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Investigation of the response to salinity of transgenic potato plants overexpressing the transcription factor StERF94

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Salinity is one of the most important constraints threatening the cultivation of potato plants (*Solanum tuberosum* L.). It affects plant growth and leads to significant yield loss. Consequently, it is important to improve the tolerance of potato plants to salinity. In this context, we investigated the involvement of a potato ethylene responsive factor (StERF94) in plant response to salinity, since our previous genome-wide analysis showed that it may be related to biotic and abiotic stress response. ERF proteins belong to a large family of transcription factors that participate in plant response to abiotic stresses. We have previously identified the StERF94 gene which shows increased expression in potato plants submitted to salt treatment. In this study, transgenic potato plants overexpressing StERF94 were produced and submitted to salt treatment (100 mM NaCl) *in vitro* and under greenhouse culture conditions. StERF94 transgenic lines showed lower decrease of stem elongation under salt treatment in comparison to non-transgenic wild-type plants. Moreover, these plants showed a low level of H_2O_2 and Malondialdehyde content, and an increase in catalase and GPX (Gluthation peroxidase) activities compared to non-transgenic plants. In a second step, enhanced expression of some target genes for example CuZn-SOD, DHN25 (Dehydrin) and ERD (Early Responsive to Dehydration) was noted in the StERF94 transgenic plants, submitted to salt treatment. The StERF94 factor was also involved in the activation of osmoprotectant synthesis. Taken together, all these data suggest that overexpression of the StERF94 transcription factor increases the tolerance of potato plants to salinity by improving plant growth, osmoprotectant synthesis and antioxidant activityleading to low oxidative stress damage.

Keywords. Ethylene responsive factor; Salinity; *Solanum tuberosum*; transcription factor; transgenic plants

1. Introduction

Soil salinity is one of the most important global problems that negatively affects crop productivity (Isayenkov and Maathuis 2019). Indeed, about 8 million hectares of land throughout the world are saline. They cover 6 % of the total land area (Munns, 2005; Kikuchi *et al.* 2015). Moreover, 50 % of irrigated lands suffer from salinity (Geilfus *et al.* 2010; Parihar *et al.* 2015). High salinity generally causes osmotic stress, ionic imbalance, and water loss (Nazar *et al.* 2011; Khan *et al.* 2012; Iqbal *et al.* 2014; Rodziewicz *et al.* 2014). The first clear symptom of salt toxicity is inhibition of the shoot and root growth (Beck *et al.* 2007) in addition to enhanced oxidative stress (Iqbal *et al.* 2014).The overaccumulation of reactive oxygen species (ROS) like the hydroxyl radical (OH.), superoxide radical (O_2^{-}) and singlet oxygen ($^1O_2$), causes cell and tissue alteration by damaging membranes by lipid peroxidation and alters normal cell metabolism. It can also lead to DNA and protein alteration (Baby and Jini 2011).

To cope with these stresses, plants possess different response pathways involving hormones, protein kinases and several other signal molecules and effectors.

Hormones, such as ethylene, play an essential role in the control of plant growth and development, but they are also implicated in response to biotic and abiotic stresses. Besides its crucial role in regulating physiological mechanisms such as cell elongation, seed germination, ripening, senescence, wounding and abscission, ethylene is also involved in plant response to biotic and abiotic stresses by regulating the expression of several ethylene responsive transcription factors (ERF; Chen *et al.* 2012). These factors belong to the AP2/ERF (APETALA2/ERF) family, characterized by an AP2/ERF DNA-binding domain of 60 to 70 amino acids.

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This domain interacts with the dehydration responsive element (DRE)/C-repeat element (CRT) and/or the GCC box in the promoter region of ethylene inducible genes to regulate their expression (Lindemose *et al.* 2013; Golldack *et al.* 2011; Hussain *et al.* 2011).

Sakuma *et al.* (2002) classified the AP2/ERF transcription factors into three subfamilies based on the number and similarities of the AP2 domain. The AP2 factors possess two AP2/ERF domains, while the RAV factors (related to ABI3/VP1) possess one AP2/ERF domain, in addition to a B3 domain. Finally, the ERF family contains one AP2/ERF domain (Sharma *et al.* 2010). This latter family was also subdivided into two major subfamilies; the CBF/DREB (cold binding factor/dehydration-responsive element binding) and the ERF subfamily (Sakuma *et al.* 2002). The ERF family was also classified into 10 groups based on their conserved motifs (Nakano *et al.* 2006). The DREB factors correspond to group I to V, while the ERF factors belong to groups VI to X.

The ERF transcription factors were first isolated from tobacco plants (Ohme-Takagi and Shinshi 1995) and then from different other plants species (Zhang *et al.* 2016; Müller and Munné-Bosch 2019).

Some ERF genes overexpressed in plants conferred resistance to bacterial and fungal pathogens (Xu *et al.* 2008) and improved response to freezing, drought and salt (Lee *et al.* 2004; Zhang *et al.* 2010a, b; Xu *et al.* 2011).

In a previous study, we identified 155 AP2/ERF encoding genes in potato (Charfeddine *et al.* 2015), they correspond to 90 ERF and 65 DREB. The overexpression of the StDREB1 and StDREB2 factors in potato plants improved their tolerance to salinity, drought and oxidative stress (Bouaziz *et al.* 2012, 2013, 2015).The StERF94 factor belongs to group IX, which was mainly related to biotic stress response (Xu *et al.* 2011). However, this transcription factor seems to be activated by both biotic (*Phytophtora infestans*) and abiotic stresses (salt, drought, heat; Charfeddine *et al.* 2015). This gene seems also to be upregulated by ABA (abscissic acid), Jasmonic acid and ethylene treatment (Bouaziz *et al.* 2015).Therefore, it may be a good candidate gene for ectopic expression in potato plants in order to improve their tolerance to biotic and abiotic stresses. The StERF94 gene of 621bp harbors zero intron and is located on chromosome 5 (Charfeddine *et al.* 2015).

Potato (*S. tuberosum L.*) is an essential food crop grown around the world. It is classified as a sensitive species to salinity, drought and infection by pathogens (Daami-Remadi and El Mahjoub 2006) due to its shallow and sparse root system.

One of the main mechanisms adopted by plants to respond to abiotic stress is the antioxidant system based on enzymatic and non-enzymatic pathways (Gill and Tuteja 2010; Noctor *et al.* 2012). The enzymatic pathway implicates superoxide dismutases (SODs), which catalyzes the dismutation of superoxide anion radicals (O_2^-) to hydrogen peroxide (H_2O_2). Catalases and peroxidases remove the bulk of hydrogen peroxide generated (Simova-Stoilova *et al.* 2008). Several studies showed the involvement of ERF factors in oxidative stress tolerance. Indeed, the StDREB1 gene regulates the expression of some antioxidant enzymes and improves potato plant response to oxidative stress (Bouaziz *et al.* 2015). Similarly, Wu *et al.* (2008) revealed that the overexpression of tomato JERF3 in tobacco improved tolerance to salinity by regulating the oxidative and osmotic stress-related gene expression. The ectopic expression of pepper CaPF1 in potato significantly enhanced plant tolerance to oxidative stress (Youm *et al.* 2008). On the other hand, the production of osmoprotectants such as soluble sugars and proline is regulated by ERF transcription factors. For example, the P5CS (δ1-pyrroline-5 carboxylate synthase) gene, implicated in proline synthesis under salt stress conditions is regulated by StDREB factors (Bouaziz *et al.* 2012; 2013).

In the current study, we concentrated on StERF94 overexpression in transgenic potato plants (cv Spunta) and investigated the plant response to salinity under, both in *in vitro* and greenhouse culture conditions.

2. Materials and methods

2.1 *Plant material*

Potato plants (*S. tuberosum* L. cv. Spunta) were cultivated *in vitro* in MS basal medium (Murashige and Skoog 1962) supplemented with vitamins (Morel and Wetmore 1951) and 8 g/l agar in a growth chamber at 25°C under 250 $\mu E/m^2/s$ light intensity and for 16/8 hours photoperiod. The leaves and stems of these plants were used for gene transfer. These plants were also used as negative control (wild -type: WT).

2.2 *Extraction of total RNA and cDNA synthesis*

Total RNA was isolated from liquid nitrogen ground material (Vaewoerd *et al.* 1989) followed by a DNaseI treatment. The concentration and purity of the extracted RNA were checked by measuring the absorbance at 260 nm and 280 nm and determining the A260/A280 ratio. RNA quality was verified by electrophoresis on 1.5% (w/v) agarose gels.

cDNA synthesis was carried out in a final volume of 20 µl using 2 µg of total RNA and MMuLV Reverse Transcriptase (200U; BIO BASIC INC) (Charfeddine *et al.* 2015).

2.3 *Production of StERF94 transgenic potato plants*

The recombinant pMDC32 plasmid harboring the StERF94 cDNA (PGSC0003DMG400016004; Bouaziz *et al.* 2015) was transferred into *Agrobacterium tumefaciens* (LB4404 strain) as described by Hmida Sayari *et al.* (2005). The resulting recombinant *Agrobacterium tumefaciens* was used for the transformation of potato leaves and internodes using

the method of Bouaziz *et al.* (2012). Transgenic plant regeneration was performed in MS medium supplemented by with zeatin 2 mg l^{-1}, gibberellic acid (GA3) 0.02 mg l^{-1}, naphthalene acetic acid (NAA) 0.2 mg l^{-1}, 250 mg l^{-1} cefotaxime and 2 mg l^{-1} hygromycin (Bouaziz *et al.* 2012; 2013; Charfeddine *et al.* 2018). Potato seedlings that grow and develop roots in MS medium supplemented with 2 mg l^{-1} hygromycin were multiplied by node culture. The final screening and identification of transgenic potato plants were performed by PCR on genomic DNA using primers specific of the CaMV35S promoter and the pMDC32 sequences flanking the StERF94 cDNA.

2.4 *Semi-quantitative RT-PCR analyses*

Total RNA was extracted from potato plants of the StERF94 transgenic lines (SP1, SP4, SP5) and WT plants cultivated *in vitro* and submitted to salt treatment (100 mM NaCl) for 20 days. The expression of the StERF94 and three target genes were determined by semi quantitative RT-PCRs (Charfeddine *et al.* 2015). The early responsive genes to dehydration (ERD; PGSC0003DMT400042374), dehydrine (DHN25; PGSC0003DMG400009968) and CuZnSOD (PGSC0003DMG400000417) were monitored. The elongation factor (ef1α) gene (GenBank ID: AB061263) was used as constitutive gene marker. PCR-amplified products were visualized on 1.5 % agarose gels and quantified using the Gel Documentation System (BioRad), that determines the average band intensity, which was graphed using Microsoft Excel. All primer sequences used in this study are presented in table 1.

2.5 *In vitro stress treatment*

Salt stress was applied on 3-week-old plants cultivated *in vitro*. Transgenic (SP1, SP4 and SP5) and non-transgenic (WT) plants were cultivated in aqueous MS medium added with 100 mM NaCl. Plants cultivated in salt-free MS medium were used as controls (0 mM NaCl).

2.6 *Greenhouse culture*

Transgenic potato plants overexpressing StERF94 (SP1, SP4 and SP5) and non-transgenic plants (WT) were transferred to a greenhouse. Two weeks later, the plants were irrigated every two days with tap water supplemented with 100 mM NaCl. Plants irrigated with tap water were used as controls.

2.7 *Determination of chlorophyll content*

Samples of 0.01 g of leaves from WT and transgenic lines were homogenized using 0.5 ml of 100% acetone and then 1 ml of 80% acetone was added. The chlorophyll content was measured spectrophotometrically according to Arnon (1949).

2.8 *Measurement of oxidative stress parameters*

Leaf and root samples (0.05g) were ground in 0.1% trichloroaceticacid (TCA). The surpernatant obtained after centrifugation at 12000 rpm at 4°C for 30 min was added to 0.5% TBA (thiobarbituric acid) in 20% TCA. The homogenate was then incubated in a water bath at 95°C for 15 min and quickly cooled on ice and the absorbance was measured at 532 nm and 600 nm. After subtracting the non-specific absorbance (600 nm), the MDA content was determined as μmol MDA g^{-1} fresh weight (FW), according to a standard calibration curve (Hodges *et al.* 1999).

2.9 *Determination of H_2O_2 content*

Samples (0.1 g) of leaves and roots were ground in 2 ml 0.1% TCA at 4°C. The mixture was centrifuged at 12000 g at 4°C for 15 min, then 1 ml of KI (1M) and 0.5 ml of potassium phosphate buffer (10 mM pH 7.0) were added to 0.5 ml supernatant. The absorbance at 390 nm was measured and the H_2O_2 concentration was calculated using a standard curve (Alexieva *et al.* 2001).

2.10 *Determination of soluble sugar concentration*

Fresh material (0.1 g) was mixed with 5 ml ethanol, heated for 30 min at 70°C and centrifuged for 15 min at 8000 rpm at room temperature. Then, 5 ml of concentrated sulfuric acid and 1 ml phenol 5% were mixed with 1 ml of the supernatant. The soluble sugar content was calculated by

Table 1. Primer sequences of StERF94 and target genes used for semi-quantitative RT-PCR analyses

Genes	Forward primer (5′-3′)	Reverse primer (5′-3′)	Amplicon size (bp)	Tm (°C)
StERF94	TCACCCAATTCCTCATACCC	GGAAATCGGAGTGGAGATTG	621	60
Ef1α	ATTGGAAACGGATATGCTCCA	TCCTTACCTGAACGCCTGTCA	240	60
DHN25	ACGAAGACCAAATGCAGCAG	CTTCTTCCTCCGACCACCTT	448	60
ERD	GGGATCAATCAAAACGGCCT	GCAGGCATGGTAGACTTTGG	600	60
CuZnSOD	ATCCAACGGTGGTACCCAT	CCTGCGTTTCCAGTTGTCTT	600	60
pMDC32	TGTTTGAACGATCGGGGAAATTCGAGCTCC	GGATCCCCGGGTACCGGGCC	744	60

measuring the absorbance at 490 nm (Dubois *et al.* 1956) and expressed as glucose equivalent.

2.11 *Determination of proline content*

Fresh tissues (0.1 g) were ground in liquid nitrogen and 5 ml sulphosalicylic acid (3%) was then added to obtain a powder. The homogenate was centrifuged for 15 min at 4°C at 12,000 rpm, and 1 ml ninhydric acid and 1 ml glacial acetic acid were mixed with the supernatant. The mixture was heated at 100°C for 1 hour and then treated with 2 ml of toluene. The absorbance, at 520 nm, was determined and the proline concentration was calculated using a standard proline curve (Bates *et al.* 1973).

2.12 *Protein extraction*

Samples of leaves, stems and roots were ground in liquid nitrogen to a fine powder and supplemented with 1 ml of Tris HCl 100 mM, pH 8.0, EDTA 10 mM, $MgCl_2$ 20 mM, KCl 50 mM, PMSF (0.5 mM Phenylmethylsulfonyl fluoride), DTT (1 mM dithiothreitol), PVP 10% (w/w) (polyvinylpyrrolidone) and 0.1% Triton X-100 (v/v). The mixture was centrifuged for 30 min at 12,000 rpm at 4°C, and the supernatant containing the proteins was used to measure the protein concentration using the Bradford technique (Bradford 1976). These crude proteins were used to determine the activity of antioxidant enzymes. Three replicates were used per treatment.

2.13 *Determination of the antioxidant enzyme activities*

The SOD activity was determined spectrophotometrically using the photochemical nitroblue tetrazolium (NBT) method that measures the capacity of the SOD to prevent the transformation of NBT to formazan by superoxide (Beyer and Fridovich 1987).

Briefly, 25 µl of protein extract were mixed with 946.1 µl phosphate buffer (67 mM; pH 7.8), 500 µl Na_2EDTA methionine (9.9 mM), 42.6 µl 0.025% NBT and 11.3 µl 0.0044% riboflavin. The mixture was incubated for 20 min in the presence of light and the photo-reduction of NBT was determined by measuring the absorbance at 560 nm.

CAT activity was determined by monitoring the decrease in the absorbance at 240 nm, as H_2O_2 was consumed (Aebi 1984). Crude protein extract (100 µl) was mixed with 50 mM sodium phosphate buffer (pH 7.0) and 30% H_2O_2 in a final volume (2 ml). The CAT activity was measured as follows: CAT U/ml: [((3.45 × slope)/0.05) × (1000 /50)].

The GPX activity was determined spectrophotometrically according to the method of Flohé and Günzler (1984). The GPX allows glutathione oxidation (GSH) by H_2O_2 in the presence of DTNB (5–5'-dithio-bis 2-nitrobenzoic acid). Protein extract (200 µl) was mixed with 400 µl of a GSH solution (0.1 mM) in 200 µl of 67 mM phosphate buffer (pH 7.8).

After incubation of the mixture at 25°C for 5 min, 200 µl of H_2O_2 (1.3 mM) were added and the incubation was then continued for 10 min. After that 1 ml of 1% TCA was added to the reaction mixture followed by a centrifugation for 10 min at 12,000 rpm at 4°C, and 2.2 ml of Na_2HPO_4 (0.32 M), and 320 µl of DTNB (1 mM) were added to 480 µl of the supernatant. The absorbance at 412 nm was determined. The blank tube contained the reaction mixture without enzyme extract. The GPX activity was measured using the following formula: $\mu M\ GSH_{\text{Reduced disappeared/min/mg of protein}}$

$$\left(\frac{ODsample - ODwhite}{ODwhite}\right) \times \left(\frac{0.04 \times 5}{X \times 10}\right)$$

2.14 *Statistical analysis*

The results were examined by the GraphPad Prism version 5 using one-way analysis of variance (ANOVA), and $P<0.05$ was used for significant differences among means between salt-treated and non-treated plants.

3. Results

3.1 *Production of transgenic potato plants overexpressing StERF94*

Leaves and internodes of vitro-plants from the Spunta cultivar were used for *Agrobacterium tumefaciens*-mediated StERF94 cDNA transfer. The CaMV 35S promoter (Cauliflower mosaic virus) and the Nos (nopaline synthase) terminator (figure 1a) flanked the StERF94 cDNA. Regenerated plantlets that successfully rooted in the MS basal medium containing 2 mg l^{-1} hygromycin, were propagated *in vitro.* The presence of the transgene was determined by PCR analyses using a pair of primers that corresponds to the CaMV35S promoter leading to a 300 bp fragment and primers specific of the pMDC32 vector sequence flanking the StERF94 cDNA leading to 744 bp fragment (figures 1b and c). The analysis of the transgene expression was carried out by semi-quantitative RT-PCR using the pMDC32 primers (table 1; figure 1d). The StERF94 expression level increased significantly in transgenic lines under standard conditions compared to untransformed lines. The SP1 transgenic line showed the highest expression level of StERF94 (figures 1e and f).

3.2 *Evaluation of plant growth and development*

The response of StERF94 transgenic plants to salinity was investigated *in vitro* and under greenhouse culture

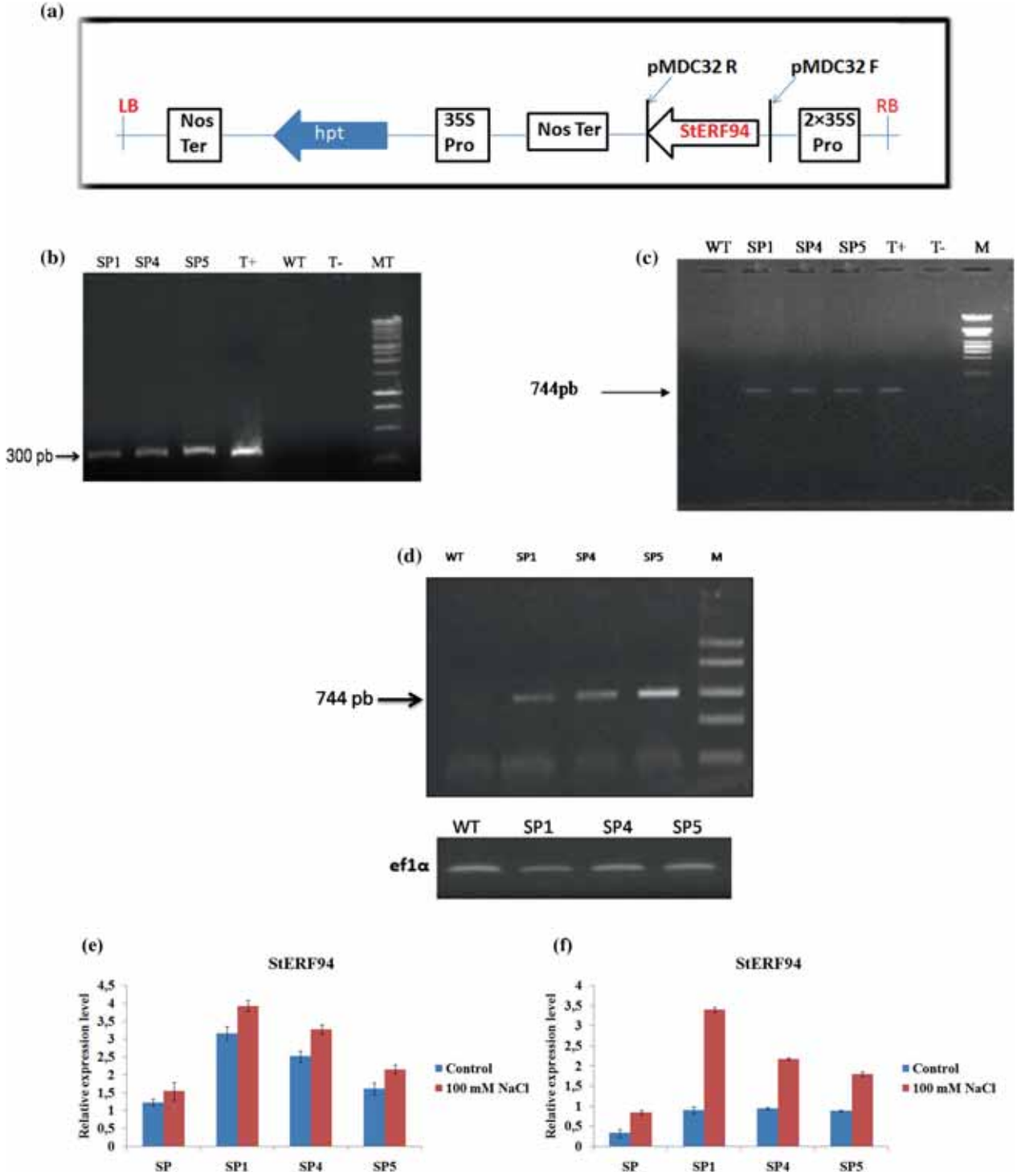

Figure 1. (**a**) Construct used to transform potato plants. 2X35S Pro: cauliflower mosaic virus (CaMV) 35S RNA doubled promoter, Nos-Ter: terminator from the nopaline synthase gene, and Agrobacterium T-DNA borders (LB left border and RB right border), pMDC32F and pMDC32R: specific primers of the binary vector, hpt: hygromycin phosphotransferase gene. **(b, c)** PCR amplification profiles on genomic DNA of the hygromycin resistant plants (SP1, SP4 and SP5) using 35S promoter and pMDC32 primers respectively. WT: untransformed wildtype plant; (T-): negative control without DNA; (T+); amplification using pMDC32 vector M: 100 bp DNA Ladder. (**d**) RT-PCR analysis using pMDC32 vector primers. The ef1α was used as control to normalize the amount of template in the PCR. **(e)** Relative expression level of the StERF94 gene after 14 days of NaCl treatment *in vitro*. (**f**) After 30 days of salinity treatment under greenhouse conditions. The relative expression was calculated using ef1α as internal reference. Band densities in the gels are expressed in arbitrary units calculated by the Gel DocXR software.

conditions. The transgenic potato plants treated by salt (100 mM NaCl) for 21 days *in vitro* or for 30 days in the greenhouse, showed better growth than non-transgenic plants (figure 2). Indeed, salt stress treatment led to a significant reduction of WT stem elongation *in vitro* as well as in the greenhouse culture conditions (figures 3a and b). However, the reduction of stem elongation of transgenic plants was much less important (figures 3a and b). Similarly, a significant alteration of the fresh weight of WT plants was observed *in vitro* (figure 3c) and under greenhouse conditions (figure 3d) due to salinity stress. However, a low decrease of FW (Fresh Weight) of transgenic plants treated by NaCl, *in vitro,* as well as in the greenhouse was measured (figures 3c and d). The SP1 StERF94 transgenic line showed the best growth rate after NaCl treatment in both culture conditions. This SP1 transgenic line also showed the highest expression level of StERF94 (figure 1).

The NaCl treatment generated an important loss of leaf greenness in WT plants, with significant decrease in chlorophyll content after salt treatment, both *in vitro* and in greenhouse conditions. This decrease was more significant *in vitro* than in greenhouse conditions. In contrast, leaves of transgenic plants remained green (supplementary figure 1), and an increase of chlorophyll content was noticed in transgenic plant leaves submitted to salt treatment in both culture conditions. The highest chlorophyll increase *in vitro* was observed in SP1, while SP4 showed the highest increase in the greenhouse. The SP5 transgenic line showed a

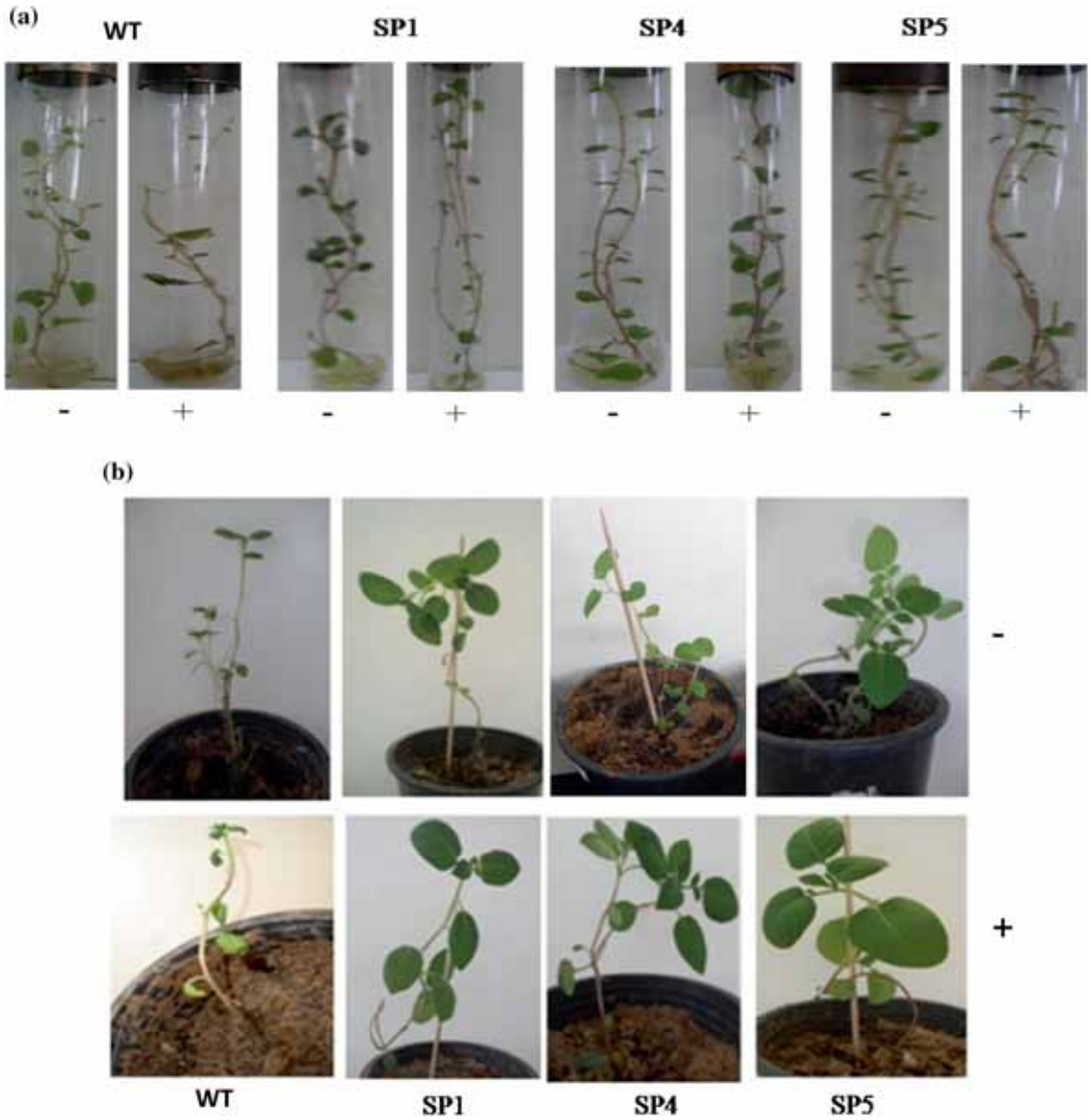

Figure 2. Morphology of transgenic plants (SP1, SP4 and SP5) and WT plants were grown in the absence (−) or in the presence (+) of 100 mM NaCl, for 21 days *in vitro* conditions (**a**) and for 30 days in greenhouse conditions (**b**).

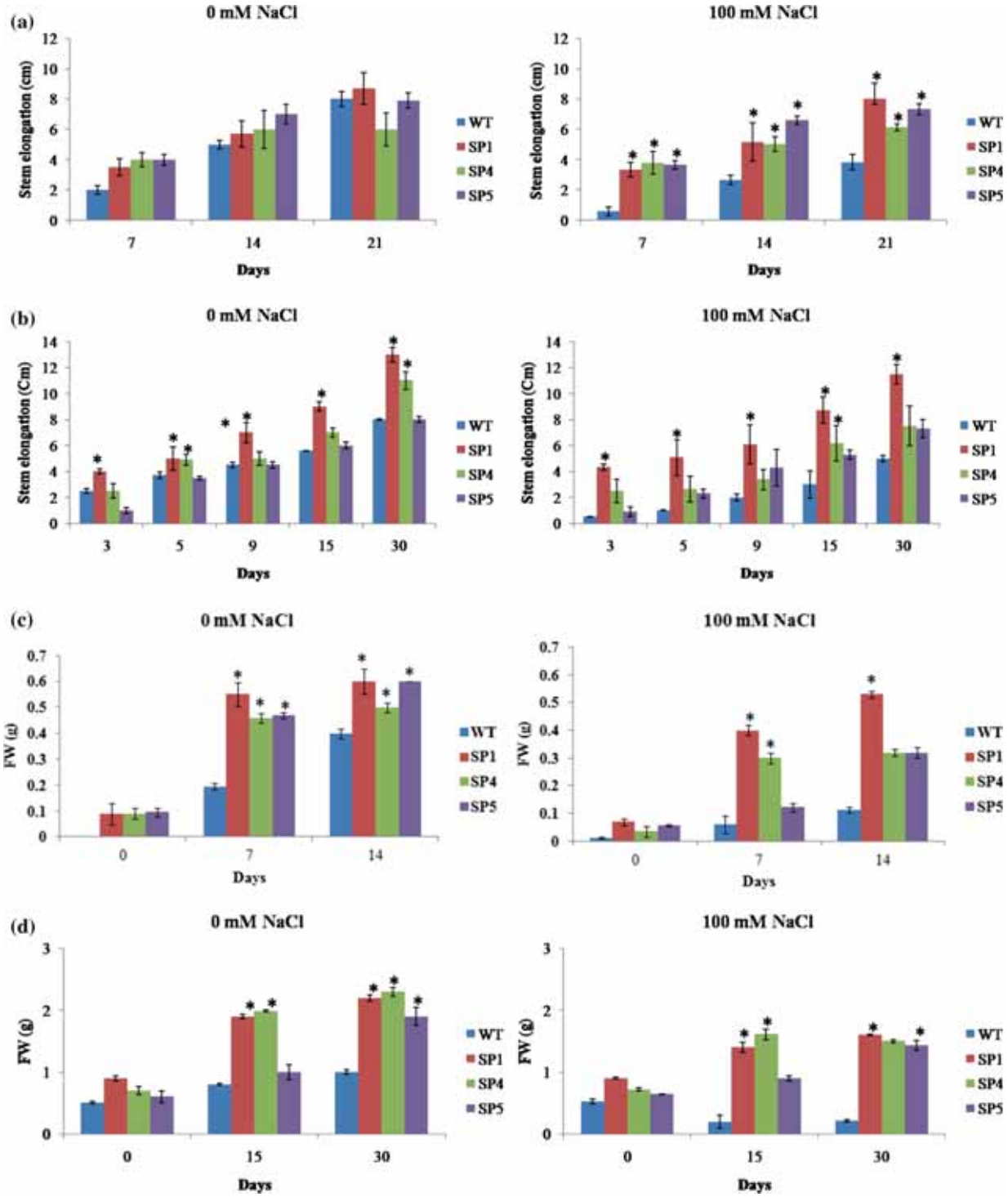

Figure 3. (**a**) Stem elongation of transgenic plants (SP1, SP4 and SP5) and non-transgenic WT plants, taken at different times of culture under standard (0 mM NaCl) and salt treatment (100 mM NaCl) conditions *in vitro*. Asterisk (*) indicates a significant difference between salt treated and non-treated plants at $p<0.05$. **(b)** Stem elongation of transgenic plants (SP1, SP4 and SP5) and the non-transgenic WT plants, taken at different times of culture under standard (0 mM NaCl) and salt treatment (100 mM NaCl) under greenhouse conditions. Asterisk (*) indicates a significant difference between salt treated and non-treated plants at $p<0.05$. **(c)** Fresh weight determination of transgenic plants (SP1, SP4 and SP5) and non-transgenic (WT) plants cultivated under standard (0 mM NaCl) or salt stress conditions (100 mM NaCl) *in vitro*. Asterisk (*) indicates a significant difference between salt treated and non-treated plants at $p<0.05$. **(d)** Fresh weight determination of transgenic plants (SP1, SP4 and SP5) and non-transgenic plants (WT) cultivated under standard (0 mM NaCl) or salt stress conditions (100 mM NaCl) under greenhouse culture conditions. Asterisk (*) indicates a significant difference between salt treated and non-treated plants at $p<0.05$.

decrease in chlorophyll content after 7 days of *in vitro* salt treatment, followed by a recovery and a chlorophyll increase after 14 days of salt treatment.

These results indicate that the overexpression of StERF94 may increase chlorophyll content in transgenic plants submitted to salt stress.

3.3 *Determination of MDA and H_2O_2 levels*

In roots, low MDA content was measured in plants cultivated *in vitro* (figure 4a) and no MDA was detected in plants cultivated in the greenhouse (figure 4b). However, in leaves the *in vitro* application of salt treatment led to an increase of MDA in WT and in the SP4 and SP5 transgenic plants; it then decreased after 14 days of culture (figure 4a).

Under greenhouse culture conditions (figure 4b), the highest content of MDA was measured in leaves of the WT lines, after 15 days of salt stress. The MDA level increased in leaves of SP4 and SP5 transgenic plants, followed by a recovery for SP5. The lowest MDA accumulation was measured in the SP1 line.

Another oxidative stress parameter was also followed in WT and transgenic lines after salt treatment *in vitro* and in the greenhouse. Indeed, the H_2O_2 level (figure 5a) showed a significant increase in WT plants in leaves after 14 days and in roots after 7 days of salt stress, while these levels remained stable in leaves of the SP1 and SP4 transgenic lines cultivated *in vitro*. An increase in H_2O_2 content was measured in all plants leaves at day 15 of the treatment under greenhouse conditions, but it was followed by a significant reduction when salt stress was pursued (figure 5).

3.4 *Measurement of the activity of some antioxidant enzymes*

The response to ROS accumulation caused by high salinity was investigated in transgenic plants by the analysis of the activity of three antioxidant enzymes, SOD, CAT and GPX.

3.4.1 *SOD activity:* The application of salt treatment *in vitro* or in the greenhouse led to a greater increase of SOD activity (figure 6) in leaves, than in roots, in all plant lines. A high increase in SOD was observed in the leaves of StERF94 transgenic plants cultivated *in vitro* after 7 days of salt stress and the highest SOD was measured in the SP1 and SP4 lines (figure 6a). However, lower increase of SOD was noticed in WT plants. A similar response was shown in plants cultivated under greenhouse conditions and treated with salt (figure 6b). An increase in the SOD activity was observed after 15 days of salt treatment in leaves and roots in all plants. However, the SP1 line showed the highest increase in leaves. These results may explain the decrease in MDA content in transgenic plants leaves.

In roots, (figure 6a) the SOD activity increased in all plants and the highest level was measured in the SP1 and SP5 lines. This activity decreased after 30 days of stress in all lines.

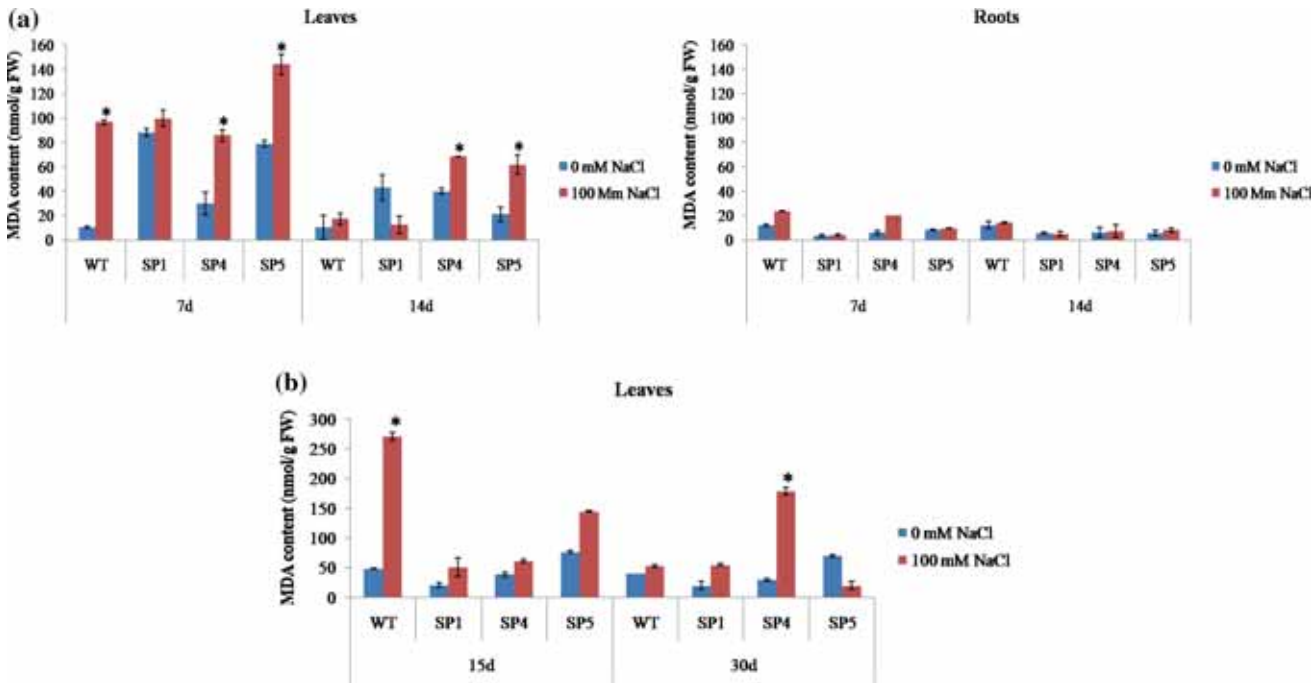

Figure 4. Measurement of MDA contents in the leaves and roots of different transgenic lines (SP1, SP4 and SP5) and of WT plants cultivated under standard or salt stress conditions *in vitro* (**a**) and in greenhouse conditions (**b**). Each value is represented by the mean ± SD. Error bars represent the standard error of triplicate analyses. Asterisk (*) indicates a significant difference between salt treated and non-treated plants at $p<0.05$.

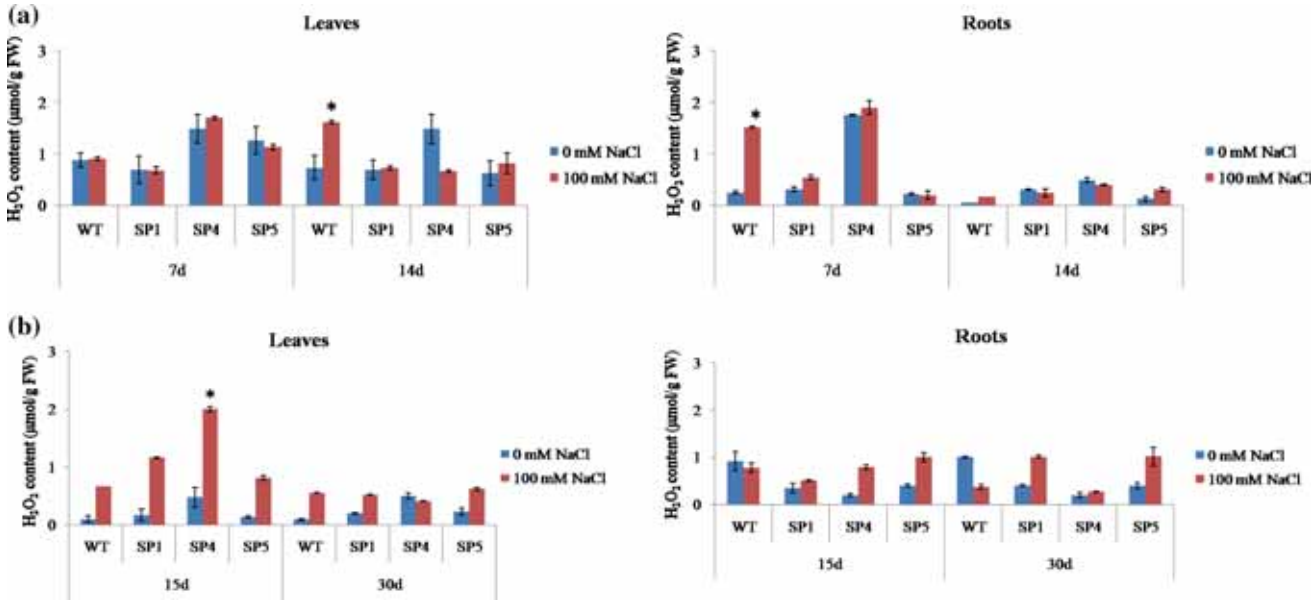

Figure 5. H_2O_2 accumulation in plants submitted to salt treatment *in vitro* (**a**) and in greenhouse conditions (**b**). SP1, SP4 and SP5 transgenic lines and WT plants. Each experiment was repeated three times. Error bars represent the standard error of triplicate analyses. Mean values ± SD are presented. Asterisk (*) indicates a significant difference between salt treated and non-treated plants at $p<0.05$.

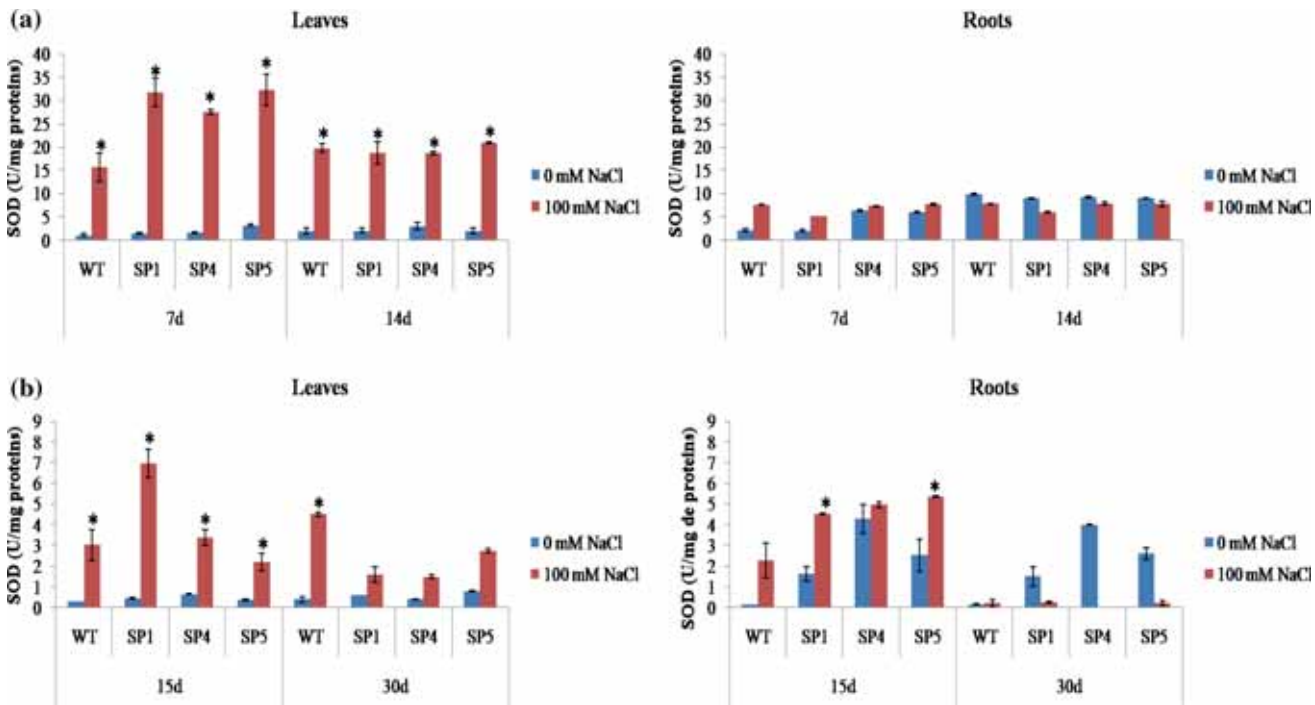

Figure 6. Estimation of SOD activity in WT and transgenic lines (SP1, SP4and SP5) cultivated under standard or saline conditions (100 mM NaCl) *in vitro* (**a**) or in greenhouse culture conditions (**b**). Error bars represent the standard error of triplicate analyses. Mean values ± SD are presented. Asterisk (*) indicates a significant difference between salt treated and non-treated plants at $p<0.05$.

These data corroborate the low MDA level (figure 4a) measured in the plant roots submitted to salt treatment.

3.4.2 *CAT activity:* The effectiveness of the H_2O_2 removal mechanism generated by SOD was determined by evaluating the CAT and the GPX activities in the leaves and roots of plants submitted to salt treatment. As observed for SOD, higher activation of CAT activity was measured in leaves in comparison to roots, for all plant lines. Furthermore, higher CAT activities (figure 7) were also determined in transgenic plant leaves, in comparison to WT plants. Indeed, the SP1 line showed the highest CAT

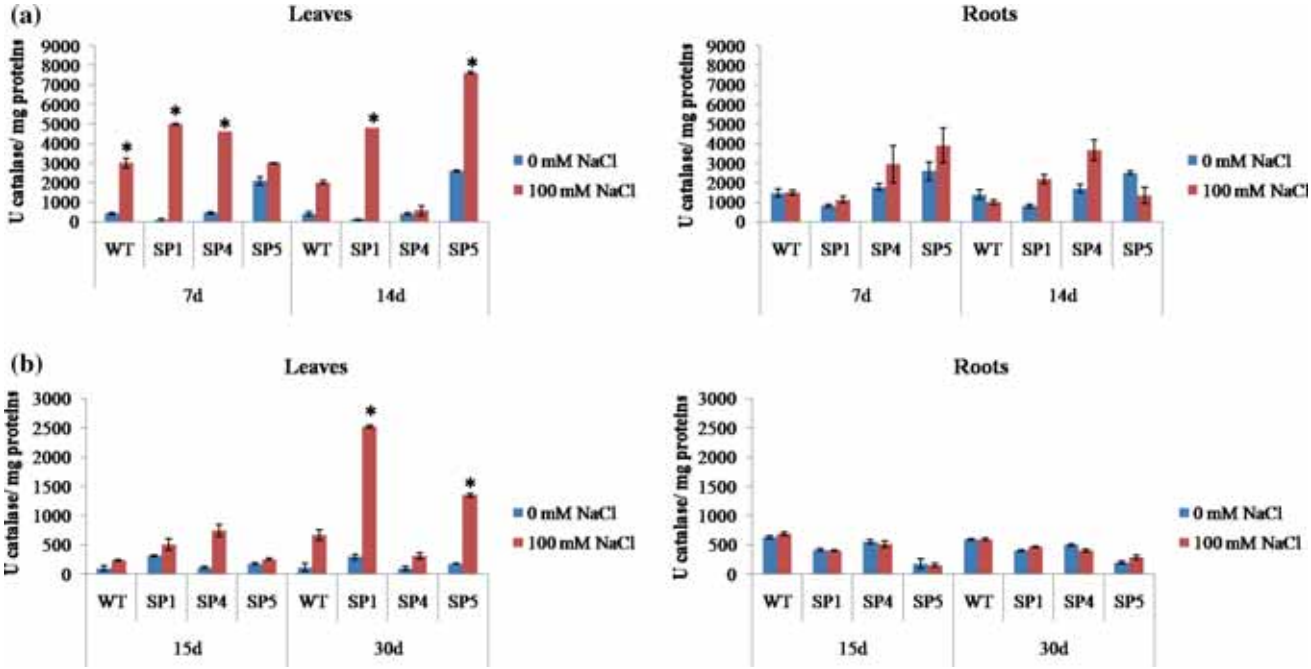

Figure 7. Analysis of CAT activities in WT and transgenic lines (SP1, SP4 and SP5) cultivated under standard conditions or after salinity treatment with 100 mM NaCl, *in vitro* (**a**) and in greenhouse culture conditions (**b**). Each experiment was repeated three times. Error bars represent the standard error of triplicate analyses. Mean values ± SD are presented. Asterisk (*) indicates a significant difference between salt treated and non-treated plants at $p<0.05$.

level in the leaves after 7 days of salt treatment *in vitro* and after 30 days of salt treatment in the greenhouse. For the SP4 line, a high increase of CAT activity was observed at day 7 *in vitro* and at day 15 under greenhouse culture conditions. The SP5 line showed an increase of the CAT activity after 14 days of salt treatment *in vitro* and 30 days in the greenhouse. In roots, a low increase of CAT activity was measured in transgenic plants submitted to salt treatment (figure 7a) *in vitro,* especially for SP4, while no CAT activity increase was determined in all plants roots treated by salt in the greenhouse (figure 7b). These results corroborate with the low production of H_2O_2 in roots (figure 5b).

3.4.3 *GPX activity:* In contrast to the CAT, the highest GPX activity (figure 8a) was measured in the roots of plants treated with NaCl *in vitro*. However, very low GPX activity was measured in plants cultivated under greenhouse conditions. The GPX activity increased also in leaves of plants cultivated either *in vitro* or in the greenhouse and submitted to salt stress. This increase was much more significant in transgenic lines compared to WT plants. The highest level was measured in leaves of the SP5 line cultivated *in vitro* (figure 8a) and in the leaves of SP1 and SP4 cultivated in the greenhouse (figure 8b).

Even if there are some differences in antioxidant enzyme activation depending on the culture conditions, a better response was generally detected in transgenic plants, in comparison to WT plants.

3.5 *Evaluation of the osmoprotectant accumulation*

Since osmoprotectants play crucial roles in plant response to salinity, we measured soluble sugars and proline contents in transgenic plants submitted to 100 mM NaCl treatment. A high increase of the soluble sugar level was measured in the SP1 transgenic line submitted to salinity both *in vitro* and under greenhouse conditions (figure 9a, b). The SP5 also exhibited the highest rise of this osmoprotectant in leaves after salt addition *in vitro*, while the SP4 line exhibited the highest level in roots after 7 days of saline treatment. Almost no soluble sugar accumulation was measured in WT plants after salt treatment under greenhouse conditions, whereas, the highest increase of soluble sugar content was measured in the roots of the SP5 plants and in the leaves of the SP1 plants.

Proline content showed a significant increase in all tissues of transgenic plants after salinity treatment, either *in vitro* or in the greenhouse conditions (figures 9c and d). The highest increase was noticed after 14 days of NaCl addition, *in vitro,* and 30 days in the greenhouse.

3.6 *Ectopic expression of StERF94 induces the stress responsive target genes*

ERF transcription factors can regulate the expression of target genes by binding to abiotic stress-related elements DRE/CRT (Yao *et al.* 2016). Among these target genes,

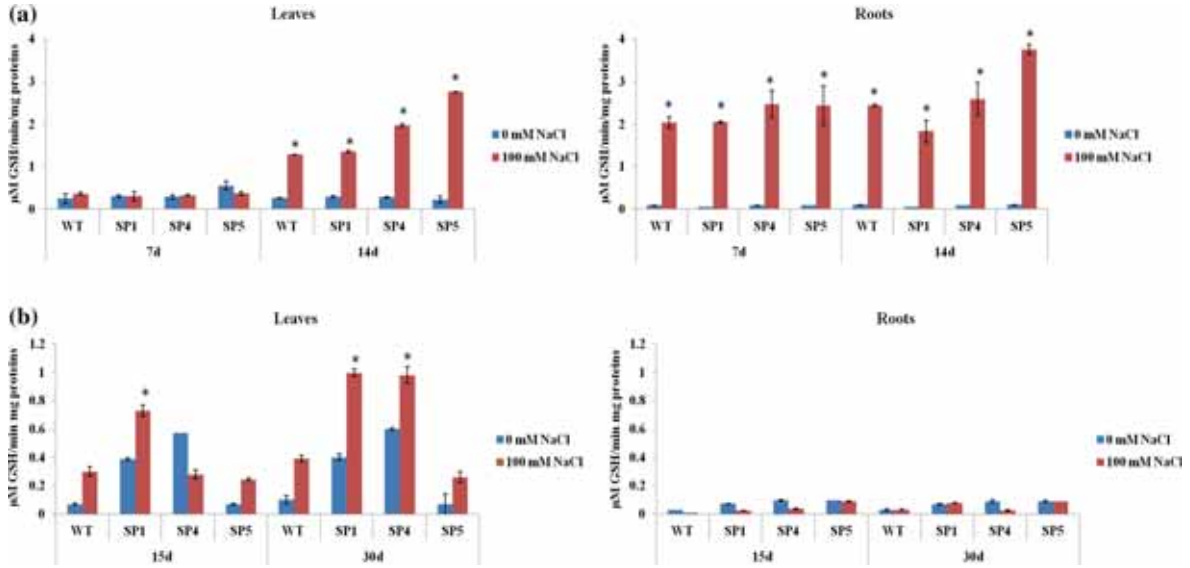

Figure 8. Measurement of GPX activity in the leaves and roots of transgenic lines (SP1, SP4, and SP5) and WT plants cultivated under standard or salt stress conditions *in vitro* (**a**) and in greenhouse culture conditions (**b**). Each value is presented by the mean ± SD. Error bars represent the standard error of triplicate analyses. Mean values ± SD are presented. Asterisk (*) indicates a significant difference between salt treated and non-treated plants at $p<0.05$.

those encoding superoxide dismutase (CuZn-SOD) and early response to dehydration-10 (ERD10) were shown to be upregulated by such ERF factors in tobacco (Kim *et al.* 2012). Here, we chose three putative target genes, which encode CuZn-SOD, a dehydrine (DHN25), and an ERD protein. Their expression was determined by semi-quantitative RT-PCR analysis performed on RNA from WT and transgenic plants cultivated under standard conditions or treated with 100 mM NaCl *in vitro* (figure 10). Higher transcription levels of these genes were detected in almost all transgenic lines in the absence of salt stress than in non-transgenic lines. This expression increased after salt stress application (figure 10). The SP4 line showed the highest transcription level of all these genes.

4. Discussion

Increasing evidence indicates that the AP2/ERF transcription factors play crucial regulatory roles in plant response to stresses. They interact with cis-acting elements, such as DRE/CRT or GCC boxes, to regulate the target stress-responsive gene expression. In this context, several studies showed that the ERF genes are involved in abiotic stress response in Arabidopsis (Zhu *et al.* 2010; Zhang *et al.* 2012), rice (Quan *et al.* 2010), tomato (Zhang *et al.* 2010a, b; Tian *et al.* 2011), tobacco (Nishiuchi *et al.* 2002), soybean (Zhang *et al.* 2009) and barley (Jung *et al.* 2007). We have also demonstrated that the StDREB proteins play vital roles in abiotic stress tolerance in potato (Bouaziz *et al.* 2012; 2013; 2015). The role of ERF factors on disease response was described in different plant species (Wang *et al.* 2016).

In a previous study, we characterized the ERF genes from potato (Charfeddine *et al.* 2015). The present work focused on the StERF94 gene that belongs to group IX of the ERF family according to the Nakano *et al.* (2006) classification, and to the B3 group (Sakuma *et al.* 2002).

Transcription factors belonging to group IX were mainly related to plant biotic stress response (Xu *et al.* 2011). However, our previous genome-wide analysis of StERF genes showed that this transcription factor seems to be upregulated by salt, drought, and fungi infection (Charfeddine *et al.* 2015). Similarly, other reports showed that some ERF factors from group IX can be involved in plant tolerance to different biotic and abiotic stresses (Nakano *et al.* 2006; Quan *et al.* 2010; Xu *et al.* 2011).

We focused here on the effect of the overexpression of the StERF94 in potato plants on salt tolerance *in vitro* and under greenhouse culture conditions. The StERF94 transgenic plants showed a better growth capacity when compared to WT plants and an increase of chlorophyll content (supplementary figure 1) under salt stress conditions, both *in vitro* and in the greenhouse. These results corroborate other reports, which showed that salt-tolerant plants exhibited an increase in the chlorophyll content after NaCl treatment (Bouaziz *et al.* 2012; Syed *et al.* 2017; Charfeddine *et al.* 2018).

In both culture conditions, the StERF94 overexpression activated the transcription of different target genes involved in salt stress response. These genes encode CuZn-SOD, DHN and ERD proteins playing different roles in protecting

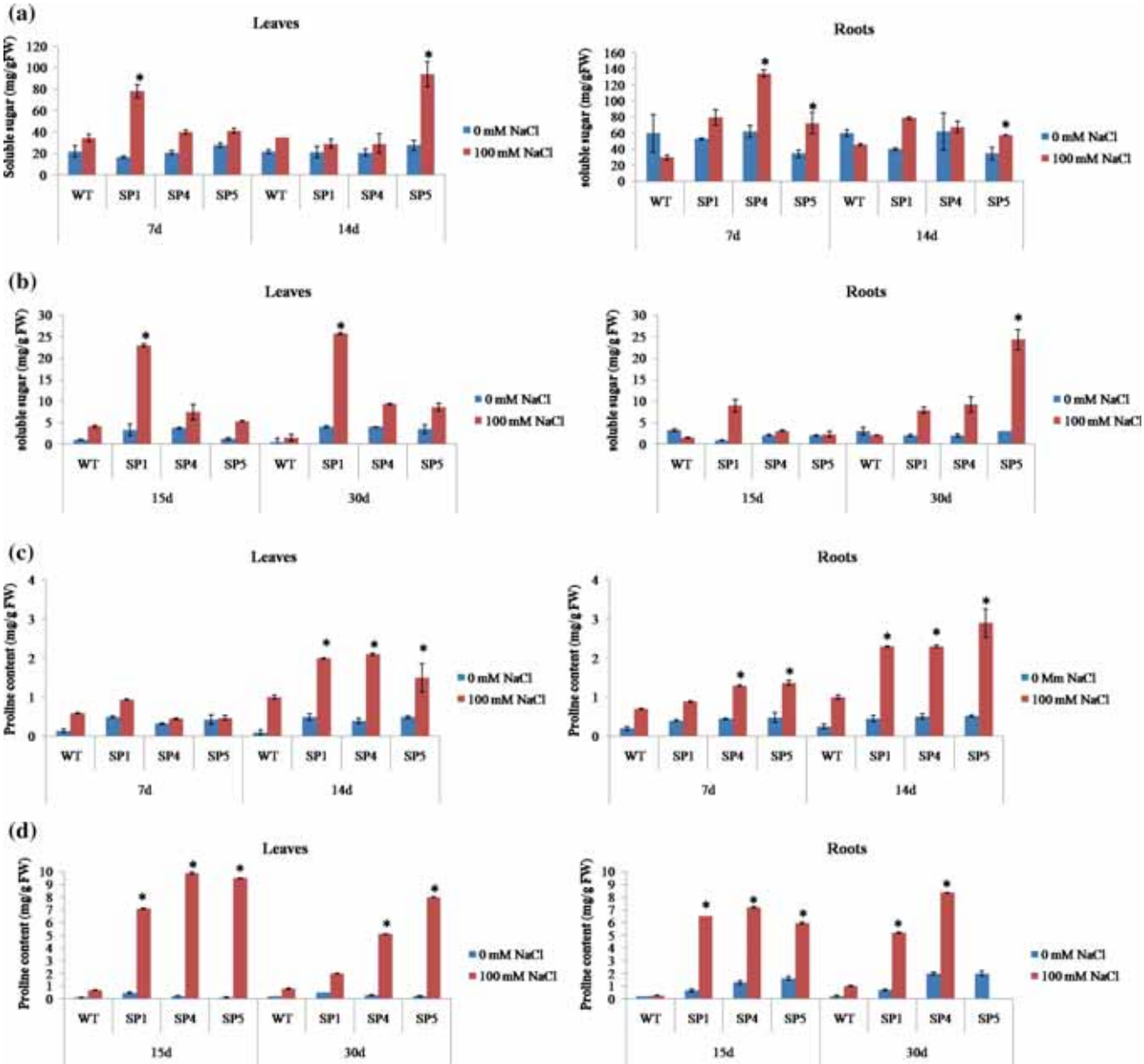

Figure 9. Evaluation of the soluble sugar (**a, b**) and proline (**c, d**) contents in the leaves and roots of plants cultivated under standard conditions and in 100 mM NaCl supplemented medium. a, c: *in vitro* b, d: in greenhouse culture conditions. Each value is presented by the mean ± SD. Error bars represent the standard error of triplicate analyses. Mean values ± SD are presented. Asterisk (*) indicates a significant difference between salt treated and non-treated plants at $p<0.05$.

cells against damages due to salinity, contents of soluble sugars and proline (Zhang *et al.* 2009).

These results are in agreement with other findings that showed that members of the ERF family could regulate the expression of a number of stress-related genes mainly CuZn-SOD, CAT and ERD10 (Kim *et al.* 2012). The StERF94 transcription factor also seems to activate enzymes involved in osmoprotectant production, such as proline and soluble sugars. These data also suggest that the StERF94 factor may control chlorophyll biosynthesis or/and degradation as previously reported by Zhai *et al.* (2013).

Similar findings were described in other transgenic plants overexpressing the ERF factors that exhibited better growth than WT after salinity treatment (Lee *et al.* 2010; Dong *et al.* 2012; Kim *et al.* 2012).

Biological membrane stability was considered here a screening tool to determine the consequence of salinity damage. MDA, a secondary breakage product of lipid peroxidation, was used as an indicator of oxidative stress (Baby and Jini 2011; Jbir-Koubaa *et al.* 2015). Our data showed that StERF94 transgenic plants produced lower MDA contents than non-transgenic plants submitted to salinity

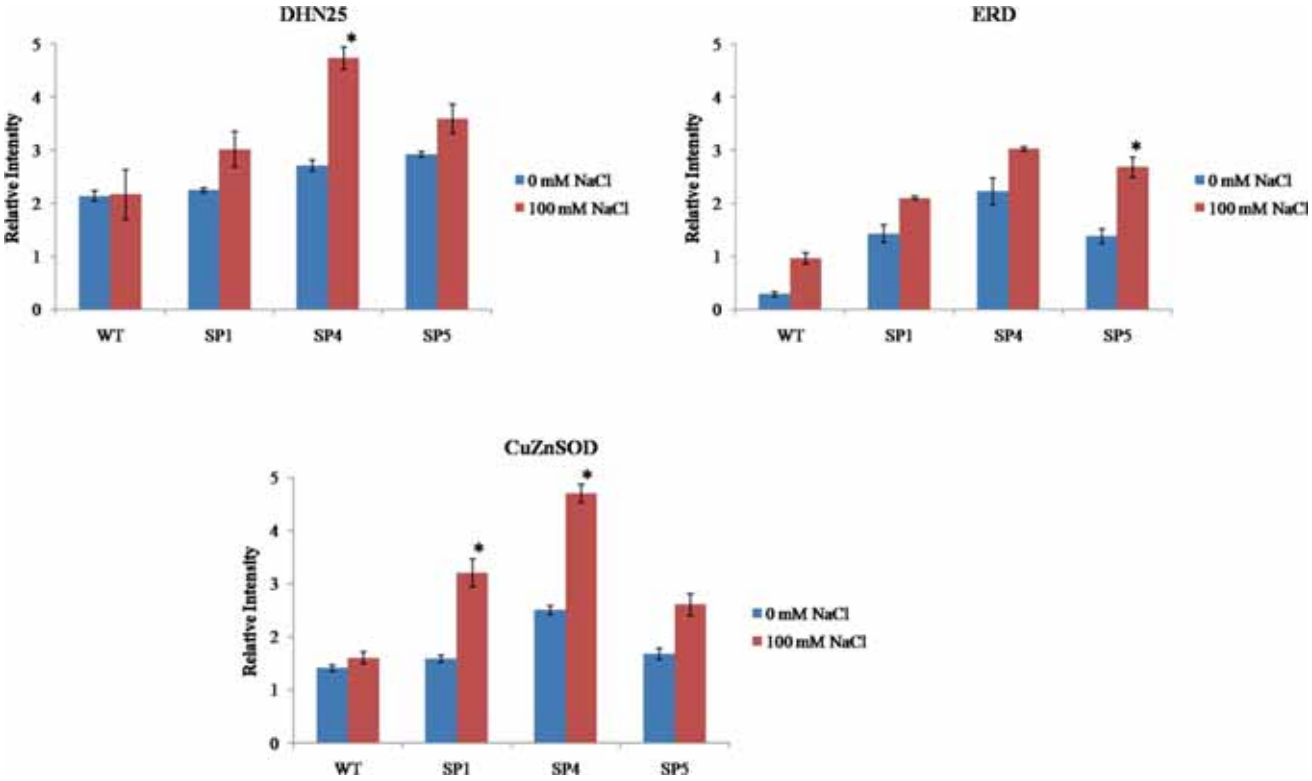

Figure 10. RT-PCR analysis of the expression of the stress-induced genes, DHN25, ERD and CuZn-SOD in transgenic lines (SP1, SP4, SP5) and WT plants under standard and salt stress conditions. Band densities in the gels are expressed in arbitrary units calculated by the Gel DocXR software. Asterisk (*) indicates a significant difference between salt treated and non-treated plants at $p<0.05$.

especially under greenhouse conditions (figure 4b). These results can be related to the significant increase of ROS scavenging enzyme activities such as catalase, SOD and GPX. These enzymes are interesting components in preventing oxidative damage by eliminating ROS in plants.

The high SOD activities determined in transgenic lines can alleviate damage of plasma membranes induced by ROS accumulation due to salt stress (Laloi *et al.* 2004). Such SOD increase was also reported in transgenic plants overexpressing a citrus CsERF. These plants also showed increased tolerance to abiotic stress (Ma *et al.* 2014). Similar to SOD activity, a significant increase of GPX and CAT activities was observed in transgenic plants submitted to 100 mM NaCl, either *in vitro* or in greenhouse culture conditions.

All these findings propose that the StERF94 factor may be implicated in the regulation of potato plant response to oxidative stress by activating the expression of antioxidant enzymes that contribute to protect plant cells and tissues against the ROS devastating effect. Similar results were described by Zhai *et al.* (2013) on the ectopic expression of the GmERF7 factor in tobacco.

The StERF94 transgenic potatoes exhibited an increase in the production of osmoprotectant molecules such as proline and soluble sugars in comparison to WT potatoes. These results are in agreement with those of Zhai *et al.* (2013), who reported that the ectopic expression of GmERF7 in tobacco plants improved their tolerance to salinity, increased the accumulation of soluble sugar contents and lowered the MDA contents (Zhai *et al.* 2013). Similarly, Yao *et al.* (2016) revealed that the overexpression of poplar ERF76 enhanced tolerance to salinity of tobacco transgenic plants by increasing the proline level. Another report showed that overexpression of ERF factors increased the accumulation of proline in transgenic plants (Ma *et al.* 2014) and improved tolerance to dehydration by increasing contents of soluble carbohydrates and proline.

These data suggest that despite small differences observed between the *in vitro* and greenhouse conditions, the results obtained led to the same conclusion – which is the improved salt tolerance of StERF94 transgenic plants. Indeed, StERF94 transgenic plants behaved almost similarly in both culture conditions by increasing antioxidant enzyme expression and osmoprotectants accumulation. However, the plant response was observed earlier *in vitro* since the NaCl was added in the culture medium, while under greenhouse culture condition, NaCl treatment was made by irrigation. In this latter case, the effect of salinity is delayed in comparison to *in vitro* conditions. Several irrigation events are needed to create a stressful condition for plants. The greenhouse culture is closer to the field conditions than *in vitro* ones. Moreover, in the greenhouse photoperiod, light intensity and temperature are not controlled parameters.

5. Conclusion

The enhancement of salt stress tolerance by the overexpression of StERF94 in potato plants supports the idea that ERF factors from group IX regulate the activation of signaling pathways that are required for plant tolerance to abiotic stresses. Moreover, this study also shows that results obtained under *in vitro* conditions can be confirmed under greenhouse conditions where temperature and light were not controlled.

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