

RESEARCH PAPER

Salt and genotype impact on plant physiology and root proteome variations in tomato

Arafet Manaa^{1,2}, Hela Ben Ahmed¹, Benoît Valot³, Jean-Paul Bouchet², Samira Aschi-Smiti¹, Mathilde Causse² and Mireille Faurobert^{2,*}

¹ Unité d'Ecophysiologie et Nutrition des Plantes, Département de Biologie, Faculté des Sciences de Tunis, Université Tunis El Manar, 1060 Tunisie

² Institut National de la Recherche Agronomique, Unité de Génétique et Amélioration des Fruits et Légumes, UR 1052, Domaine Saint-Maurice, BP 94, F-84143 Montfavet cedex, France

³ Institut National de la Recherche Agronomique, Unité de Recherches en Génétique Végétale, UMR320, F-91190 Gif sur Yvette, France

* To whom correspondence should be addressed. E-mail: Mireille.Faurobert@avignon.inra.fr

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Abstract

To evaluate the genotypic variation of salt stress response in tomato, physiological analyses and a proteomic approach have been conducted in parallel on four contrasting tomato genotypes. After a 14 d period of salt stress in hydroponic conditions, the genotypes exhibited different responses in terms of plant growth, particularly root growth, foliar accumulation of Na⁺, and foliar K/Na ratio. As a whole, Levovil appeared to be the most tolerant genotype while Cervil was the most sensitive one. Roma and Supermarmande exhibited intermediary behaviours. Among the 1300 protein spots reproducibly detected by two-dimensional electrophoresis, 90 exhibited significant abundance variations between samples and were submitted to mass spectrometry for identification. A common set of proteins (nine spots), up- or down-regulated by salt-stress whatever the genotype, was detected. But the impact of the tomato genotype on the proteome variations was much higher than the salt effect: 33 spots that were not variable with salt stress varied with the genotype. The remaining number of variable spots (48) exhibited combined effects of the genotype and the salt factors, putatively linked to the degrees of genotype tolerance. The carbon metabolism and energy-related proteins were mainly up-regulated by salt stress and exhibited most-tolerant versus most-sensitive abundance variations. Unexpectedly, some antioxidant and defence proteins were also down-regulated, while some proteins putatively involved in osmoprotectant synthesis and cell wall reinforcement were up-regulated by salt stress mainly in tolerant genotypes. The results showed the effect of 14 d stress on the tomato root proteome and underlined significant genotype differences, suggesting the importance of making use of genetic variability.

Key words: Genetic variability, proteome, root, salt stress, *Solanum lycopersicum*, tomato.

Introduction

Salinity is one of the major abiotic stresses in plant agriculture worldwide, and an excess amount of salt in the soil adversely affects plant growth, development, and crop productivity. It induces a wide range of perturbations at the cell and whole plant levels. The detrimental effects of salts result not only from a water deficit with relatively high solute concentrations in the soil but also from specific Cl⁻ and Na⁺ stresses. The result is a wide variety of physiological and biochemical

changes in plants that inhibit growth and development, reduce photosynthesis, respiration, and protein synthesis, and disrupt nucleic acid metabolism (Levine *et al.*, 1990; Zhang and Blumwald, 2001; Sairam *et al.*, 2002). Growth reduction is linked with the time over which the plant has grown in saline conditions. At the beginning, the plant suffers from osmotic stress and later it is affected by salt-specific effects (Munns *et al.*, 2002). Several cellular processes

involved in salt-stress tolerance (osmotic adjustment, osmo-protection, ion homeostasis, elimination of oxygen scavengers, stress response etc.) are related to the duration of the stress. A large number of genetic traits have been shown to be linked with these mechanisms (Cuartero *et al.*, 2006). Plant species and cultivars within a crop species may differ greatly in their response to salinity (Marschner, 1995). In tomato, which is one of the most important and widespread vegetables in the world, many genes and QTL with epistatic interactions have been described for salt resistance. Most of them were highly influenced by environment (Cuartero and Fernández-Muñoz, 1999; Monforte *et al.*, 1999; Dasgan *et al.*, 2002; Juan *et al.*, 2005; Cuartero *et al.*, 2006; Al-Busaidi *et al.*, 2009).

Due to the highly complex physiological and genetic basis of plant responses to salt, discrimination between tolerant and sensitive genotypes within a species is clearly a challenging but tricky issue. Genotype tolerance should be evaluated taking into account the overall behaviour of the plant, including different stages of development, ideally from seed germination to fruit yield. In tomato, salt tolerance is known to increase with plant age, plants being usually most tolerant at the fruit maturation stage (Bolarin *et al.*, 1993). As demonstrated by Cuartero *et al.* (2002), screening for genotype tolerance should involve the determination of several physiological parameters such as Na⁺ accumulation and transport parameters, in addition to plant growth parameters (Ashraf and Harris, 2004; Munns *et al.*, 2002; Sairam *et al.*, 2002).

Screening genotypes in natural saline soils is a strategy that presents some drawbacks related to local variability in salt content in the field and is also related to a high potential for interactions with other environmental factors, such as soil fertility, drainage, field temperature, and light flux density (Richards, 1983; Shannon and Noble, 1990; Daniells *et al.*, 2001). Hydroponic culture is a widely used system to screen genotypes using a high NaCl level (Cuartero *et al.*, 2002; Agong *et al.*, 2003).

Roots are the primary site of salinity perception and injury for several types of stress, including nutrient deficiency and heavy metals. In many circumstances, the stress sensitivity of the root limits the productivity of the entire plant (Atkin *et al.*, 1973; Steppuhn and Raney, 2005). An improved understanding of molecular responses of roots to NaCl treatment is therefore necessary to further improve crop tolerance to NaCl and other stresses. Providing a description of the molecular mechanisms active in the response of roots to NaCl treatment, is necessary to characterize the components of these mechanisms, including proteins. A global protein expression overview can be obtained using the high resolution of protein separation by two-dimensional gel electrophoresis (2-DE) coupled with protein identification by mass spectrometry and database search (Lee *et al.*, 2004; Ndimba *et al.*, 2005). Such a proteomic approach has become a powerful tool to study plant development in general (Faurobert *et al.*, 2007; Rossignol *et al.*, 2006) and, in particular, the responses to salt stress (Sha Valli Khan *et al.*, 2007). Variation of the plant proteome under salt stress has already been studied in several plants, among others in *Arabidopsis* (Jiang *et al.*, 2007),

soybean (Aghaei *et al.*, 2009; Sobhanian *et al.*, 2010), wheat (Caruso *et al.*, 2008), rice (Yan *et al.*, 2005, 2006; Cheng *et al.*, 2009), and grapevine (Jellouli *et al.*, 2008). All these works were dealing with the short-term effects of salt stress, from a few hours to 7 d. To date, there have only been a few reports on proteomic analyses after longer periods of stress in tomato and genetic variability needed to be taken into account. Chen and Plant (1999) showed a transient synthesis of unidentified tomato salt-responsive proteins in response to less than 4 d stress. They also demonstrated that abscisic acid doesn't play a major role in the synthesis of most of these proteins. Amini *et al.* (2007) could only identify five tomato proteins regulated by a 24 h salt stress, while Chen *et al.* (2009) applying a 7 d stress identified 23 salt-stress-responsive proteins comparing a sensitive and a tolerant genotype. Some anti-oxidant proteins, heat shock proteins and carbohydrate metabolism-associated proteins were shown to be up-regulated.

Our work aimed at comparing four *Solanum lycopersicum* genotypes contrasted for both plant and fruit characteristics. The question of the physiological response of these genotypes to 14 d of salt stress was addressed first in order to evaluate the sensitivity of each genotype to salt stress at the plantlet stage. Then, modifications of their root proteome were explored in order to characterize the respective and combined effects of both genotype and treatment factors on protein expression.

Materials and methods

Plant material and growth conditions

Four *Solanum lycopersicum* L. genotypes were used: Roma with oblong fruits and determinate growth, Super Marmande with large fruits and semi-determinate growth, Cervil a small-fruited cherry tomato type with indeterminate growth, and, finally, Levovil a large-fruited tomato genotype with indeterminate growth.

Tomato 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 mmol 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 mmol m⁻² s⁻¹). They were grown in hydroponic nutrient solution continuously aerated containing: KNO₃ 3 mM, Ca(NO₃)₂ 1 mM, KH₂PO₄ 2 mM, MgSO₄ 0.5 mM, Fe-K-EDTA 32.9 μM, and micronutrients: H₃BO₃ 30 μM, MnSO₄ 5 μM, CuSO₄ 1 μM, ZnSO₄ 1 μM, and (NH₄)₆Mo₇O₂₄ 1 μM for 10 d. At this time, plants were at the three-true leaf stage and NaCl was added in increments of 25 mM d⁻¹ up to a final concentration of 200 mM for physiological experiments and 100 mM for proteomic analysis while control plants were maintained in nutrient solution without NaCl. Salt stress was maintained for 14 d.

Physiological parameters measurements

For plant growth and ion analysis, 20 independent dry matter measurements and ion analysis were performed on separated leaves, stems, and roots. For the measurement of cations, plant material was dried at 80 °C and digested with nitric acid (1% (v/v) HNO₃) according to the method of Wolf (1982). K⁺, Ca²⁺, and

Na^+ were analysed by flame emission using an Eppendorf spectrophotometer. Cl^- was quantified by a colorimetric method using a Digital Chloridometer HaakeBuchler (Buchler instruments Inc., New Jersey, USA).

Proteomic methods

One biological sample was obtained by pooling the roots from five plants. In total, four biological repeats were analysed. Roots were frozen in liquid nitrogen, ground to a fine powder, and stored at -80°C . For protein content measurement, 200 mg of root powder material were directly extracted in 1.2 ml of Laemmli sample buffer (Laemmli, 1970) for 15 min at room temperature. After 15 min centrifugation at 5500 g the protein content of the supernatant was assayed using the Bio-Rad RC-DC kit with bovine serum albumin (BSA) as a standard according to the manufacturer's instructions. All procedures for protein extraction, separation by two-dimensional electrophoresis, image analysis, and mass spectrometry were performed as previously described by Page *et al.* (2010). Briefly, proteins were extracted using a phenol extraction procedure (Faurobert *et al.*, 2006). Before 2-DE proteins were solubilized 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) and SDS-PAGE was carried out with 11% acrylamide gels in the Bio-Rad Protean Plus Dodeca cell electrophoresis chamber. 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 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 nano-electrospray 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) was used. The tryptic peptides identified were filtered according to (i) their cross-correlation score (Xcorr), superior to 1.7, 2.2, and 3.3 for mono-, di-, and tricharged peptides, respectively, and (ii) 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 (SE) were obtained from of at least 20 replicates and analysed

using Duncan's multiple range test or Student's *t* test. A *P* value of <0.05 was considered to be 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 and *q* <0.015 . On the deduced set of spots, a two-way ANOVA was performed to detect genotype, treatment, and interaction effects, a *P* value less than 0.01 was considered statistically significant.

Results and discussion

Genotypic variation for physiological parameters related to salt tolerance

A comparison of genotype tolerance or sensitivity to salt stress requires appropriate physiological traits, such as plant growth parameters and ion (e.g. Na^+ , Cl^- , K^+) accumulation (Cuartero *et al.*, 2006), to be measured. Plant growth and biomass yield are classically used to evaluate plant tolerance to abiotic stress (Munns *et al.*, 2002; Sairam *et al.*, 2002; Ashraf and Harris, 2004).

Hydroponic culture of young tomato seedlings of four genotypes Cervil, Levovil, Roma, and Super Marmande was therefore performed and the biomass obtained was compared after growth on control medium or on salt-supplemented medium. In control conditions, differences were detected between the genotypes: Roma had the higher dry weight while the other three genotypes exhibited roughly the same growth (Fig. 3A). Salt stress treatment significantly decreased plant growth for each genotype. Levovil presented the least decrease in whole plant growth (46%) compared with the control. For the other genotypes, growth reduction was 60%, 64%, and 65%, respectively, for Roma, Cervil, and Super Marmande. The magnitude of the response was also linked to the plant organ considered, salt treatment inducing a strong reduction of stem and leaf DW (Fig. 1D, B) for all genotypes. In leaves, for example, the reduction was of 55, 61, 63, and 67.5%, for Levovil, Roma, Super Marmande, and Cervil, respectively. The root DW (Fig. 1C) was less sensitive to salt treatment, Roma, Cervil, and Super Marmande exhibiting respectively 25, 40% and 43% DW reduction. The root organ also showed the highest difference among genotypes as Levovil's root DW reduction was only 4%. Such variability between tomato cultivars for biomass reduction after salt stress has been stated previously (Alian *et al.*, 2000), although some other studies did not report a relationship between biomass production and salt tolerance at the seedling stage of the tomato genotypes (Dasgan *et al.*, 2002), underlying the necessity to add other criteria to evaluate tomato tolerance to salt stress.

Our results showed the accumulation of Na^+ and Cl^- (Fig. 1) in leaves, stems, and roots. When the four cultivars tested were compared, significant differences in Na^+ and Cl^- concentrations were found. Concerning the Na^+ ion, Cervil exhibited the highest concentration at the root level, while for Cl^- , the concentrations in the roots were similar in Cervil and Levovil, and higher than those of Roma and Super Marmande. Levovil showed the lowest Na^+ foliar accumulation. The highest concentration of foliar Na^+ was detected in Super

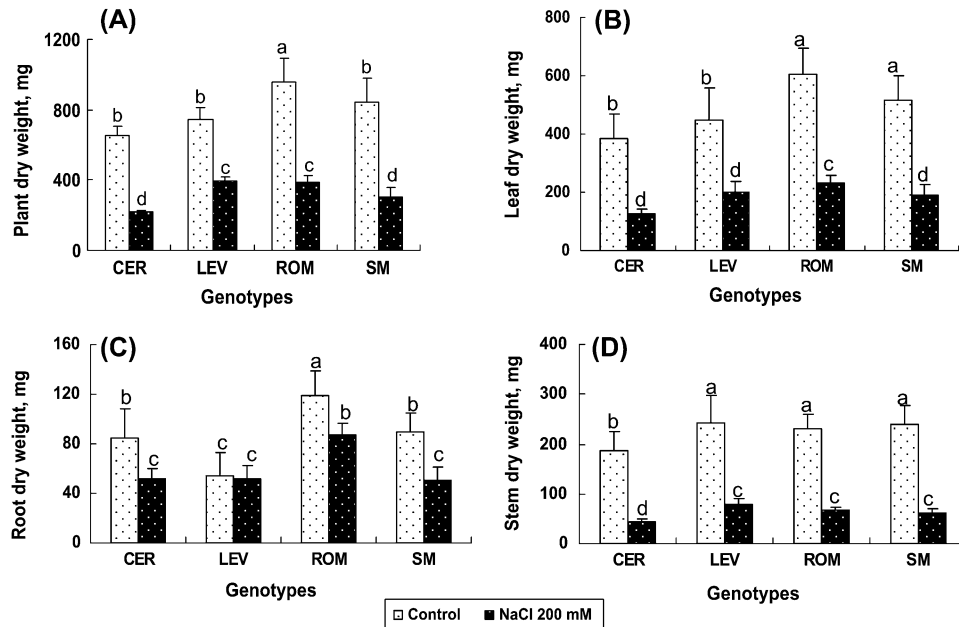


Fig. 1. Effect of salinity on plantlets growth for the four tomato lines. Dry weight comparison of control plants and plants submitted to salinity (0 mM and 200 mM of NaCl) in (A) whole plants, (B) leaves, (C) roots, and (D) stems. Data are means of 32 replicates \pm SE at $P > 0.05$. Letters correspond to Duncan's multiple range test at 95%.

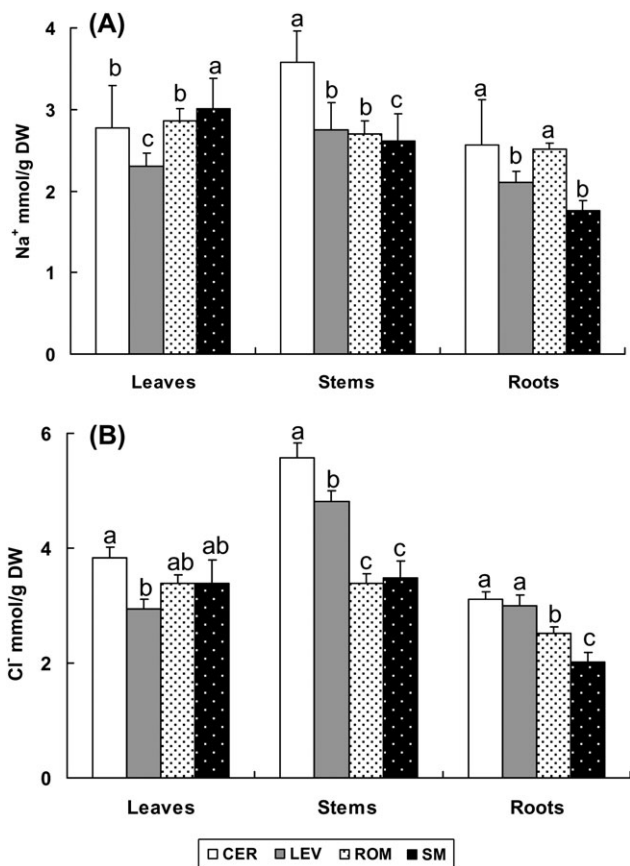


Fig. 2. Na^+ and Cl^- ions accumulation within tomato plants. Leaf, stem, and root Na^+ (A) and Cl^- (B) content of the four tomato cultivars after 14 d with 200 mM NaCl in the nutrient medium while, in control plants, no NaCl was added to the medium. Data are means of 32 replicates \pm SE at $P > 0.05$. Letters correspond to Duncan's multiple range test at 95%.

Marmande while Cervil exhibited the highest quantity of Cl^- in the leaves. Na^+ root content was higher in Cervil and Roma than in the other varieties. The highest concentrations of both Na^+ and Cl^- had previously been recorded in the shoots and the lowest in the roots (reviewed in Munns and Tester, 2008). One of the harmful effects of salinity on plant growth is the excessive accumulation of Na^+ and Cl^- in the leaves (Zhang and Blumwald, 2001; Munns et al., 2002; Ashraf and Harris, 2004). This accumulation under saline conditions depends on the plant's capacity to limit the uptake of these elements (Koval and Koval, 1996). Alian et al. (2000) showed that, in tomato, salt stress induced the uptake of considerable amounts of sodium and chloride and these accumulations were cultivar-dependent and organ-specific. In the present work, $\text{K}^+:(\text{K}^++\text{Na}^+)$ and $\text{Ca}^{2+}:(\text{Ca}^{2+}+\text{Na}^+)$ ratios have been used as nutritional indicators for the salt tolerance of tomato plants according to previously published works (Pérez-Alfocea et al., 1996; Cuartero and Fernández-Muñoz, 1999; Maathuis and Amtmann, 1999; Asch et al., 2000; Sairam et al., 2002). In some cases, these ratios have been demonstrated to be in direct proportional relationship with biomass production and it has been suggested that the control of Na^+ accumulation and high shoot $\text{K}^+:(\text{K}^++\text{Na}^+)$ and $\text{Ca}^{2+}:(\text{Ca}^{2+}+\text{Na}^+)$ ratios may enhance salt tolerance or resistance in tomato crops (Cuartero and Fernández-Muñoz, 1999; Al-Karaki, 2000; Dasgan et al., 2002).

Our results on the variation of the physiological parameters among the genotypes confirmed that tolerance to salt is a complex trait and that clustering the genotypes according to their salt tolerance is a tricky issue. In this experiment, the dose effect on the variation in physiological parameters was not studied. Nevertheless, in our test conditions, i.e. 200 mM NaCl and the young plantlet stage, cultivar Levovil could be classified as the most tolerant to

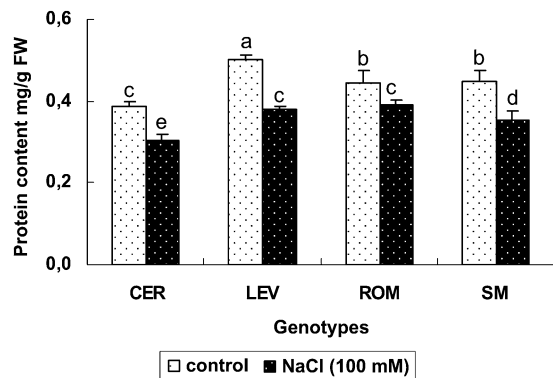


Fig. 3. Effect of salinity on root protein content in four tomato genotypes. Effect of NaCl treatment (0 mM and 100 mM) on protein content in roots. Data are means of four replicates \pm SE at $P > 0.05$. Letters correspond to Duncan's multiple range test at 95%.

salt stress because of the low foliar accumulation of Na^+ (Fig. 2) and the high foliar $\text{K}^+:(\text{K}^++\text{Na}^+)$ ratios (Table 1) and a lower root growth reduction compared with the other genotypes. Levovil and Roma also presented the highest foliar $\text{Ca}^{2+}:(\text{Ca}^{2+}+\text{Na}^+)$ ratios. By contrast, Cervil and Super Marmande which had the lower foliar $\text{K}^+:(\text{K}^++\text{Na}^+)$ ratios and a high dry matter decrease appeared as the most sensitive.

For the proteomic part of the work, use was made of these four genotypes showing differences in response to salt stress, in order to investigate the proteomic bases of the variability of the tomato response to stress in salt concentrations similar to those that can be found in salty soils (100 mN NaCl). At this dose the differences in plant growth were maintained between genotypes (see Supplementary Fig. S1 at *JXB* online). For breeding purposes, it would also be valuable to analyse further the behaviour of these four genotypes during the fruit production phase.

Tomato root proteome was modified by salt stress

The plant root is the first organ to suffer from exposure to salt stress (Steppuhn and Raney, 2005). Actually, in our experiment, the roots showed the greatest contrasting response among all the organs. For these reasons, the root proteome was investigated in response to prolonged salt stress by performing 2-DE analysis of the total proteins from three or four biologically independent replicate experiments.

Protein content measurement (Fig. 4) showed genotype-dependent variations. Levovil presented the highest root protein content and Cervil the lowest in the control and salt treatments. Salt stress led to a decrease in root protein content for all genotypes from 12–25%; Levovil exhibited the highest reduction (25%), while in other genotypes the reduction was of 21, 22, and 12%, for Super Marmande, Cervil, and Roma, respectively. A higher protein content in tolerant cultivars in control conditions has already been reported in several plants as well as a decrease in the total protein content due to salt treatment (Ashraf and Harris, 2004; Debouba *et al.*, 2006).

Table 1. Comparison between genotypes sensitivity/tolerance to salt stress

K^+/Na^+ and Ca^+/Na^+ ratio were calculated in leaves, stems, and roots of tomato plants submitted to salinity stress (200 mM). Letters correspond to Duncan's multiple range test at 95%.

Genotypes	Leaf $\text{K}/(\text{K}+\text{Na})$	Stem $\text{K}/(\text{K}+\text{Na})$	Root $\text{K}/(\text{K}+\text{Na})$
Cervil	0.19 b	0.23 a	0.21 a
Levovil	0.24 c	0.40 c	0.29 b
Roma	0.20 b	0.38 c	0.25 a
Super Marmande	0.13 a	0.32 b	0.30 b
	Leaf $\text{Ca}/(\text{Ca}+\text{Na})$	Stem $\text{Ca}/(\text{Ca}+\text{Na})$	Root $\text{Ca}/(\text{Ca}+\text{Na})$
Cervil	0.23 a	0.12 a	0.03 a
Levovil	0.32 b	0.17b	0.03 a
Roma	0.37 b	0.22c	0.06 a
Super Marmande	0.21 a	0.15 b	0.07 a

A representative 2-DE Coomassie brilliant blue-stained gel of the Cervil genotype is presented in Fig. 2. The root 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 1300 detected spots, a total of 90 protein spots exhibited significant spot abundance variation ($P \leq 0.05$). These spots were excised from the gels for LC-MS/MS analysis to determine protein identity. In total, it was possible to obtain good quality protein identification results with a high probability score for 80 out of the 90 protein spots. For the ten remaining spots, it was not possible to determine the protein spot identity (see Supplementary Table S1 at *JXB* online) because of a lack of matching unigene sequences. All the proteins identified in this study have enriched the proteomic database of the tomato fruit SOLstIS developed at INRA Avignon (<http://w3.avignon.inra.fr/solstis/>). Some functions were common to different spots (Table 2). For example, Elongation factor 1B alpha-subunit 2 (SGN-U313292) was identified from six spots, peroxidase (SGN-U315420) was identified from four spots, thioredoxin H-type 1 (SGN-U313399) and caffeoyl-CoA O-methyltransferase 6 (SGN-U315544) were identified, respectively, in two spots. Some of the proteins identified had different pI and M_r values from their theoretical values. This phenomenon is commonly observed in proteomic analyses, and is probably a consequence of post-translational modifications such as glycosylation, phosphorylation, or proteolytic cleavage, but it can also be related to the presence of gene isoforms and to allelic polymorphism (Rossignol *et al.*, 2006).

The identified proteins were distributed into nine classes according to their function including energy and carbon metabolism (23 spots), oxidative stress (14 spots), stress and defence response (15 spots), protein translation, processing, and degradation (11 spots), cell wall-related (five spots), hormone-related and amino acid metabolism (nine spots), and miscellaneous (three spots) (see Supplementary Fig. S2 at *JXB* online). Among them, proteins of the first functional group accounted for more than 30% of the total number of spots identified. As recapitulated in Table 2 (and in

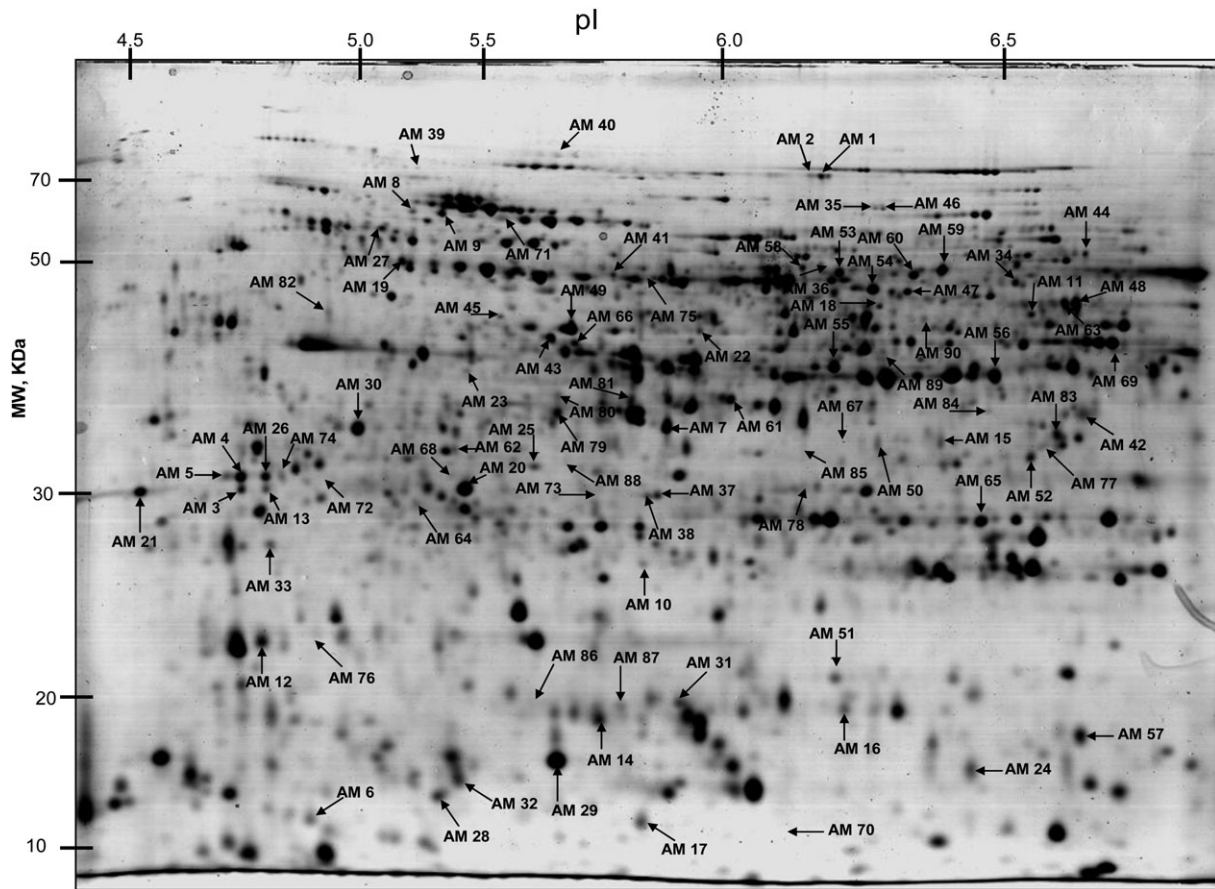


Fig. 4. Representative two-dimensional electrophoresis gel of tomato root proteins of Cervil, in control growth conditions. The positions and numbers of the 80 identified protein spots are indicated by arrows.

Supplementary Table S1 at *JXB* online for the 10 unidentified spots), only seven (plus two unidentified) spots showed variation strictly related to salt stress whatever the genotype, while many more spots (30 plus three) only varied according to the genotype. The remaining major part (43 plus five) of the spots exhibited either up- or down-regulation according to both factors (genotype and salt treatment), with or without an interaction effect between these two factors.

Some spots exhibited abundance variations only linked to the tomato genotype and were not regulated by salt stress

One of the important outputs of our work relies on the impact of the genetic factor on spot intensity. This effect was more widespread than the salt effect alone. In total, 30 spots exhibited significant differences among the genotype. These differences were mainly associated with the tomato fruit type, Cervil spot intensity being, most of the time, different from the three other variety spot intensities. Cervil is a cerasiforme type of tomato and the three other genotypes are classical large-fruited tomatoes (*S. lycopersicum*). The *S. lycopersicum* cerasiforme group evolved through hybridization between *S. lycopersicum* and a wild relative species *S. pimpinellifolium* (Ranc et al., 2008). This

distant phylogenetic origin may explain the differences observed in the Cervil proteome pattern (see Supplementary Fig. S3 at *JXB* online). Actually, as many as 19 spots among 30 were more intense in Cervil than in the other three genotypes (AM41, 11, 42, 17, 14, 13, 26, 5, 15, 12, 8, 9, 10, 24, 49, 3, 4, 37, and 45). In several cases, the genetic effect relied on the quantity of the protein isoforms. Aldehyde dehydrogenase, elongation factor, and subtilisin (P69C) showed several isoforms with contrasting patterns of expression according to the genotype. The functions of the proteins varying between Cervil and the other genotypes were mainly related to protein translation, processing, and protection: thioredoxin, disulphide isomerase, subtilisin, elongation factor, peroxidase, and HSP.

In the case of glutamine synthetase (AM62) the spot was more intense in the two tolerant genotypes, Levovil and Roma, so the quantity of this cytoplasmic protein isoform might be associated with the genotype's constitutive tolerance to salt stress. Indeed, glutamine synthetase is reported to play a pivotal role in nitrogen assimilation through reassimilation of NH_4 from photorespiration and proteolysis processes in plants subjected to salt stress and water deficiency (Tsai and Kao, 2002; Ouyang et al., 2007) via the synthesis of glutamine and the precursors of proline. However, it is important to notice that another spot of glutamine synthetase (AM43, chloroplastic) was

Table 2. Tomato root proteins varying among genotypes and salt conditions identified by LC-MS/MS

Spot ID ^a	Accession no. ^b	Protein identification	Organism	PS ^c TargetP	Experimental pI/M _r ^d	Theoretical pI/M _r ^e	PN ^f	C % ^g	Salt effect	Geno effect ^h	Int G×E	Cervil ⁱ	Levovii ⁱ	Roma ⁱ	SM ⁱ
Energy and carbon metabolism															
AM19	SGN-U312672	Vacuolar ATPase subunit B	<i>Mesembryanthemum crystallinum</i>	cyt	5.52/56	4.96/54.13	4	10	***	***	***	9.6/-3.2	16.8/-10.5	19.3/-12.7	11.4/-3.8
AM59	SGN-U316882	ATP synthase F1 subunit 1	<i>Nicotiana tabacum</i>	SP	6.37/54	5.84/55.23	27	53	**	–	–	19.1/+7.2	24.3/+4.1	20.4/+5.6	21.6/+5.8
AM41	SGN-U319484	Aldehyde dehydrogenase (ALDH1a)	<i>Oryza sativa</i>	cyt	5.91/53	6.29/54.89	13	54	–	***	–	5.3/+1.3	5.4/-0.8	4.0/+0.1	3.0/+0.1
AM53	SGN-U316642	Methylmalonate semi-aldehyde dehydrogenase	<i>Arabidopsis thaliana</i>	mTP	6.24/54	7.54/58.01	29	57	***	***	**	8.2/+1.1	6.1/+3.5	5.8/+4.0	6.8/+2.3
AM56	SGN-U312385	Malate dehydrogenase cytosolic	<i>Solanum chilense</i>	pTP	6.44/38	6.10/35.74	13	47	***	***	–	25.6/+10.4	26.8/+9.1	22.8/+7.0	24.1/+9.0
AM58	SGN-U318400	NADP quinone oxidoreductase	<i>Solanum lycopersicum</i>	cyt	6.19/55	5.87/59.10	17	41	***	–	–	3.8/+1.2	4.1/+1.1	4.0/+0.5	4.1/+0.7
AM75	SGN-U319484	Aldehyde dehydrogenase (ALDH1a)	<i>Oryza sativa</i>	cyt	5.96/52	6.29/54.89	13	57	–	**	–	3.5/+1.7	5.6/+0.5	7.4/+0.5	5.7/+0.4
AM89	SGN-U312518	Phosphoglycerate kinase	<i>Solanum tuberosum</i>	cyt	6.30/40	6.06/42.54	6	21	***	**	–	5.1/-0.1	5.0/-0.4	5.3/-1.46	8.4/-3.4
AM25	SGN-U315096	Putative 6-phosphogluconolactonase	<i>Oryza sativa</i>	pTP	5.78/31	5.44/28.02	15	50	**	***	–	4.8/-1.6	2.3/+0.4	1.9/+0.4	2.1/+0.1
AM88	SGN-U315096	Putative 6-phosphogluconolactonase	<i>Oryza sativa</i>	pTP	5.85/32	5.44/28.02	3	17	***	***	***	1.5/+0.3	1.4/+0.1	1.3/+0.4	3.1/-1.2
AM68	SGN-U315098	6-phosphogluconolactonase-like protein	<i>Arabidopsis thaliana</i>	pTP	5.61/31	5.44/28.02	18	55	–	***	–	1.6/+0.8	4.5/-0.4	4.2/+0.9	4.9/+0.4
AM34	SGN-U319405	Phosphogluconate dehydrogenase	<i>Arabidopsis thaliana</i>	cyt	6.47/52	7.02/53.58	16	56	***	****	–	4.6/+1.6	2.7/+3.4	3.4/+1.8	5.1/+2.2
AM35	SGN-U312322	Transketolase 1	<i>Capsicum annuum</i>	cyt	6.28/73	6.16/80.11	10	37	*	–	–	2.1/+0.5	1.4/+0.8	1.4/-0.0	1.6/+0.7
AM46	SGN-U312320	Transketolase 1	<i>Capsicum annuum</i>	cyt	6.29/72	6.16/80.11	12	58	***	***	–	2.7/+1.4	2.1/+2.1	2.2/+0.3	3.9/+0.3
AM54	SGN-U312830	UDP-glucose pyrophosphorylase	<i>Solanum tuberosum</i>	cyt	6.28/50	5.70/51.87	42	73	***	***	–	13.7/+2.7	10.6/+5.9	9.8/+4.5	12.6/+3.1
AM11	SGN-U315474	Beta-ketoacyl-ACP synthase	<i>Ricinus communis</i>	SP	6.49/45	6.76/49.37	6	22	–	**	–	2.6/-0.3	1.2/+0.2	1.0/+0.1	1.2/-0.3
AM23	SGN-U321873	Fructokinase	<i>Solanum lycopersicum</i>	cyt	5.65/39	5.20/37.31	9	32	–	*	–	0.6/-0.02	0.3/+0.3	.3/-0.0	0.4/-0.1
AM44	SGN-U314200	Dihydroliipoamide dehydrogenase precursor	<i>Solanum lycopersicum</i>	mTP	6.56/58	6.90/52.81	17	48	***	***	–	3.8/+2.0	5.4/+1.3	5.4/+2.6	6.4/+2.2
AM47	SGN-U312378	Enolase (2-phosphoglycerate dehydratase)	<i>Solanum lycopersicum</i>	cyt	6.33/50	5.68/47.80	17	52	***	***	–	6.2/+1.1	4.2/+2.5	4.3/+2.0	6.7/+2.0
AM50	SGN-U315298	Esterase D	<i>Arabidopsis thaliana</i>	cyt	6.29/33	5.91/31.66	7	45	***	–	–	4.6/+1.3	3.4/+2.7	3.1/+2.4	4.2/+1.2
AM51	SGN-U319205	Beta-hydroxyacyl-ACP dehydratase	<i>Arabidopsis thaliana</i>	pTP	6.23/19	8.61/24.24	5	25	***	**	–	8.3/+2.4	7.4/+3.6	9.1/+3.9	7.8/+3.9
AM69	SGN-U315305	Pyruvate dehydrogenase	<i>Solanum lycopersicum</i>	pTP	6.60/40	6.87/43.37	28	54	***	***	–	1.5/+1.7	4.4/+1.7	4.0/+3.2	5.5/+1.8
AM84	SGN-U317665	1,3-Beta-glucanase	<i>Solanum lycopersicum</i>	SP	6.42/36	6.61/37.57	17	49	***	***	***	1.6/-0.4	4.7/-3.1	3.8/-2.8	5.6/-4.2
Oxidative stress															
AM64	SGN-U316119	Glutathione S-transferase	<i>Pisum sativum</i>	cyt	5.55/29	5.53/27.26	13	49	–	*	–	4.7/+0.3	6.1/+1.8	7.4/+0.6	6.9/+0.6
AM76	SGN-U322657	Glutathione peroxidase	<i>Arabidopsis thaliana</i>	cyt	5.32/20	5.11/19.07	5	35	–	**	–	5.7/+1.1	10.8/-2.6	11.1/-1.1	8.5/+1.3

Table 2. Continued

Spot ID ^a	Accession no. ^b	Protein identification	Organism	PS ^c Target	Experimental pI/M _r ^d	Theoretical pI/M _r ^e	PN ^f	C % ^g	Salt effect ^h	Geno effect ^h	Int G×E	Cervil ⁱ	Levovil ⁱ	Roma ⁱ	SM ⁱ
AM65	SGN-U312581	Ascorbate peroxidase 1, cytosolic (APX1)	<i>Solanum lycopersicum</i>	cyt	6.42/28	5.86/27.32	35	76	**	***	***	16.7/-1.8	10.7/+3.9	11.4/+0.8	15.1/+2.1
AM79	SGN-U315420	Peroxidase	<i>Populus alba</i> / <i>Populus tremula</i>	SP	5.82/35	5.17/35.16	21	61	***	***	-	15.2/-5.4	19.9/-9.9	22.3/-9.2	12.5/-5.6
AM80	SGN-U315420	Peroxidase	<i>Populus alba</i> / <i>Populus tremula</i>	SP	5.83/37	5.17/35.16	15	60	***	***	***	5.6/-1.2	7.1/-2.7	8.5/-4.1	4.9/-1.8
AM81	SGN-U315420	Peroxidase	<i>Populus alba</i> / <i>Populus tremula</i>	SP	5.94/36	5.17/35.16	23	61	***	**	-	6.1/-3.0	6.6/-3.1	8.6/-4.7	6.9/-3.8
AM83	SGN-U315420	Peroxidase	<i>Populus alba</i> / <i>Populus tremula</i>	SP	6.52/34	5.17/35.16	22	57	***	***	-	10.6/-4.9	7.6/-2.6	11.1/-4.8	10.9/-5.0
AM42	SGN-U314124	Peroxidase	<i>Nicotiana tabacum</i>	SP	6.56/35	6.10/38.53	20	58	-	***	-	14.8/-2.2	7.8/+1.3	8.4/-1.3	7.2/+0.2
AM17	SGN-U313399	Thioredoxin H-type 1 (TRX-H-1)	<i>Nicotiana tabacum</i>	cyt	5.95/14	5.62/13.96	7	51	-	***	-	14.5/-3.9	7.3/-1.2	5.0/-0.6	4.7/+0.7
AM70	SGN-U313399	Thioredoxin H-type 1 (TRX-H-1)	<i>Nicotiana tabacum</i>	cyt	6.17/14	5.62/13.96	6	51	**	**	*	6.6/+11.6	24.7/-6.4	24.0/-5.5	19.6/+2.8
AM77	SGN-U328536	NADPH-dependent thioredoxin reductase	<i>Arabidopsis thaliana</i>	cyt	6.50/33	5.82/35.31	8	61	-	***	-	3.6/+0.9	5.9/-0.5	6.8/-0.4	5.5/+0.2
AM67	SGN-U313302	Lactoylglutathione lyase	<i>Brassica oleracea</i>	cyt	6.25/34	7.63/31.55	20	58	-	***	-	4.8/+5.4	23.1/-5.0	25.5/-2.2	19.9/-0.9
AM07	SGN-U313302	Lactoylglutathione lyase	<i>Citrus paradisi</i>	cyt	5.99/34	5.46/32.64	21	68	**	***	-	21.5/-7.2	4.9/+3.2	5.9/-1.9	4.2/-0.1
AM85	SGN-U315784	Cysteine synthase	<i>Solanum tuberosum</i>	cyt	6.19/33	5.93/34.31	4	24	***	***	***	2.0/-0.6	1.8/-0.0	2.0/+0.2	6.2/-4.2
Stress defence and heat shock															
AM08	SGN-U313773	P69C protein	<i>Solanum lycopersicum</i>	mTP	5.54/70	5.27/70.68	4	11	-	***	-	1.9/-0.8	0.7/+0.1	0.5/-0.1	0.4/-0.1
AM09	SGN-U313773	P69C protein	<i>Solanum lycopersicum</i>	mTP	5.60/69	5.27/70.68	14	18	-	***	-	5.6/-2.4	1.8/+0.3	1.9/-0.8	1.5/-0.4
AM71	SGN-U313773	P69C protein	<i>Solanum lycopersicum</i>	mTP	5.72/67	5.27/70.68	13	18	-	***	-	4.3/+1.6	12.8/-3.6	13.6/-3.0	10.6/-2.1
AM01	SGN-U327796	Serine protease	<i>Solanum lycopersicum</i>	cyt	6.22/96	6.00/81.45	14	52	**	***	***	6.8/-3.6	2.5/+0.3	1.9/+0.3	1.7/-0.2
AM10	SGN-U321169	Class I small heat shock protein HSP 26.5	<i>Arabidopsis thaliana</i>	mTP	5.95/25	6.86/26.5	8	59	-	***	-	1.5/-0.5	0.6/-0.0	0.5/-0.1	0.4/-0.0
AM24	SGN-U316987	Class II small heat shock protein HSP17.6	<i>Solanum lycopersicum</i>	SP	6.41/16	6.32/17.62	9	63	-	***	-	14.5/-3.3	7.1/+1.6	5.6/+2.2	5.5/-0.3
AM39	SGN-U316401	Hsp90 protein	<i>Ricinus communis</i>	cyt	5.55/100	5.19/90.67	10	28	**	-	-	0.8/+0.4	0.7/+0.4	0.7/+0.4	0.6/+0.5
AM40	SGN-U314389	Heat shock hsp70 protein	<i>Ricinus communis</i>	cyt	5.83/109	5.22/93.59	5	29	**	***	-	2.9/+0.6	1.9/+1.2	1.6/+0.3	2.6/-0.0
AM27	SGN-U312542	60 kDa chaperonin subunit alpha	<i>Solanum lycopersicum</i>	SP	5.48/64	5.08/62.07	12	41	**	***	***	4.5/-2.0	1.8/+0.3	2.1/+0.1	2.2/-0.1
AM14	SGN-U312368	Pathogenesis-related protein 10/ PR 10	<i>Solanum virginianum</i>	cyt	5.89/17	5.29/17.59	13	75	-	***	-	19.9/+0.7	10.4/+5.3	10.3/-2.7	11.4/-5.0
AM87	SGN-U312368	Pathogenesis-related protein 10/ PR 10	<i>Solanum virginianum</i>	cyt	5.92/18	5.29/17.59	7	48	***	***	*	22.4/-5.9	27.1/-6.7	31.6/-8.6	41.2/-18.6
AM86	SGN-U312370	TSI-1	<i>Solanum lycopersicum</i>	cyt	5.77/18	5.61/20.22	6	45	***	***	***	4.7/-0.1	4.8/+0.1	5.4/-1.7	10.0/-5.9
AM20	SGN-U313542	Plasma membrane polypeptide	<i>Nicotiana tabacum</i>	cyt	5.64/30	5.06/24.41	22	73	***	***	***	27.5/+0.2	10.2/+14.8	11.9/+7.8	17.0/+8.8
AM49	SGN-U314753	Actin	<i>Gossypium hirsutum</i>	cyt	5.85/43	5.24/41.70	32	76	-	***	-	28.8/+2.3	18.8/+7.8	19.3/-1.8	25.8/+3.5
AM61	SGN-U314161	Annexin p35	<i>Solanum lycopersicum</i>	cyt	6.09/36	5.84/36.18	24	61	***	-	-	13.9/+5.4	14.2/+4.2	15.1/+4.7	15.4/+1.9
Protein translation, processing and degradation															
AM03	SGN-U313292	Elongation factor1B alpha-subunit 2	<i>Solanum demissum</i>	mTP	5.10/30	4.57/24.58	13	56	-	***	-	3.2/-1.2	0.8/+0.4	0.7/+0.3	0.5/+0.1

Table 2. Continued

Spot ID ^a	Accession no. ^b	Protein identification	Organism	PS ^c TargetP	Experimental pI/M _r ^d	Theoretical pI/M _r ^e	PN ^f	C % ^g	Salt effect	Geno effect ^h	Int G×E	Cervil ⁱ	Levovil ⁱ	Roma ⁱ	SM ⁱ
AM04	SGN-U313292	Elongation factor1B alpha-subunit 2	<i>Solanum demissum</i>	mTP	5.10/31	4.57/24.58	14	71	–	***	–	8.6/-3.7	1.8/+1.3	1.9/+0.1	1.5/+0.3
AM13	SGN-U313292	Elongation factor1B alpha-subunit 2	<i>Solanum demissum</i>	mTP	5.20/26	4.57/24.58	11	52	–	***	–	2.2/-0.2	1.1/+0.1	1.2/-0.1	0.8/-0.1
AM26	SGN-U313292	Elongation factor1B alpha-subunit 2	<i>Solanum demissum</i>	mTP	5.19/31	4.57/24.58	15	68	–	***	–	8.0/-0.2	3.8/+1.0	4.3/-0.8	4.0/+0.4
AM72	SGN-U313292	Elongation factor1B alpha-subunit 2	<i>Solanum demissum</i>	mTP	5.36/30	4.57/24.58	19	67	–	***	–	4.5/+3.4	13.3/-3.5	14.3/+1.4	13.3/-1.1
AM74	SGN-U313292	Elongation factor1B alpha-subunit 2	<i>Solanum demissum</i>	mTP	5.24/31	4.57/24.58	20	62	–	***	–	3.8/+1.7	8.0/-1.8	10.1/-0.9	9.5/-1.5
AM31	SGN-U312968	Transcription factor BTF3	<i>Solanum tuberosum</i>	cyt	6.01/18	6.31/17.48	6	56	***	*	–	7.0/-2.8	5.6/-1.6	7.6/-2.7	6.5/-1.7
AM57	SGN-U312969	Transcription factor BTF3	<i>Solanum lycopersicum</i>	SP	6.54/19	6.85/17.41	19	57	***	**	***	16.4/-2.5	11.6/+2.1	11.8/+5.1	10.8/+3.4
AM05	SGN-U314972	Ripening regulated protein DDTFR10-like	<i>Solanum tuberosum</i>	cyt	5.04/31	4.57/25.32	9	43	–	***	–	2.4/-0.9	0.4/+0.7	0.7/-0.1	0.6/-0.0
AM15	SGN-U328536	Disulphide oxidoreductase, putative	<i>Ricinus communis</i>	cyt	6.37/33	8.20/39.34	4	46	–	***	–	4.1/-1.4	2.2/-0.5	1.4/-0.1	1.6/-0.1
AM33	SGN-U315989	Remorin	<i>Solanum lycopersicum</i>	SP	5.20/26	5.64/21.85	3	13	–	*	–	7.3/-1.0	5.2/+0.4	5.3/+0.2	4.2/+1.4
Cell wall-related															
AM28	SGN-U313020	Glycine-rich RNA-binding protein (GRP7)	<i>Solanum tuberosum</i>	cyt	5.59/15	5.59/17.52	14	53	***	***	***	12.9/-4.9	6.7/+0.8	5.2/+1.0	9.3/-0.9
AM29	SGN-U313020	Glycine-rich RNA-binding protein (GRP7)	<i>Solanum tuberosum</i>	cyt	5.82/17	5.60/17.60	17	73	***	***	***	45.3/-11.0	20.3/+9.9	23.5/+7.2	33.0/-1.4
AM55	SGN-U313049	UDP-glucose: protein transglucosylase-like protein SIUPTG1	<i>Solanum lycopersicum</i>	SP	6.23/39	5.61/41.16	39	73	***	***	–	18.2/+1.2	12.0/+6.5	13.5/+4.7	15.3/+3.6
AM37	SGN-U315544	Caffeoyl-CoA O-methyltransferase 6	<i>Nicotiana tabacum</i>	cyt	5.98/30	5.30/27.80	4	28	–	***	–	6.6/-1.3	3.0/+0.9	2.6/+0.4	3.1/-0.1
AM73	SGN-U315544	Caffeoyl-CoA O-methyltransferase 6	<i>Nicotiana tabacum</i>	cyt	5.88/29	5.30/27.80	15	59	**	***	–	2.4/+0.2	3.9/+0.5	3.8/+0.7	3.7/+0.8
Amino acid metabolism and Hormone-related proteins															
AM60	SGN-U312375	Neutral leucine aminopeptidase preprotein	<i>Solanum lycopersicum</i>	pTP	6.33/53	7.92/60.28	38	69	***	–	–	13.3/+3.9	12.8/+5.2	12.4/+3.1	14.4/+3.6
AM62	SGN-U313256	Glutamine synthetase	<i>Nicotiana plumbaginifolia</i>	SP	5.62/33	5.29/38.55	13	47	–	***	–	3.4/+0.9	6.4/-0.6	6.8/-1.6	4.2/-0.3
AM43	SGN-U314517	Glutamine synthetase GS58	<i>Nicotiana attenuata</i>	pTP	5.81/41	6.68/47.59	31	52	***	***	***	12.5/+3.3	7.6/+8.3	9.5/+4.6	11.8/+5.3
AM63	SGN-U315500	DNA-binding protein GBP16	<i>Oryza sativa</i>	cyt	6.53/47	6.48/43.15	15	41	***	***	***	10.5/-2.3	4.2/+3.1	6.7/+1.0	7.4/-0.0
AM21	SGN-U315472	RNA-binding protein precursor	<i>Nicotiana tabacum</i>	mTP	4.75/30	4.64/28.34	28	55	***	**	–	14.6/+2.1	7.7/+7.4	14.9/+8.5	15.8/+6.9
AM48	SGN-U312580	S-adenosylmethionine synthetase	<i>Nicotiana tabacum</i>	cyt	6.55/47	5.96/42.60	13	42	***	***	***	20.7/-4.2	9.9/+7.0	13.0/+4.8	17.7/+0.4
AM66	SGN-U317294	Isopropylmalate dehydrogenase	<i>Brassica napus</i>	pTP	5.86/40	5.89/43.48	8	27	***	**	–	4.0/+0.3	3.0/+1.0	4.1/-0.0	3.8/+0.4
AM36	SGN-U312795	Ketol-acid reductoisomerase	<i>Capsicum annuum</i>	pTP	6.23/55	6.50/63.35	3	37	***	***	–	1.7/+1.1	3.0/+0.4	2.6/+1.2	3.5/+1.0
AM45	SGN-U329087	Aminomethyltransferase	<i>Zea mays</i>	pTP	5.71/45	5.93/44.38	4	37	–	***	–	2.2/-0.4	1.0/+0.6	1.2/-0.0	1.2/+0.1

Table 2. Continued

Spot ID ^a	Accession no. ^b	Protein identification	Organism	PS ^c TargetP pI/M _r ^d	Experimental pI/M _r ^d	Theoretical pI/M _r ^e	PN ^f	C % ^g	Salt effect ^h	Geno effect ⁱ	Int G×E	Cervilj ^j	Levovilj ^j	Roma ^j	SM ^j
Miscellaneous															
AM12	SGN-U314933	Metallothionein-like protein type 2 A (LeMT(A))	<i>Solanum lycopersicum</i> cyt		5.18/20	5.56/19	14	75	-	***	-	9.6/-1.3	3.4/+1.5	3.8/-0.2	2.8/+1.3
AM90	SGN-U313556	Ana1 domain-containing protein similar to Protein C14orf3 (HSPC322)	<i>Arabidopsis thaliana</i> mTP	mTP	6.03/45	5.70/40.17	13	36	**	***	-	2.3/+0.4	2.7/+0.5	2.6/+1.2	5.0/-0.1
AM78	SGN-U318707	Uncharacterized protein At2g37660	<i>Arabidopsis thaliana</i> cyt	cyt	6.20/30	6.77/27.61	11	45	***	**	-	2.7/+0.7	3.7/-0.0	3.4/+1.7	3.6/+0.8

^a Spot ID corresponds to position of the spot in the gel as illustrated in Fig. 3.

^b Database unigene/EST accession numbers.

^c Peptide signal according to 'TargetP' software; mTP, mitochondrial transit peptide; pTP, plast transit peptide; cyt, cytoplasm; SP, secreted protein.

^d Experimental pI and mass (kDa) of identified proteins. Experimental values were computed by Progenesis Software.

^e Theoretical pI and mass (kDa) of identified proteins.

^f Number of peptides matching to the unigene sequence.

^g Sequence coverage percentage.

^h ANOVA results for salt, genotype, and interaction effects. NS non-significant effect, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

ⁱ Normalized volume of identified proteins: control/increased or decreased in average volume after salt treatment.

demonstrated to be variable in our experiment. This spot was more intense in salt-sensitive genotypes and exhibited a decrease in abundance following NaCl treatment for all the genotypes. The exact role of these two forms in the resistance of tomato to salt stress has to be investigated further.

The other spot showing a genetic effect that might be correlated to salt sensitivity is actin (AM49) which was more intense in salt-sensitive genotypes. Recent studies have indicated a close relationship between the microfilament cytoskeleton and salt stress (Wang *et al.*, 2009). However, the existence of a genetic variability for actin cell content is, to our knowledge, unexplored.

Attempts to establish a genetic link between the observed genotypic variations and constitutive sensitivity or tolerance of the genotypes to adverse conditions such as salt stress would require complementary investigations such as a genetic association study (Gupta *et al.*, 2005). For such experiments, the polymorphism of the genes coding for the varying proteins identified here would have to be investigated and related to the response to salt stress.

Salt stress led to up-regulation of a common set of genetically non-variable tomato root proteins

Our results brought into light a set of proteins that were up-regulated by 14 d of salt stress whatever the tolerance or sensitivity of the genotype. The function of these proteins may correspond to a basic response of tomato roots to salt stress, including stress signalling (annexin P35, AM61), further up-regulation of energy production (ATP synthase AM59, transketolase AM35), detoxification and oxidative stress response (esterase D AM50, NADP quinone oxidoreductase AM58), and protein turnover and protection (neutral leucine amino peptidase AM66, HSP 90 AM39). Annexin expression, abundance, and cellular position are known to respond to osmotic stress, salinity, drought, and ABA (Watkinson *et al.*, 2003; Lee *et al.*, 2004; Buitink *et al.*, 2006; Vandeputte *et al.*, 2007). Annexins could form ROS-stimulated passive Ca²⁺ transport pathway and be central regulators or effectors of plant growth and stress signalling (Clark *et al.*, 2001; Bassani *et al.*, 2004; Cantero *et al.*, 2006; Mortimer *et al.*, 2008). Annexins also exhibit peroxidase activity in soluble or membrane-bound form (Hofmann *et al.*, 2000; Gorecka *et al.*, 2007; Konopka-Postupolska *et al.*, 2009; Laohavisit *et al.*, 2009). Stress induced membrane peroxidation has been linked to changes in plasma membrane H⁺-ATPase activity (Veselov *et al.*, 2002; Cheng *et al.*, 2009), which could affect hyperpolarization and annexin-mediated Ca²⁺ influx. In our experiment, the up-regulation of the ATP synthase F1 subunit 1 (AM 59), which increased in all NaCl-treated tomato roots, was also detected. Vacuolar H⁺-ATPase can generate a proton electrochemical gradient, which is the driving force utilized by the tonoplast Na⁺/H⁺ antiporter, to compartmentalize Na⁺ into the vacuole (Chinnusamy *et al.*, 2005). Variations of energy

transport-related proteins have already been reported following salt stress in tomato but with some contradictory data (reviewed in Ashraf and Harris, 2004). As a matter of fact, a second spot of ATPase was also identified (vacuolar ATPase subunit B, AM19, see below) which exhibited a general decrease in intensity under salt stress. This spot was more intense in the case of the two stress-tolerant genotypes. Moreover, Barkla *et al.* (2009) recently demonstrated the association of some glycolytic enzymes (aldolase and enolase) with the tonoplast and their effect on the regulation of the H⁺-pump activity.

The increase in NADP quinone oxidoreductase abundance may be related to its antioxidant property. This enzyme of the respiratory process is induced by oxidative stress and utilizes either NADH or NADPH as electron donors which might be produced by activation of the pentose phosphate pathway as discussed below.

If acidic leucine aminopeptidase is already known to be induced in response to wounding and may respond to signals generated during water deficit and salinity stress (Chao *et al.*, 1999), the regulation of neutral leucine aminopeptidase (LAP-N) by salt stress is a new hypothesis raised by our work. LAP-N is ubiquitous and has a role in protein turnover by participating in the regulation of ubiquitin-dependent protein degradation by exposing penultimate residues that influence protein half-life or by processing peptides released by the proteasome (Walling, 2006).

Finally, up-regulation of HSP 90 (spot AM39) in all treated tomato genotypes might be linked to its role in preventing aggregation of the denatured proteins and in facilitating the refolding under salt stress (Chang *et al.*, 2000; Wang *et al.*, 2008, 2009; Chen *et al.*, 2009). The over-expression of the HSP 90 gene in tobacco was found to improve salt stress tolerance (Liu *et al.*, 2006).

The observation that salt factor treatment alone explained only a very limited part of the total protein variations was a main result of our work. Indeed, about half of the spots identified varied both according to genetic and treatment factors, underlying the importance of the plant genetic composition in its response to abiotic conditions and showing a significant diversity in the tomato response that was not strictly related to the salt tolerance level.

Most of the carbon metabolism and energy-related proteins were up-regulated by salt stress and exhibited abundance variations with salt tolerance level

The majority of proteins, whose function was related to carbon metabolism, exhibited an up-regulation under salt-stress. Some of them also showed variation which could be related to the degree of genotype tolerance. If the central carbohydrate metabolism is known to be rapidly re-adjusted after salt stress, most of the studies dealing with short-term salt or osmotic stress reported the down-regulation of carbohydrate metabolism genes (Wang *et al.*, 1999; Kerepesi and Galiba, 2000; Ndimba *et al.*, 2005; Jiang and Deyholos, 2006). However, in the present work a general up-regulation of proteins related to primary

metabolism was found: glycolysis and citric acid circles (spots AM44, AM47, AM56, and AM69), pentose phosphate (spots AM25, AM34, AM46, AM68, and AM88), starch synthesis (AM54). It is likely that, in case of 14 d of salt stress, plant metabolism is differently affected than in case of a few hours of stress. This hypothesis is also supported by the results of Kempa *et al.* (2008) in *Arabidopsis thaliana*. Spots AM34, AM46, AM47, AM53, and AM54 exhibited similar patterns: they were less abundant in tolerant genotypes in control conditions but were up-regulated whatever the genotype following salt stress. Enolase (spot AM47) is one of the most important enzymes in glycolysis catalysing the dehydration of 2-phosphoglycerate to phosphoenolpyruvate. This enzyme is responsive to many environmental stresses, including salt stress, drought, cold, and anaerobic stress in different plant species (Umeda, 1994; Riccardi *et al.*, 1998; Yan *et al.*, 2005) and may act as a positive regulator of some stress responsive genes (Lee *et al.*, 2002). The pentose phosphate pathway which produces the NADPH needed by different ROS-scavenging systems, was clearly affected in our conditions. As an example, transketolase is known to play an important role in the stress-induced production of cytosolic NADPH. One of the two transketolase isoforms (spots AM35 and AM46) identified and a phosphogluconate dehydrogenase exhibited the same pattern of variation. An increase in transketolase activity was previously detected under conditions of salt and oxidative stress in maize (Rapala-Kozik *et al.*, 2008).

Methylmalonate semi-aldehyde dehydrogenase (spot AM53), involved in energy production, and UDP-glucose pyrophosphorylase (spot AM54) which is related to sucrose and starch metabolism were, in control conditions, also less abundant in tolerant genotypes than in sensitive ones but were more up-regulated by salt stress. The UDP-glucose pyrophosphorylase protein was previously shown to be up-regulated in *Arabidopsis* leaves after long-term salt treatment (Kempa *et al.*, 2008). UDP-glucose is used, directly or indirectly, as a precursor in the biosynthesis during cell wall biogenesis (Gibeaut, 2000) or in the synthesis of the carbohydrate moiety of glycolipids and glycoproteins (Flores-Diaz *et al.*, 1997; Bishop *et al.*, 2002). It may play important roles in the root morphological adaptation to salt stress.

Dihydrolipoamide dehydrogenase (spot AM44) and pyruvate dehydrogenase (spot AM69), are partners in the pyruvate dehydrogenase complex which transforms pyruvate into acetyl-CoA and links cytosolic glycolytic metabolism with the tricarboxylic acid cycle. Their patterns of variation were, actually, very similar and marked by an overall up-regulation in stressed tomato roots, but with a markedly lower expression in Cervil than in the other genotypes.

Our results showed that some proteins associated with energy production or with transport, malate dehydrogenase cytosolic (AM56) and methylmalonate semi-aldehyde dehydrogenase (AM53), also exhibited genotype- and salt-related abundance variations. Cytosolic malate dehydrogenase was reported to be responsive to salinity stress in root

and suspension cells of *Arabidopsis* (Ndimba *et al.*, 2005; Jiang *et al.*, 2007), and pea roots as well (Kav *et al.*, 2004). In addition, cytosolic malate dehydrogenase was up-regulated by long-term salinity stress in *Thellungiella halophila* leaves (Fei *et al.*, 2008).

By contrast with this general up-regulation of carbohydrate metabolism, two spots (AM19 vacuolar ATPase and AM89 phosphoglycerate kinase) were less abundant after salt stress. V-ATPase, which may provide the driving force for Na⁺ transport, via Na⁺-H⁺ exchangers, to isolate toxic ions within the vacuole, has been associated, in many studies, to the ability of the plant to resist salty conditions. The higher abundance of this protein in Levovil and Roma genotypes could, therefore, be linked to their higher salt tolerance level compared with the other genotypes. However, the decrease in the quantity of protein after a 2-week stress might indicate that different mechanisms occur during salt stress. A time-dependent effect of salt stress was also observed by Kabala *et al.* (2008) on vacuolar ATPase activity in *Cucumis sativus*.

Some proteins putatively involved in osmoprotectant synthesis and cell wall reinforcement were up-regulated by salt stress mainly in tolerant genotypes

Hyperosmotic stresses have been shown to induce the accumulation of various free amino acids in plant cells, among them glutamine, asparagine, and proline (Fougere *et al.*, 1991; Wang *et al.*, 1999; Di Martino *et al.*, 2003; Ashraf and Harris, 2004). In this experiment, amino acid content was not measured but the variations of various enzymes playing a role in amino acid biosynthesis could be identified. *S*-adenosylmethionine synthetase (SAMS, AM48), isopropylmalate dehydrogenase (spot AM66), and glutamine synthetase (spot AM43) are all involved in amino acid biosynthesis and could play a role in osmotic adjustment in roots under salt stress by protecting enzymes and membranes (Yancey *et al.*, 1982; Ouyang *et al.*, 2007). Glutamine synthetase is reported to play a pivotal role in nitrogen assimilation through reassimilation of NH₄ from photorespiration and proteolysis processes in plants subjected to salt stress and water deficiency (Tsai and Kao, 2002) via the synthesis of glutamine and precursors of proline. In our experiments, one spot of glutamine synthase, being addressed to the chloroplast (spot AM43), increased in abundance following NaCl treatment for all the genotypes, but to a greater extent in the Levovil genotype. The abundance of *S*-adenosylmethionine synthetase, SAMS (spot AM48) was higher in salt-sensitive genotypes but only increased in salt-tolerant treated roots. In the same way, the up-regulation of UDP-glucose pyrophosphorylase (spot AM54) in all genotypes under salt stress, but mainly in the most tolerant genotypes, may signal an adjustment by sucrose as an osmotic protectant (see above).

Three cell wall-related proteins putatively involved in cell wall reinforcement were identified: glycine-rich RNA-binding protein GRP7 (AM28, AM29), caffeoyl-CoA *O*-methyltransferase 6 (AM37 and AM73), and UDP-

glucose:protein transglucosylase-like protein SIUPTG1 (AM55). All these proteins were more expressed in the Cervil genotype. The first one, namely glycine-rich RNA-binding protein, plays certain roles in the post-transcriptional regulation of gene expression in plants under various stress conditions and its synthesis is part of the plant's defence mechanism (Mousavi and Hotta, 2005). In this study, GRP7 appeared to be only down-regulated in roots of the sensitive genotype (Cervil), but its abundance was increased by salt stress in roots of Levovil and Roma. The second protein, namely caffeoyl-CoA *O*-methyltransferase 6 (AM37 and AM73) plays a vital role in lignin biosynthesis of the cell wall (Pakusch *et al.*, 1989; Martz *et al.*, 1998). AM73 varied only according to genotype effect as discussed previously, being less abundant in Cervil than other genotypes. The second form of this protein, AM37, was by contrast in higher abundance in Cervil where it was down-regulated by salt stress. AM37 was up-regulated in the roots of the most tolerant genotype. Previous studies demonstrated that caffeoyl-CoA *O*-methyltransferase was present in control conditions and increased with the NaCl stress treatment in foxtail millet seedlings (Veeranagamallaiah *et al.*, 2008) and was more up-regulated in the salt tolerant rice variety Pokkali than in the salt-sensitive variety IR29. Furthermore, it was suggested that increasing this protein which is involved in lignin biosynthesis may help to reduce the bypass water flow that allows Na⁺ ions to enter the root via the apoplastic route (Yeo *et al.*, 1999) which would be coherent with the low accumulation rate of sodium in our most salt-tolerant genotype (Levovil). The concerted up-regulation of SIUPTG1 and UDP-glucose pyrophosphorylase in the tolerant genotypes suggested a role if these proteins in cell wall biosynthesis in response to salt stress. A similar behaviour of these two proteins was described in tomato roots subjected to iron deficiency (Li *et al.*, 2008).

Some antioxidant and defence proteins were unexpectedly down-regulated by salt stress

Previously, the tomato root antioxidative system of the wild salt-tolerant species *S. pennellii* was shown to be up-regulated by salt (Mittova *et al.*, 2004). Exposure of plants to salt-stress increased the production of reactive oxygen species (ROS) that cause oxidative damage to different cellular components, including membrane lipids, proteins, and nucleic acids (Zhu *et al.*, 2000; Apel and Hirt, 2004; Tanaka *et al.*, 2006).

Plants can regulate the ROS by scavenging them with antioxidant enzymes such as ascorbate peroxidase, glutathione peroxidase, and glutathione *S*-transferase. Other enzymes such as thioredoxin act as a major defence system against oxidative damage by reducing the disulphide bonds of oxidized proteins. Such proteins (AM65, AM76, AM64, and AM70) were detected in our experiment, all of them being considered as very important salt adaptation proteins in plants (Apel and Hirt, 2004; Askari *et al.*, 2006; Fulda *et al.*, 2006; Bhushan *et al.*, 2007). However, only ascorbate peroxidase exhibited variations related to salt and genotype

factors as Levovil, the most tolerant genotype showed a significantly increased abundance of ascorbate peroxidase. Similar results were obtained by Mittova *et al.* (2004) in the case of the wild salt-tolerant tomato species *S. pennellii*. Four spots (AM79, AM80, AM81, and AM83) corresponding to the same cell wall peroxidase protein were down-regulated after salt stress but with variations according to the genotype. These data are in opposition to previous studies that demonstrated the up-regulation of peroxidase by salt stress in rice (Yan *et al.*, 2005) and in *Arabidopsis* (Jiang *et al.*, 2007) roots. Moreover, thioredoxin H-type 1 (TRX-H-1) was up-regulated under salt treatment in Cervil and Super Marmande and down-regulated in Levovil and Roma. Such a pattern of variations suggested that, in sensitive genotypes, a prolonged oxidative stress occurred while, in tolerant genotypes, it was more rapidly overcome.

Among the 4 HSP identified in our study, HSP 70 (AM40) was the only one which varied with both genotype and salt factors. This cytosolic protein was more abundant in sensitive varieties and up-regulated in all genotypes by salt stress. It plays a role in a variety of cellular processes by keeping proteins in a competent state and also for their translocation to subcellular compartments (Sung *et al.*, 2001) and has already been found to be up-regulated in tomato fruit after cold stress (Page *et al.*, 2010).

TSI-1 (tomato stress induced-1) protein (AM86), and pathogenesis-related protein PR 10 (AM87), are two PR proteins constitutively present in all genotypes but mainly in Super Marmande. These proteins appeared to be strongly down-regulated by salt treatment while they were reported to be up-regulated in the case of a short stress (Moon *et al.*, 2003; Jellouli *et al.*, 2008).

A transcription factor and a membrane protein may play a role in the tomato salt response

Two spots of BTF3 were found (AM31, AM57) corresponding to two unigene accessions. This protein controls translation initiation and the first step of elongation. AM31 mainly varied according to the salt factor, being down-regulated in all genotypes, while AM57 varied according to the combined effect of the genotype and salt factors. This isoform was down-regulated in Cervil and up-regulated in all other genotypes. It was shown (Yang *et al.*, 2007) that down-regulation of BTF3 expression in *Nicotiana benthamiana* reduced the chloroplast size, and the expression of many chloroplast and mitochondria-encoded genes. The affected cells produced excessive amounts of reactive oxygen species. In our study, 14 mitochondrial and 11 plastidial proteins were detected. Lastly, a plasma membrane protein showed interesting variations under salt stress. This DREPP (developmentally regulated polypeptide) has the property to bind calcium and may be associated with the Ca²⁺ signal transduction pathway under salt stress stimuli. It was also found to increase in rice after salt stress (Cheng *et al.*, 2009). The exact role of these two proteins deserves a deeper analysis.

Conclusion

Comparing the response to 14 d stress of four tomato genotypes at the young plantlet stage, it was possible to characterize different levels of sensitivity among the genotypes and a variability of physiological parameters among the tomato organs. It is proposed that Cervil and Super Marmande are the most sensitive genotypes while Levovil and Roma are the most tolerant ones. 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. Part of the observed variations, were unexpected and might be linked to the duration of the stress in our experiment. The variability observed for the proteome variation underlines the necessity to take into account the genetic variation when analysing the tomato response to salt stress.

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