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BRIEF COMMUNICATION

Effect of salt stress on gene expression of superoxide dismutases and copper chaperone in *Arabidopsis thaliana*

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Abstract

Arabidopsis thaliana plants (wild type accessions Col and N1438) were grown in nutrient solution for 34 d with or without 50 mM NaCl. Salt stress inhibited plant growth rate more in Col than in N1438 and a decrease in K^+, Ca^{2+} and nitrogen contents was observed in both accessions. NaCl diminished accumulation of malate, fumarate and citrate only in Col accession. To measure the effect of NaCl on transcript level of superoxide dismutase (SOD) isoforms and copper chaperone for SOD genes, a semi-quantitative polymerase chain reaction (RT-PCR) method was developed using cDNA normalized against the *EF1a* gene in parallel with quantitative real time RT-PCR (Q-PCR) technique. Both methods gave the same results. The abundance of transcripts of the three genes coding for Cu/Zn-SOD responded similarly to NaCl in both accessions: *CSD1* gene was overexpressed, and *CSD2* and *CSD3* genes were repressed. However, the genes coding for Fe-SOD (*FSD1*), Mn-SOD (*MSD1*) and Cu-chaperone for SOD (*CCS*) responded to NaCl differently in Col and N1438: the former gene was overexpressed in Col and repressed in N1438, and the opposite behaviour was observed for the latter two genes.

Additional key words: citrate, fumarate, intraspecific variability, malate, mineral nutrition, RT-PCR.

Salt stress leads to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity (Wang *et al.* 2001). Abiotic stresses promote the formation of reactive oxygen species (ROS) which may cause serious damages to the cell structures. The superoxide dismutases (SODs) reduce O_2^- to H_2O_2, which is then decomposed into H_2O and O_2 by catalase. SODs are classified into three groups according to their metal co-factor: Fe-SOD, Mn-SOD, and Cu/Zn-SOD. Seven SODs are encoded in *Arabidopsis* genome (Kliebenstein *et al.* 1998): three Fe-SODs (*FSD1* - *3*), one Mn-SOD (*MSD*) and three Cu/Zn-SODs (*CSD1* - *3*). The *CSD1* and *CSD2* activities are detected in roots, leaves, stems, and siliques, and their proteins are localized in the cytosol and chloroplast, respectively. *CSD3* is though to be a peroxisomal protein (Kliebenstein *et al.* 1998).

In yeast and mammals, a copper chaperone for SOD (*CCS*) is required to transfer Cu to Cu/Zn-SOD proteins and activate the resulting metallo-enzymes *via* formation of a disulfide bond (Arnesano *et al.* 2004). The *Arabidopsis* genome contains a single *CCS* gene, which codes for a protein with a chloroplast targeting sequence. An alternative localization in cytosol seems possible if alternative initiation codon is used (Wintz and Vulpe 2002). *CCS* genes have also been found in tomato (Zhu *et al*. 2000), potato (Trindade *et al.* 2003) and maize (Ruzsa and Scandalios 2003). Northern blot study indicated that the *A. thaliana CCS* gene is expressed both in root and shoot tissues. Increase in *CCS* mRNA abundance has been observed in senescing *Arabidopsis* shoot (Abdel-Ghany *et al.* 2005).

In the present work, we studied the effect of NaCl on growth, mineral nutrition and expression of five SOD genes and *CCS* gene in rosette leaves of two natural accessions of *A. thaliana*, namely Col and N1438.

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Abbreviations: CCS - copper chaperone for SOD; Q-PCR - quantitative real time RT-PCR; ROS - reactive oxygen species; RT-PCR - reverse transcription polymerase chain reaction; SOD - superoxide dismutase.

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Seeds of *Arabidopsis thaliana* L. accessions Columbia (Col) and N1438 were purchased form *NASC* (Nottingham, UK), and *ABRC* (Ohio State University, USA). Seedlings were grown in a culture chamber with 8-h photoperiod (150 µmol m^{-2} s^{-1} PAR), day/night temperature of 22/18°C and relative humidity 60/80 %. Seedlings were grown in ¼ strength Gay and Hauck (1994) nutrient solution. At the age of 34 d, the 50 mM NaCl was added to half of plants. Plants were harvested 15 d after the start of the treatment. Fresh mass was rapidly measured and 3 fully expanded rosette leaves were collected, frozen in liquid nitrogen and stored at -80 °C for PCR analyses. Another leaf was sampled and digested in 0.5 % (v:v) HNO_3 for ion analysis. Total dry mass was determined after 72 h at 70 °C. Cations were assayed by flame photometry (*Jenway PFP7, Spectronic Analytical Instruments*, Garforth, UK). Organic and inorganic anions were assayed by high performance ion chromatography *ICS-2500* (*Dionex*, Sunnyvale, USA) in aqueous extracts.

Harvested rosettes were immediately ground with liquid nitrogen into a fine powder, which was then mixed with 1 cm^3 of Trizol (guanidium thiocyanate-phenol-chloroform) reagent, stirred for 5 min at room temperature; then 0.2 cm^3 of chloroform were added. The solution was centrifuged for 10 min at 14 000 *g* at 4 °C. The RNAs present in the aqueous phase were precipitated by the addition of 0.5 cm^3 of isopropanol. After a 15 min centrifugation at 14 000 *g* at 4 °C, the pellet was rinsed with 75 % ethanol, dried and suspended in 0.09 cm^3 of ultrapure water. The RNA samples (40 µg) were treated with 1500 U DNase (RNase-free DNase set, *Qiagen*, Courtaboeuf, France) and purified (RNeasy R *MinElute*™ cleanup kit; *Qiagen*) according to the supplier's recommendations. A 4 µg aliquot was incubated with 20 pmol of anchored dT oligonucleotides and 0.011 cm^3 of sterile water, and denatured for 5 min at 75 °C. The mixture temperature was progressively lowered to 20 °C by 1 °C steps, 10 s each, to allow hybridization. The reverse transcription was then performed at 42 °C for 1 h 30, in the presence of 200 U *M-MLV* reverse transcriptase (*Promega*, Madison, WI, USA). The reaction was stopped by adding 0.18 cm^3 of *T10E1* (10 mM Tris-HCL, 1 mM EDTA).

PCR reactions were conducted using an *Eppendorf* thermal cycler at 94 °C for 2 min to denature the samples, followed by 30 PCR cycles (30 s at 94 °C, 45 s at 60 °C, 1 min at 72 °C), and a 5 min final extension step at 72 °C. The number of amplification cycles (30) was determined following an optimisation, which ensured that all amplification reactions were stopped in the exponential phase. The PCR reaction was resolved by electrophoresis on a 1 to 2 % (m/v) agarose gel stained with ethidium bromide. Gel images were photographed.

Primers were defined using *Primer3* software, and synthesized by *Eurobio* (Les Ulys, France). Amplifications were achieved in 0.01 cm^3 using 0.001 cm^3 of matrix, 10 pmol of oligonucleotides and 0.001 cm^3 of *LC mix* in a *Light Cycler*™ (*Roche Diagnostics,* Mannheim, Germany). A 10 min activation step at 95°C was followed by 45 cycles, each one with a 5 s denaturation step at 95 °C, a 7 s hybridisation step at 65 °C and an 8 s elongation step at 72 °C. The relative quantification of the gene expression was achieved with the help of the software *Light Cycler*, using several references: *EF1a*, *CLATHRIN* and *ACTINE2*. *CLATHRIN* and *ACTINE 2* systematically led to identical results for all the studied genes. The results below were normalized using *ACTINE2*. Primers used were as follows. For *FSD1*: direct 5'-CCTCAAGAAACAGGTTCTTGGAACCG-3', reverse 5'-GGTCATGAATGTCTTTATGTAATC-3'; for *CSD1*: direct 5'-GAAAACAAGTAACCAAAGAGAG CG-3', reverse 5'-CCCTCACTGCTGTTCAAAACTGC-3'; for *CSD2*: direct 5'-GCAGCAGCCATGGCT CCACC-3', reverse 5'-GGCCCTGGAGTGAGACCA TG-3'; for *CSD3*: direct 5'-CGGGAGGGCGGTTGTTG TGCATGGG-3', reverse 5'-CTGAGTGTGGCT CTGTCCGTTGATCTCAAAAGCTAT-3'; for *MSD*: direct 5'-CCACCAAAAGGATCTCTTGGTAGTGCC-3', reverse 5'-CCTCGCTTGCATATTTCCAGTTGAT CAC-3'; for *CCS*: direct 5'-CGATCGTCTCCA CGTCTCTTG-3', reverse 5'-GAGGAACACCTTGTCC AATTAAGCGAGC-3'; for *ACTINE2* (At3g18780): reverse 5'-CTGAGGCTGATGATATTCAACC-3', direct 5'-ACACTGGGAAAAACAGCCC-3'; for *EF1α* (At1g07920): reverse 5'-GTCGATTCTGGAAAGTCG ACC-3', direct 3'-AATGTCAATGGTGATA CCACGC-5'.

Analyses of variance (*ANOVA*) with orthogonal contrasts and mean-comparison procedures were used to detect differences between treatments. Mean-separation procedures were carried out using the multiple range tests with Fisher least significant difference ($P < 0.05$).

After 15 d of culture with 50 mM NaCl, the rosette biomass was 66 % (N1438) to 50 % (Col) of control plants (0 mM NaCl). The leaves of NaCl-treated plants accumulated large amounts of Na^+, while Cl^- accumulation was two fold less than that of Na^+ in N1438 plants. However, direct Na^+ and Cl^- toxicity was not regarded as the main cause of salt-induced growth reduction (Hu *et al.* 2005). The accumulation of K^+ and NO_3^- in both accessions was strongly inhibited by NaCl treatment, in contrast, accumulation of Ca^{2+}, SO_4^{2-} and $H_2PO_4^-$ was only slightly depressed. Salt has also been shown to depress the chloroplast content in K^+, NO_3^- and SO_4^{2-} (Schröppel-Meier and Kaiser 1988). Organic acid content decreased under salinity in Col accession, but in N1438 no appreciable differences between the treatments for malate, fumarate and citrate were observed (Table 1).

The abundance of SOD gene transcripts in rosette leaves was assessed using semiquantitative RT-PCR and Q-PCR (Fig. 1, Table 2). The expression levels of five SOD genes was normalized using *EF1a* probe. As expected, the intensity of the *EF1a* gel bands did not vary between the treatments, justifying the use of this gene for normalization of the results (Fig. 1). The most abundant transcripts in control conditions were those of *FSD1* and *CSD2,* as in the Northern analysis of Kliebenstein *et al.* (1998). *MSD* transcript was also present at a relatively

Table 1. Effect of salt stress on rosette growth, ion and organic acid contents in two *Arabidopsis* accessions Col and N1438. Fifteen days before the harvest, NaCl was added to the medium at the indicated concentrations. Means ± SE, *n = 10*. The means sharing the same letter are not significantly different at *P* = 0.01.

Parameters	Col		N1438	
	0 mM	50 mM	0 mM	50 mM
Leaf biomass [g plant^{-1}]	0.10 ± 0.02^a	0.04 ± 0.01^b	0.11 ± 0.02^a	0.07 ± 0.01^b
Na^+ [mmol g^{-1} (d.m.)]	0.03 ± 0.01^a	3.38 ± 0.30^b	0.05 ± 0.01^a	3.08 ± 0.38^b
K^+ [mmol g^{-1} (d.m.)]	1.45 ± 0.09^a	0.62 ± 0.08^b	1.63 ± 0.15^a	0.60 ± 0.09^b
Ca^{2+} [mmol g^{-1} (d.m.)]	0.54 ± 0.03^a	0.36 ± 0.02^b	0.44 ± 0.05^a	0.27 ± 0.03^b
Cl^- [mmol g^{-1} (d.m.)]	0.02 ± 0.00^a	2.81 ± 0.50^b	0.03 ± 0.00^a	1.45 ± 0.16^b
NO^{3-} [mmol g^{-1} (d.m.)]	2.35 ± 0.33^a	0.76 ± 0.22^b	2.31 ± 0.50^a	0.95 ± 0.19^b
SO_4^{2-} [mmol g^{-1} (d.m.)]	0.31 ± 0.06^a	0.22 ± 0.02^b	0.32 ± 0.04^a	0.27 ± 0.04^a
$H_2PO_4^-$ [mmol g^{-1} (d.m.)]	0.51 ± 0.06^a	0.48 ± 0.03^a	0.42 ± 0.03^a	0.37 ± 0.06^a
Malate [mmol g^{-1} (d.m.)]	0.09 ± 0.01^a	0.06 ± 0.01^b	0.10 ± 0.01^a	0.07 ± 0.01^a
Fumarate [mmol g^{-1} (d.m.)]	0.03 ± 0.01^a	0.01 ± 0.00^b	0.05 ± 0.01^a	0.04 ± 0.01^a
Citrate [mmol g^{-1} (d.m.)]	0.12 ± 0.01^a	0.09 ± 0.02^b	0.08 ± 0.01^a	0.07 ± 0.04^a

Table 2. Quantitative PCR analysis of some SOD gene expression and *CCS* expression in rosette leaves as affected by 50 mM NaCl. Means ± SE, *n* = 3. Within each accession, means sharing the same letter are not significantly different at *P* = 0.01.

	Genes	Col		N1438	
		0 mM	50 mM	0 mM	50 mM
Fe-SOD	*FSD1* (At4g25100)	1.5^a	0.9^b	1.0^a	2.4^b
Cu/Zn-SOD	*CSD1* (At1g08830)	1.2^a	2.8^b	0.9^a	1.3^b
Cu/Zn-SOD	*CSD2* (At2g28190)	0.8^a	0.5^b	1.9^a	0.7^b
Cu/Zn-SOD	*CSD3* (At5g18100)	3.0^a	1.7^b	2.7^a	2.3^b
Mn-SOD	*MSD* (At3g10920)	1.3^a	1.6^b	1.7^a	1.2^b
CCS	*CCS* (At1g12520)	0.5^a	1.0^b	1.4^a	0.9^b

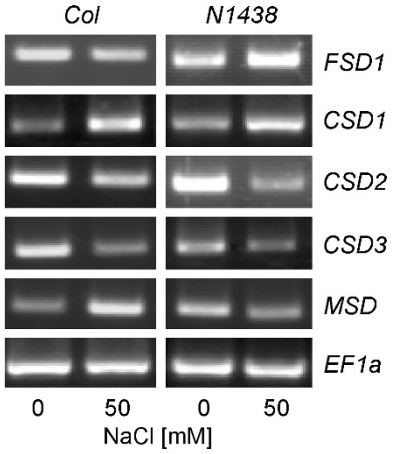

Fig. 1. Semiquantitative RT-PCR amplification of RNA extracted from Col and N1438 rosette leaves. The plants were 34-d old at the start of the experiment. Fifteen days before the harvest, NaCl was added to the medium at the indicated concentrations. Amplification conditions were as described in Materials and methods.

high abundance, in our work as in that of Kliebenstein *et al.* (1998). In control conditions, the same high expression level was observed for *CSD*3 in both accessions. A low expression level of *CSD2* and *CCS* characterized Col, the corresponding levels in N1438 being almost three times higher in N1438. For all the studied genes, both methods gave qualitatively identical results for the changes in mRNA abundances in response to NaCl treatments. The abundance of transcripts of the three genes coding for Cu/Zn-SOD isoforms responded similarly to NaCl in both accessions: *CSD1* was overexpressed, and *CSD2* and *CSD3* were repressed under salt treatment. On the contrary, the genes coding for Fe-SOD (*FSD1*), Mn-SOD *(MSD1*) and CSC (*AtCCS*) responded to NaCl differently in Col and N1438: the former gene was overexpressed in Col and repressed in N1438, and the opposite behaviour was observed for the two latter genes. These differences suggest that the two accessions have evolved different mechanisms for protection against oxidative stress.

Several factors, including irradiance, UV radiation, oxidative stress (Kliebenstein *et al.* 1998) and salinity (Hernandez *et al.* 1993, 1995, Gomez *et al.* 1999) have been shown to induce differential SOD encoding genes responses. The genes coding for Cu/Zn-SODs are generally overexpressed in response to oxidative stress

(Perl-Treves and Galun 1991, Kurepa *et al.* 1997), excess radiation (Rao *et al.* 1996), wounding (Van Camp *et al.* 1997), and dehydration (Yu and Rengel 1999, Borsani *et al.* 2001). Plants overexpressing Cu/Zn-*SOD* are protected from photo-oxidative damage caused by high irradiance (Allen *et al.* 1997). We observed different NaCl responses among the three genes encoding Cu/Zn-SODs, CSD1 being the only one to be overexpressed, and this pattern was the same in both accessions. This analysis revealed a variability of the response between *SOD* genes and between accessions.

The observed changes in *SOD* transcript abundance might be a superimposition of two opposite effects. The first effect was the induction of some genes (*CSD1* and *MSD* in Col; *CSD1* and *FSD1* in N1438) and/or the stabilization of their transcripts, which probably represented adaptative responses for protection against oxidative stress. The other effect was an apparent repression of other genes (*FSD1* and *CSD3* in Col; *CSD2*, *CSD3* and *MSD* in N1438), which might be either a specific transcriptional repression, or a global inhibition reflecting damages to the transcription machinery. In the latter case, only the genes strongly overexpressed would present increased transcript abundance, the genes with small or null induction levels appearing repressed.

The protein encoded by At1g12520 (*CCS*) is predicted to be the chaperone that delivers Cu to *CSD2* in the chloroplast stroma, in view of its sequence, the complementation data and the observed localization in plant cells (Pilon *et al*. 2006). Since *CCS* is the only gene candidate for a Cu-chaperone for SOD in the *Arabidopsis* genome, it is therefore possible that the encoded CCS protein delivers Cu to both cytosolic CSD1 and chloroplastic CSD2. Indeed, there is a theoretical possibility for targeting CCS to the cytosol, if an alternative translation start site that skips the chloroplast targeting peptide is used (Wintz and Vulpe 2002). The expression of *CSD1* and cytosolic *CSD2* is upregulated by Cu (Abdel-Ghany *et al.* 2005) and *AtCCS* is co-regulated with its *CSD1* and *CSD2* targets, indicating a role of Cu delivery in oxidative stress protection. *AtCCS*, *CSD1* and *CSD2* were found to be downregulated together in response to Cu deficiency in a study using micro-arrays (Wintz *et al.* 2003). Our work is the first quantitative analysis of the response of the *CCS* gene to salt stress in *Arabidopsis*. Our results show a variability in the abundance of transcripts of *CCS* in two accessions of *A. thaliana* differing in salt tolerance. The sensitivity of Col accession to salt stress was associated with an up-regulation of *CCS* gene. Such CSS overexpression did not appear in N1438 accession, which was more tolerant to salt by comparison with Col accession.

In conclusion, our results show variability in the response of SOD and CCS genes to NaCl treatment and between the two accessions of *A. thaliana*. Two SOD genes (*FSD1* and *MSD*) and CCS behave differently in salt-treated Col and N1438. They could be good candidates to explore the links between salt aggression and expression levels, and the associated variability.

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