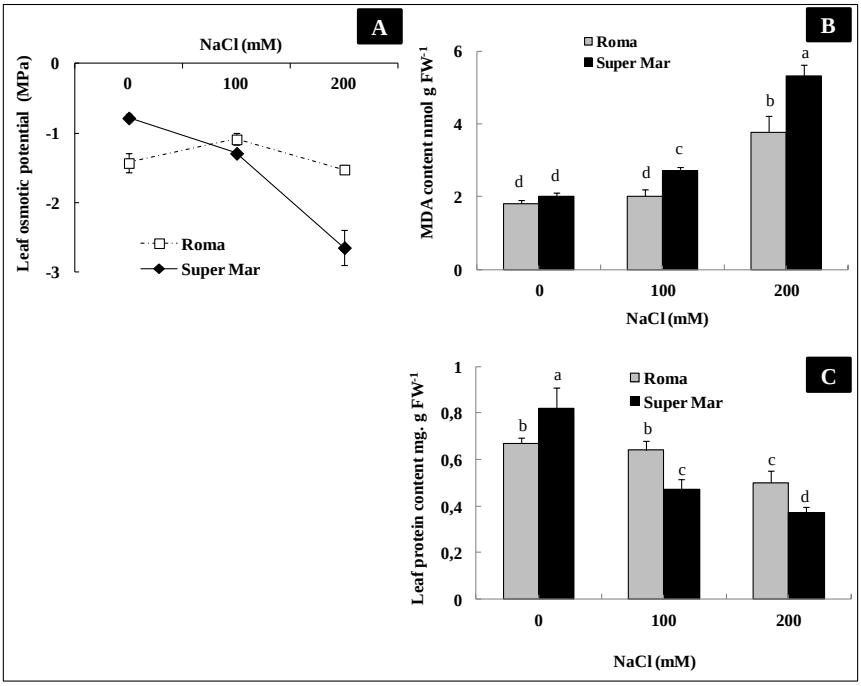


**Fig 1.** The effect of NaCl treatments (0, 100 and 200 mM) on leaf osmotic potential (A), lipid peroxidation (B) and protein content (C) of two tomato genotypes (*cv.* Roma and Supermarrmande). Data are means of 12 replicates ±SE at P >0.05. Letters correspond to Duncan's multiple range test at 95%.

detoxification (6 spots), stress defence and heat shock proteins (8 spots), amino acid metabolism (1 spot) and electron transport (1 spot). It was found that four proteins were identified in two spots, although they were excised from the same gel: Ascorbate peroxidase (spots 4 and 12), rubisco large subunit (spots 21 and 22), heat shock hsp70 protein (spots 6 and 11) and peroxidase (spots 2 and 3). Further examination of electrophoresis patterns indicated that the inferred mass or isoelectric point values of these spots differed, due perhaps to post-translational modification (like glycosylation) which can change the molecular weight and/or charge of proteins. Alternatively, proteins that were present in multiple spots could result from being translated from alternatively spliced mRNAs (Ishikawa et al., 1997). This phenomenon was also reported previously (Holmes-Davis et al., 2005; Ndimba et al., 2005). Proteomic studies have also shown that some proteins may be degraded during abiotic stress. For example, Rubisco large subunit was detected as 19 different fragments plus the intact protein in NaCl-treated rice roots (Yan et al., 2006). Similar phenomena have also been reported in pea mitochondrial proteome under chilling stress (Taylor et al., 2005). It is possible that reactive oxygen species (ROS) may also contribute to the degradation of proteins under stress conditions (Desimone et al., 1996; Kingston-Smith and Foyer, 2000).

***NaCl treatment adversely affects photosynthesis***

Exposure plant to salt stress has been shown to bring about a reduction in the overall growth and plants productivity by perturbing the function of vital components of photosynthesis, such as PSII and RuBisCO. Proteins involved in photosynthesis included the small (spot 24) and large subunits of RuBisCO (spots 21 and 22), RuBisCO activase (spot 23) and chlorophyll a/b binding protein (spot 18) were identified in our experiment.

RuBisCO activase (spot 23) exhibited a general increase in intensity under salt stress. This spot was more intense in salt-tolerant genotype (Roma). The up-regulation of RuBisCO activase under salinity stress has been reported previously (Kim et al., 2005; Bandehagh et al., 2011). Parker et al. (2006) reported that up-regulation of RuBisCO activase activity in rice leaf lamina may be required in order to tolerate salt stress, owing to a direct reduction in stomatal conductance and subsequent low $CO_2$ level. The principal role of RuBisCO activase is to mediate the release of inhibitory sugar phosphates such as ribulose-1,5-bisphosphate from the active sites of RuBisCO so that $CO_2$ can activate the enzyme by carbamylation (Portis 2003). Stress induced stomatal closure which can reduces the leaf internal $CO_2$ concentration, diminishes carbamylation and promotes dead-end binding of ribulose-1,5-bisphosphate to the active sites of RuBisCO. This enzyme may function to reactivate RuBisCO for fixing $CO_2$ or in protective energy dissipation. RuBisCO activase may also play a role as a chaperon during stress (Rokka et al., 2001). During abiotic stress, ROS may contribute to the degradation of proteins under stress conditions (Kingston-Smith and Foyer, 2000; Feller et al., 2008). It was found that two different fragments of the RuBisCO large subunit (spots 21 and 22) exhibited an increase in abundance following NaCl treatment in both genotypes. This result is in accordance with previous data (Hajduch et al., 2001) that reported an increase in the number of RuBisCO large subunit breakdown products in metal-stressed rice leaves. Caruso et al. (2008) reported that RuBisCO large subunit was up-regulated in salt-stressed leaves of wheat (*Triticum durum* L.). RuBisCO is the key enzyme of the Calvin cycle and catalyses the reaction of Dribulose 1,5-bisphosphate and $CO_2$ to two 3-phospho-Dglycerate molecules. It was found that the abundance of RuBisCO small subunit (spot 24) decreased in response to salinity whatever the genotype. This reduction could be linked to limited $CO_2$ fixation and lead to a down-regulation of enzymes that are associated with the Calvin cycle. Consequently, the down-regulation of the Calvin cycle may lead to the accumulation of ATP and NADPH in the chloroplasts, and to protect plants from photo-oxidative damage, the decrease of proteins involved in PSII would be necessary (Ranieri et al., 2001). This hypothesis is supported by our results, as well as other studies (Bohler et al., 2007; Gao et al., 2011). Our results indicated an up-regulation of the activity of pyruvate dehydrogenase (spot 25) complex involved in transferring carbon from glycolysis to Krebs cycle, correlated with an increase in the activity of Rubisco

activases (spot 23) involved in regeneration of ribulose-1,5-bisphosphate (RuBP), the substrate for Rubisco. Induction of these proteins in this study in salt-tolerant genotype (Roma) is one of the mechanisms which may confer salt tolerance. It should be noted that such enhanced resistance of PSII can be associated with the induction of chlorophyll a/b binding protein that take a central role in maintaining the structure and function of PSII (Li et al., 2011). We observed that chlorophyll a/b binding protein (spot 18) was specifically induced by salt stress extremely on tolerant genotype (Table 1).

***Primary metabolism related proteins are mainly up-regulated by salt stress***

The central carbohydrate metabolism is known to be rapidly readjusted after salt stress, most of the studies dealing with salt or osmotic stress reported the down regulation of carbohydrate metabolism genes (Ndimba et al., 2005; Jiang and Deyholos, 2006; Zhang et al., 2012). However, in the present work the majority of proteins, whose function was related to primary metabolism, exhibited an up-regulation under salt stress. Some of them also showed variation which could be linked to the degrees of genotype tolerance. Glucose-6-phosphate dehydrogenase (spot 20) exhibited an increase in abundance level following NaCl treatment in tomato leaf but to a greater extent in SuperMarmande genotype. Glucose-6-phosphate dehydrogenase is a key enzyme that catalyses a non-equilibrium reaction and thus regulates the flux of carbon through the pentose phosphate pathway. It is also a key enzyme that catalyses the first step of the oxidative pentose phosphate pathway. The main function of the pentose phosphate pathway is to provide NADPH and other intermediates, such as pentose and erythrose 4-phosphate (Levy, 1979). Malate dehydrogenase cytosolic (spot 10) also exhibited genotype- and salt related abundance variations. Previous study reported that cytosolic malate dehydrogenase was up-regulated by long-term salinity stress in tomato root (Manaa et al., 2011) and *Thellungiella halophila* leaves as well (Fei et al., 2008). Pyruvate dehydrogenase (spot 25) is partner in the pyruvate dehydrogenase complex which transforms pyruvate into acetyl-CoA and links cytosolic glycolytic metabolism with the tricarboxylic acid cycle. Our results showed that relative abundance of this protein spot (25) decreased significantly in stressed tomato leaves, but with less abundance in salt sensitive genotype. It has been reported previously that the abundance pyruvate dehydrogenase and malate dehydrogenase, generally decreased in NaCl-treated *Arabidopsis* roots (Jiang and Deyholos, 2006) and revealed lower expression in root of sensitive tomato genotype (Manaa et al., 2011). In contrast, in the present study, proteins whose function was related to primary metabolism were mainly up-regulated by salt stress in tomato leaves. It may thus be hypothesized that enhanced abundance of several enzymes involved in energy metabolism under salt stress was more prominent in leaves than in roots. Another energy-related protein, Putative ATP synthase beta chain (spot 16), was found to be down regulated in response to salinity stress. This spot was more intense in the case of stress-tolerant genotype (Roma). The decrease in ATP synthase could be associated with transiently decreased photosynthesis rates and the down-regulation of the Calvin cycle enzyme phosphoglycerate kinase (Ashraf and Harris, 2004; Barkla et al., 2009).

***Salt tolerance could be associated with ROS scavenging and detoxifying enzymes***

The series of events observed after exposure to salinity is often discussed as a consequence of a decrease in photosynthesis accompanied by accumulation of reactive oxygen species (ROS) (Dat et al., 2000). Salt stress induces an accumulation of ROS that are harmful to plant cells at high concentrations and plants enhance ROS scavenging enzymes (Apel and Hirt, 2004). Presence of these ROS-scavenging enzymes may provide protection against oxidative stress and it was suggested that the fine-tuning of various antioxidants is important in salt tolerance (Jiang et al., 2007). Interestingly, in this study six proteins were identified (Table 1): ascorbate peroxidase (APX, spots 4 and 12), glutathione peroxidase (GPX, spot 1), peroxidase (PER, spots 2 and 3) and mitochondrial peroxiredoxin (spot 14). In general, relative abundance of these proteins was increased upon NaCl treatment in both genotypes. Previous microarray results demonstrated that members of them are responsive to various stresses including NaCl, drought and cold (Seki et al., 2002; Jiang and Deyholos, 2006; Jiang et al., 2007). APX, mitochondrial peroxiredoxin and GPX can directly detoxify $H_2O_2$ to $H_2O$. Previous studies showed that APX is a central component of the reactive oxygen gene network of *Arabidopsis* (Davletova et al., 2005). Other earlier reports demonstrated that peroxidase were shown to be differentially expressed in the root of salt tolerant barley and shoots of *Salicornia europaea* in response to salinity stress (Sugimoto and Takeda, 2009; Wang et al., 2009). In the present study, major part of antioxidants was more intense in salt- tolerant (Roma) than in salt-sensitive genotype (SuperMarmande). The alleviation of oxidative damage and increased resistance to environmental stresses is correlated with an efficient anti-oxidative system (Smirnoff, 1998). In the same way, previous studies demonstrated that relative abundance of antioxidants proteins in tomato root were putatively linked to the degrees of genotype tolerance (Mittova et al., 2004; Manaa et al., 2011). The present work demonstrated that accumulation of antioxidants enzymes in the leaves of salt tolerant genotype (Roma) was well correlated with the level of lipid peroxidation of membranes. It was worth noting that other protein involved in oxidative process as lactoylglutathione lyase (spot 5) were also identified as abundant proteins in both genotypes (*cv* Roma, SuperMarmande) and exhibited down regulation under NaCl treatment. These data are in opposition to previous studies that demonstrated the up-regulation of Lactoylglutathione lyase in *Arabidopsis* roots (Jiang et al., 2007). The observed variations were unexpected and might be linked to stress application, in our experiment, which was prolonged to 14 days.

***HSP accumulation was strongly correlated with maintenance of leaf water status***

NaCl stress reduces water availability and affects plant growth by increasing the threshold pressure for wall yielding in expanding cells or inducing hydraulic limitations to water

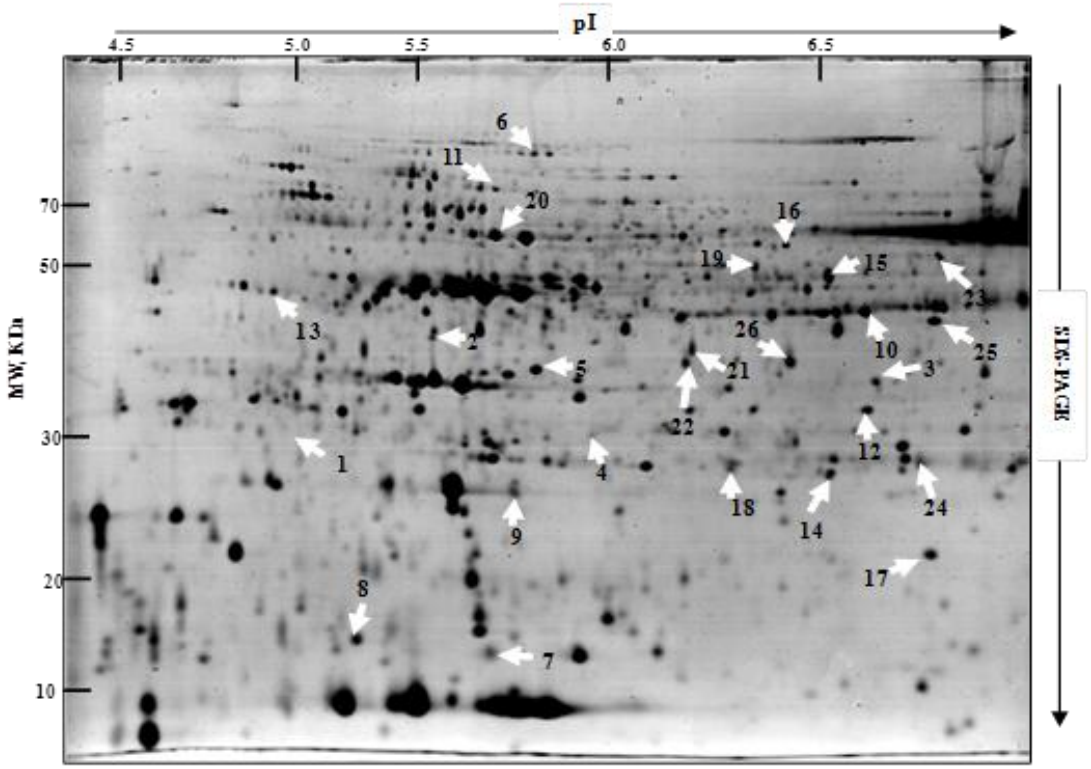


**Fig 2.** Reference two dimensional electrophoretic patterns of soluble leaf proteins obtained from tomato leaf proteins of Roma, in control growth conditions. Proteins were resolved using a linear gradient pH 4–7 in the first dimension and 12.5% Sodium Dodecyl Sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) in the second dimension. Gels were stained with Coomassie G 250. The positions and numbers of the 26 identified protein spots are indicated by arrows.

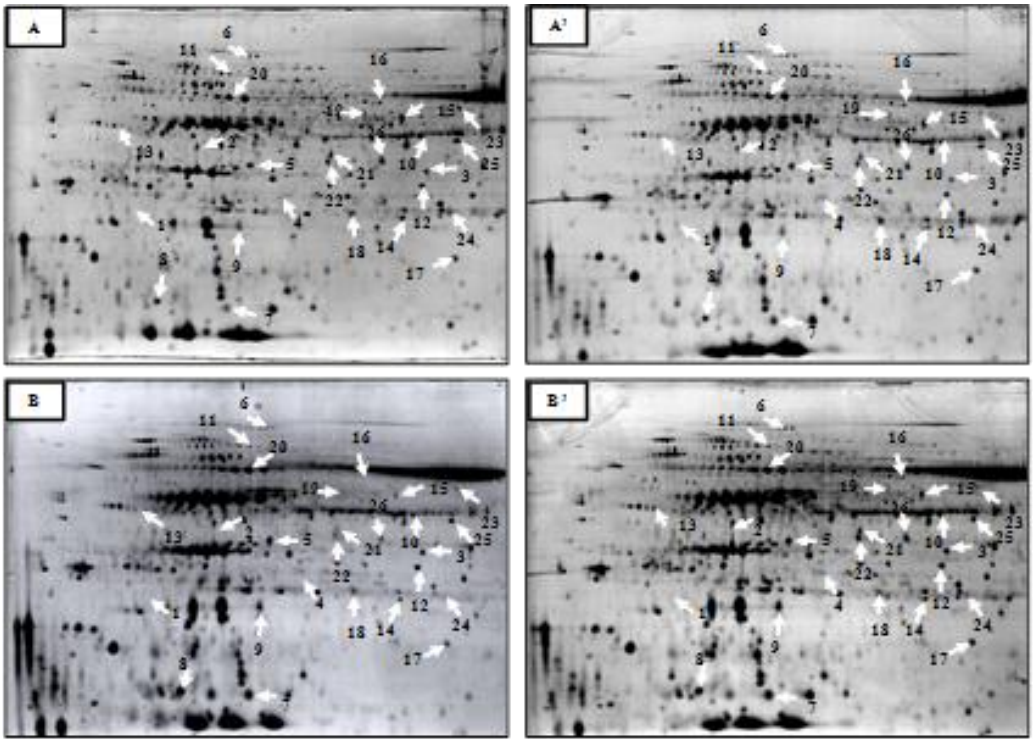


**Fig 3.** 2-DE protein profiles in tomato leaf under control condition: Roma (A), SuperMarmande (B) and with salt treatment Roma (A'), SuperMarmande (B'). The position and numbers of the twenty-six identified protein spots are indicated by arrows. The positions of the same proteins are also marked in almost the same direction and position.

uptake (Neumann et al., 1994; Steudle, 2000). To cope with this stress, plant produces some of proteins defence. Heat Shock proteins (HSP) are a highly conserved family of proteins and play a protective role against stress by stabilizing proteins and membranes and by re-establishing normal protein conformation and thus cellular homeostasis (Wang et al., 2004). They are also known to be induced under various stresses, including high salinity and hyperosmotic stress (Ndimba et al., 2005; Wang et al., 2009; Pang et al., 2010). In the current study, four heat-shock proteins were identified; Heat Shock Hsp70 protein (spots 6 and 11), Class II Small Heat Shock HSP 17.6 (spot 8) and Class I Small Heat Shock HSP 26.5 (spot 9). All these proteins exhibited an increase in abundance following NaCl treatment whatever the genotype. Similarly to that was found in previous studies (Wang et al., 2009; Manaa et al., 2011), their up-regulation was involved in preventing aggregation of the denatured proteins and in facilitating the refolding under salt stress. On the other hand, up-regulation of HSP and maintenance of water status as reflected by leaf osmotic potential (especially in salt-tolerant genotype, Roma) are considered to be strongly correlated with to the degrees of genotype tolerance in this study. In the same way we identified another cell-wall-related protein, Glycine-Rich Proteins. GRPs containing >60% glycine have been found in the cell wall of many higher plants and form a group of structural protein components of the wall in addition to extensins and Proline-Rich Proteins (Ringli et al., 2001). GRPs play roles in post-transcriptional regulation of the gene expression in plants under various stress conditions and, in most cases, they are accumulated in the vascular tissues and their synthesis is part of the plant's defence mechanism (Mousavi and Hotta, 2005). GRP-like protein 2 (spot 13) exhibited an increase in intensity under NaCl stress, but to a greater extent in Roma genotype. The increased expression of GRPs in salt-tolerant genotype (Roma) would form an independent structure-determining network within the extracellular matrix that adds to the mechanical strength of the wall and assists in proper wall assembly.

## Materials and methods

### *Plant material, salinity treatments and growth conditions*

Tomato (*Solanum lycopersicum* L. *cv*. Roma and *cv*. SuperMarmande) seeds were surface-sterilized by soaking in a 5% (v/v) sodium hypochlorite solution for 15 min followed by three washes with sterile distilled water. Seeds were then germinated in Petri dishes with moistened filter paper and were incubated under fluorescent light (90 µmol $m^{-2}s^{-1}$ with a 16 h photoperiod at 25 °C). When germinated, 20 seedlings per sample were transferred to a growth chamber (25 °C/70% relative humidity during the day and 20 °C/90% relative humidity during the night; photoperiod: 16 h daily with a light irradiance of 150 µmol $m^{-2}s^{-1}$). They were grown in hydroponic nutrient solution continuously aerated containing: $KNO_3$ 3 mM, $Ca(NO_3)_2$ 1 mM, $KH_2PO_4$ 2 mM, $MgSO_4$ 0.5 mM, Fe-K-EDTA 32.9 µM, and micronutrients: $H_3BO_4$ 30 µM, $MnSO_4$ 5 µM, $CuSO_4$ 1 µM, $ZnSO_4$ 1 µM, and $(NH_4)_6MO_7O_{24}$ 1 µM for 10 days. At this time, plants were at the third leaf stage and salt stress (0, 100, 200 mM NaCl) was initiated and maintained for 14 days. Leaves from control and NaCl-treated plants under 100 mM NaCl were harvested and washed with distilled water at least five times and immediately, frozen in liquid nitrogen and stored at −80 °C for protein extraction.

### *Leaf osmotic potential*

Osmotic potential ($\psi$s) was determined from the third leaf (numbered acropeticaly) of ten seedlings, cut in small segments, frozen in liquid nitrogen and stored at −80 °C. After being encased individually in a second intact Eppendorf tube, they were allowed to thaw for 30 min and centrifuged at 15.000xg for 15 min at 4°C. The collected tissue sap was analyzed for ($\psi$s) estimation. Osmolarity (C) was assessed with a vapour pressure osmometer (Wescor 5500; Wescor, Inc., UT, USA) and converted from mosmoles $kg^{-1}$ to MPa using the formula as follows: Ws (MPa) = - C (mosmoles $kg^{-1}$) x 2.58 x $10^{-3}$ according to the Van't Hoff equation.

### *Lipid peroxidation*

The level of lipid peroxidation was measured as 2-thiobarbituric acid-reactive substances [mainly malondialdehyde (MDA)] according to Madhava Rao and Sresty (2000). Frozen samples (0.25 g from 10 pooled plants) were homogenized with a pre-chilled mortar and pestle with 5ml of ice-cold 5% (w/v) trichloroacetic acid (TCA) and centrifuged at 12.000xg for 15 min and at 4°C. Assay mixture containing a 2 mL aliquot of supernatant and 2 mL of 0.67% (w/v) thiobarbituric acid was heated to 100 °C for 30 min and then rapidly cooled to 4°C in an ice-bath. After centrifugation (10.000xg for 1 min at 4 °C), the supernatant absorbance was read (532 nm) and values corresponding to non-specific absorption (600 nm) were subtracted. MDA concentration was calculated using its molar extinction coefficient ($155 \cdot mM^{-1} \cdot cm^{-1}$).

### *Proteomic methods*

One biological sample was obtained by pooling the leaves from five plants. In total, four biological repeats were analyzed. Leaves were frozen in liquid nitrogen, ground to a fine powder and stored at -80°C. For protein content measurement, 200 mg of leaf powder material were directly extracted in 1.2 mL of Laemmli sample buffer (Laemmli, 1970) during 15 min at room temperature. After 15 min centrifugation at 5500 g the protein content of the supernatant was assayed using the Biorad RC-DC kit with bovine serum albumin (BSA) as a standard according to the manufacturer's prescriptions. All procedures for protein extraction, separation by two-dimensional electrophoresis, image analysis and mass spectrometry were performed as previously described by Manaa et al. (2011). Briefly, proteins were extracted using a phenol extraction procedure (Faurobert et al., 2006). Before 2-DE proteins were solubilised in lysis buffer (9 M Urea, 4 % (w/v) CHAPS, 0.5 % (v/v) Triton X-100, 20 mM DTT, 1.2 % (v/v) pharmalytes pH 3-10) and protein concentration was measured according to a modified Bradford assay (Ramagli and Rodriguez, 1985) in order to load 500 µg of proteins on 24 cm long Immobiline dry strips, pH 4-7 (Amersham Bioscience, Uppsala, Sweden). Isoelectric focusing was performed with the Multiphor II (Amersham Bioscience, Uppsala, Sweden) according to the following program: 2 h at 150 V, 2 h at 400 V, 2 h to increase the voltage from 400 to 3,500 V, 25 h at 3,500 V. After migration, IEF strips were stored at -80°C or immediately incubated in equilibration buffer respectively, for 20 min. SDS-PAGE was carried out with 11 % acrylamide gels in the Biorad Protean Plus Dodeca cell electrophoresis chamber (2 h at 70 V, 15 h at 100 V).

Gels were stained with Coomassie colloidal blue and gel images were analysed using Progenesis SameSpots v3.0 software (Nonlinear Dynamics Ltd). Spot volumes were normalized by the total spot volumes per gel to avoid experimental variations among 2-D gels. To verify the auto detected results, all spots were manually inspected and edited as necessary. For the identification of the protein spots by by nano LC-MS/MS, in gel-digestion was performed with the Progest system (Genomic Solution) according to a standard trypsin protocol. HPLC was performed on an Ultimate LC system combined with a Famos autosampler and a Switchos II microcolumn switch system (Dionex). Eluted peptides were analysed on-line with a LCQ Deca XP+ ion trap (Thermo Electron) using a nanoelectrospray interface. Peptide ions were analysed using Xcalibur 1.4. A database search was performed with Bioworks 3.2 (Thermo Electron). Trypsin digestion, cys carboxyamidomethylation and Met oxidation, protein N-ter acetylation and deamination were set to enzymatic cleavage, static or possible modifications. Precursor mass and fragment mass tolerance were 1.4 and 1, respectively. The Solanaceae Genomic Networks tomato database (db34, version 20081201, http://www.sgn.cornell.edu/, 34829 tentative consensus sequences) and Uniprot (http://www.uniprot.org/) was used. Identified tryptic peptides were filtered according (1) to their cross-correlation score (Xcorr), superior to 1.7, 2.2, and 3.3 for mono-, di-, and tricharged peptides, respectively and (2) to their probability lower than 0.05. A minimum of two different peptides was required. In the case of identification with only two or three MS/MS spectra, similarity between the experimental and the theoretical MS/MS spectra was visually confirmed.

The identified proteins were classified according to the Funcat automatic classification (http://mips.gsf.de/proj/funcatDB) and according to the literature when the automatic classification failed.

### *Statistical analysis*

The statistical analyses were performed with the "Statistica" software (version 6.0). All physiological parameters mean values and standard error (S.E.) were obtained from of at least 12 replicates and analyzed using *Duncan's* multiple range test. A $P$ value of <0.05 was considered statistically significant. For gel image analysis, Samespots software was used to detect varying spots using one way ANOVA on normalized spot volume from the four gel repeats with $p < 0.01$.

## Conclusions

In this study, a leaf proteomic analysis of two contrasting genotypes (*cv*. Roma and *cv*. SuperMarmande) subjected to non-lethal NaCl treatment, with physiologically defined responses, was performed. Symptoms of salt stress were detected, such as decrease of plant growth and leaf osmotic potential with an increase of lipid peroxidation (MDA content) with variations linked to the degrees of genotype tolerance. At the proteome level, some general proteome variations linked to salt stress (whatever the genotype) were demonstrated as well as some variations combining the genotype and the salt factors. This study also indicates that identified proteins in tomato leaf are implicated in diverse processes i.e. photosynthesis, defence processes, oxidative stress, energy production and while some are probably part of a general stress response to help plants survive in suboptimal conditions, others may contribute to mitigation of salt treatment effects. Many other NaCl responsive proteins still need to be identified. However, because of post-transcriptional events and post-translational modifications such as phosphorylation and glycosylation, mRNA levels do not usually correlate with the expression levels of proteins, which are more directly related to signaling and metabolic processes under salt stress conditions. A further validation of data reported herewith would likely provide, deeper insight pertaining to the stress physiology of this important crop plant.

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